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Human Salivary Acidic Proline-Rich Proteins and Statherin Promote the Attachment of *Actinomyces viscosus* LY7 to Apatitic Surfaces

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***Actinomyces viscosus* LY7 cells adsorbed in high numbers to experimental pellicles formed on hydroxyapatite (HA) from human parotid or submandibular saliva but not to pellicles prepared from human plasma or serum. To determine the nature of the salivary components responsible for promoting adhesion, pellicles were prepared from fractions of submandibular and parotid saliva obtained by chromatography on Trisacryl GF 2000 columns. Adsorption of LY7 cells was promoted by two groups of fractions. Each group was rechromatographed on DEAE-agarose. Fractions which promoted adsorption of LY7 cells were found by polyacrylamide gel electrophoresis to contain the acidic proline-rich proteins (PRPs) and statherin. Pellicles prepared from 12-μg/ml solutions of pure PRP-1, PRP-2, or parotid isoelectric focusing (PIF-slow) variant promoted maximal adsorption of *A. viscosus* LY7 cells. Somewhat higher concentrations of PRP-3 and PRP-4 were required for maximal adsorption, indicating that the 44-residue carboxy-terminal segment of PRP-1, PRP-2, and PIF-slow enhances LY7 binding but is not essential. Much higher concentrations of statherin were required to promote LY7 adsorption. Adsorption of LY7 cells to pellicles prepared from PRP-1 was not affected over the range of pH 5 to 8. Adsorption was also not inhibited by 50 mM lactose, which is consistent with the notion that type 1 fimbriae, rather than type 2 fimbriae, were responsible. *A. viscosus* T14, *Actinomyces odontolyticus* ATCC 17982, and *Actinomyces israelii* 12597 also adsorbed to PRP-1 pellicles, whereas *Actinomyces naeslundii* ATCC 12104 did not. Although *A. viscosus* cells bind strongly to adsorbed PRP-1, the presence of PRP-1 or PRP-3 in solution did not inhibit adhesion. Similarly, [^{3}H]PRP-1 did not bind to LY7 cells, nor was it degraded when incubated with the organism. However, LY7 cells adsorbed to [^{3}H]PRP-1 pellicles. These data suggest that hidden molecular segments of PRP become exposed when the protein adsorbs to HA; these segments then react with adhesins of LY7 cells. The apparent ability of *A. viscosus* cells to recognize segments of PRPs which are exposed only in surface-adsorbed molecules provides a novel mechanism which enables the organism to attach to teeth when suspended in salivary secretions.**

Actinomyces viscosus is a prominent microorganism in human supra- and subgingival dental plaque. The organism preferentially colonizes the teeth and may actually require teeth for oral colonization, since it is usually not detected in the mouths of predentate infants (14, 17). In humans, high proportions of *A. viscosus* have been associated with gingivitis (29, 42) and with cemental dental caries (15, 41); it has also been shown to induce periodontal pathology and root surface caries in experimental animals (27).

Typical strains of *A. viscosus* possess two antigenically and functionally distinct types of fimbriae which are thought to mediate its attachment to teeth and to oral epithelial surfaces (8). Type 2 fimbriae bind to galactosyl-containing receptors on mammalian cells or on the surfaces of certain bacteria (8, 16). Reactions involving type 2 fimbriae are inhibited by galactosides, such as lactose. On the other hand, Clark and co-workers (9–11) have shown that type 1 fimbriae mediate attachment of *A. viscosus* cells to salivary pellicles formed on mineral surfaces similar to those of teeth. Interactions involving type 1 fimbriae are not inhibited by lactose or by other saccharides which have been studied. Although *A. viscosus* cells have been shown to attach avidly to salivary pellicles formed on mineral surfaces (10, 38), the salivary components which mediate its attachment have not been identified. The purpose of the present investigation was to determine the nature of the pellicle components which serve as receptors for this organism.

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MATERIALS AND METHODS

Cultures and cultural conditions. *A. viscosus* LY7 was isolated from human dental plaque. Negatively stained preparations examined by electron microscopy by Z. Skobe (Forsyth Dental Center, Boston, Mass.) showed the organism to be densely fimbriated (Fig. 1). The organism reacts with specific antibodies to both type 1 and type 2 fimbriae (11). Furthermore, antibodies to type 1 fimbriae inhibit adsorption of LY7 cells to experimental salivary pellicles, whereas anti-type 2 antibodies do not (11; W. B. Clark, personal communication). Other *Actinomyces* strains (*A. viscosus* T14, *A. naeslundii* ATCC 12104, *A. odontolyticus* ATCC 17982, and *A. israelii* ATCC 12597) and *Streptococcus sanguis* C5 were obtained from the culture collection of the Forsyth Dental Center. Stock cultures were stored in 50% glycerol at −20°C until use. The organisms were propagated on Trypticase soy blood agar plates (BBL Microbiology Systems, Cockeysville, Md.) and in Trypticase soy broth (BBL). Cultures were incubated in Brewer jars filled with 80% N_2, 10% CO_2, and 10% H_2 at 35°C.

Early-stationary-phase cells were used in all adhesion assays. For adhesion studies, the organisms were radiolabeled by growing the cells in Trypticase soy broth supplemented with 10 μCi of [^{3}H]thymidine (New England Nuclear Corp., Boston, Mass.) per ml as previously described (18). The bacteria were harvested by centrifugation, washed three times, and suspended in buffered KCl (0.05 M KCl containing 1 mM KH_2PO_4, 1 mM $CaCl_2$, and 0.1 mM $MgCl_2$ at pH 6.0) at a concentration of 5×10^7 to 10×10^7 bacteria per ml.

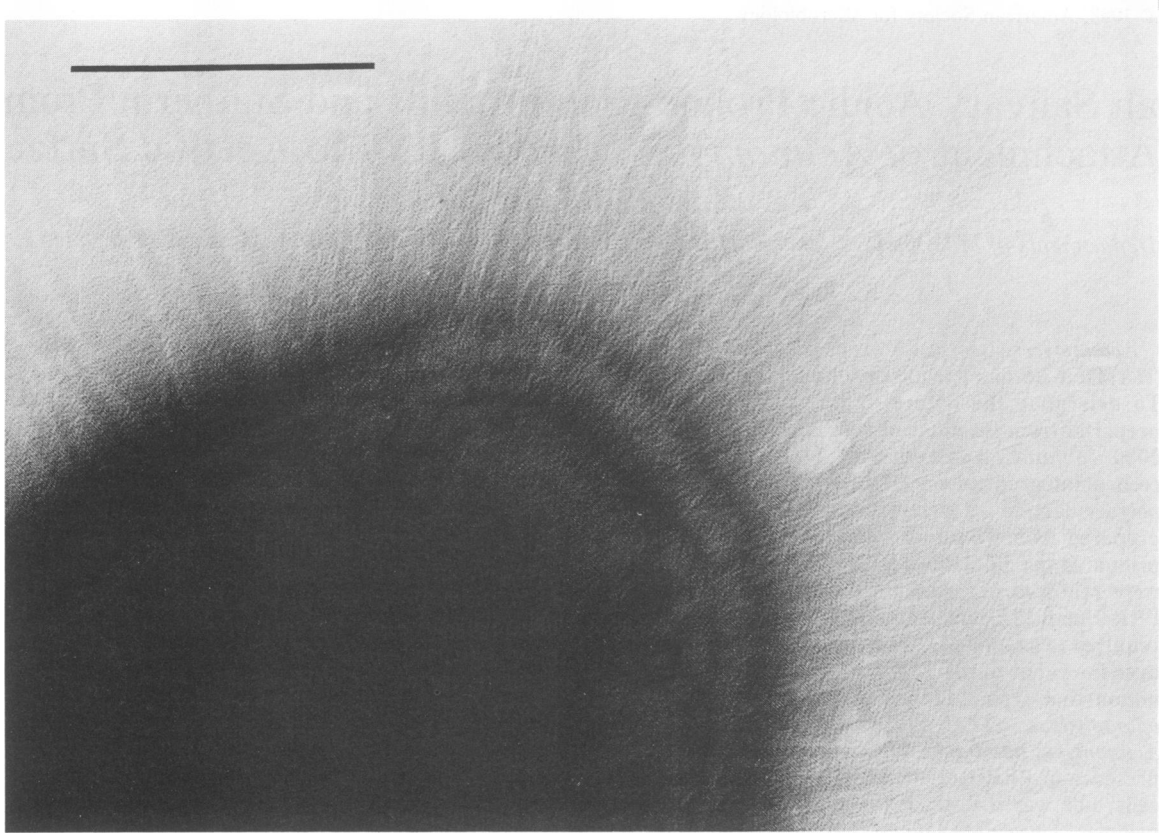

FIG. 1. Electron photomicrograph of a negatively stained cell of *A. viscosus* LY7, showing abundant fimbriae. Bar, 0.25 μm. Kindly provided by Z. Skobe at Forsyth Dental Center.

Collection and fractionation of saliva. Samples of submandibular saliva were obtained with a custom-made collecting device (35). Parotid saliva was obtained with a collecting device similar to that described by Lashley (28) as modified by Curby (12), made of silicone rubber. Acidic candy was used to stimulate salivary flow. Saliva samples were immediately dialyzed against 0.1 M NH_4HCO_3 (pH 8.0) buffer at 4°C overnight. This buffer and those used for the subsequent chromatographic steps contained 0.5% chloroform to inhibit microbial growth. The dialyzed saliva samples were fractionated on columns (5.31 cm^2 by 92 cm) of Trisacryl GF 2000 (LKB, Bromma, Sweden) at 4°C and eluted with the 0.1 M NH_4HCO_3 buffer at a flow rate of 22 ml/h; 11-ml fractions were collected and stored frozen until used.

To identify the specific salivary proteins responsible for promoting adhesion of *A. viscosus* LY7 cells to hydroxyapatite (HA), active fractions from the Trisacryl GF 2000 columns were pooled, diluted 1:1 with water to reduce the buffer concentration to 0.05 M NH_4HCO_3, and chromatographed on a column (2.0 cm^2 by 12 cm) of DEAE-agarose (Bio-Rad Laboratories, Richmond, Calif.) by using an ammonium bicarbonate gradient (0.05 to 0.5 M) at a flow rate of 14 ml/h and collecting 7-ml fractions. The proteins present in the fractions obtained were examined by polyacrylamide gel electrophoresis (PAGE) (13).

Preparation of experimental pellicles. Experimental pellicles were prepared by exposing 5-mg samples of spheroidal HA beads (BDH Chemicals, Gallard-Schlesinger Chemical Corp., Carle Place, N.Y.) to 125-μl saliva samples or each saliva fraction for 1 h (18). The beads were washed twice with buffered KCl and then treated for 30 min with 5 mg of bovine serum albumin (Sigma Chemical Corp., St. Louis, Mo.) per ml in buffered KCl to block any uncoated regions of the HA (19). The beads were then washed three times with buffered KCl and incubated with 6.25×10^6 to 12.5×10^6 [^{3}H]thymidine-labeled bacterial cells suspended in 125 μl of buffered KCl containing 5 mg of albumin per ml. The mixtures were incubated with continuous rotation at 6 rpm at room temperature for 1 h. The beads were then washed three times with buffered KCl, and the number of *Actinomyces* cells which attached to the beads was determined by scintillation counting as previously described (18, 19). Values were corrected for quench due to the HA beads. All assays were done in duplicate, and most experiments were performed at least twice.

Preparation of purified salivary proteins. Samples of the pure proline-rich proteins (PRPs) and statherin were prepared from human saliva as previously described (36). Their purity was assessed by PAGE, high-pressure liquid chromatography, and sequencing (39, 40, 43, 44).

Adsorption of [^{3}H]PRP-1 to *A. viscosus* LY7 cells. Samples of PRP-1 were radiolabeled with [^{3}H]formaldehyde by reductive methylation as previously described (26). The specific activity of typical preparations of the ^{3}H-labeled PRP-1 was approximately 1,150 cpm/μg. Reaction mixtures containing 2×10^9 *A. viscosus* cells (10 mg [wet weight]) were incubated with various quantities up to 100 μg of the ^{3}H-labeled protein for 2 h in buffered KCl. The mixtures were centrifuged, and the bacteria were washed three times to remove unbound protein and transferred to scintillation vials to count. To determine if the protein was degraded, samples of the supernatant liquor containing unbound [^{3}H]PRP-1 were chromatographed on a column of DEAE-agarose, and the labeled PRP-1 was eluted with an

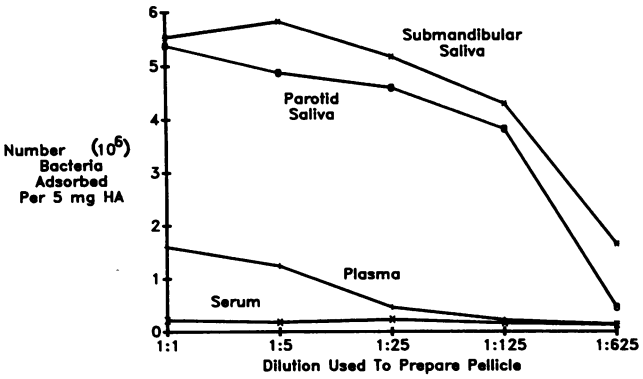

FIG. 2. Attachment of *A. viscosus* LY7 cells to experimental pellicles formed from human parotid and submandibular saliva and from serum and plasma on HA surfaces.

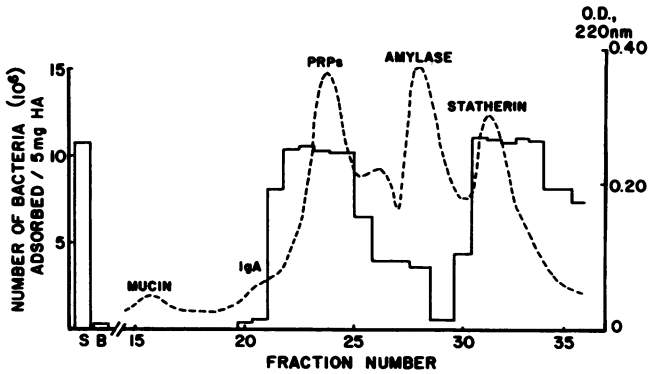

FIG. 3. Attachment of *A. viscosus* LY7 cells to HA treated with submandibular salivary fractions obtained by chromatography on columns of Trisacryl GF 2000. The A_{220} values of fractions (– – – –) and the number of *A. viscosus* LY7 cells which attached (——) are indicated. The region in which mucin, immunoglobulin A (IgA), PRPs, amylase, and statherin elute is indicated. Controls of HA treated with unfractionated saliva (S) or buffer (B) are indicated by bars. O.D., Optical density.

NH_4HCO_3 gradient. Controls of [^{3}H]PRP-1 incubated in the absence of bacteria were included for comparison.

Preparation of the amino-terminal tryptic peptide of PRP-1. PRP-1 (8.5 mg) and trypsin (0.17 mg) (TRTCPK trypsin; Worthington Diagnostics, Freehold, N.J.) were dissolved in 3.5 ml of 0.025 M (pH 8) Tris chloride which contained 1 mM $CaCl_2$ and incubated at 37°C for 2 h. The digest was chromatographed on a DEAE-agarose column with an ammonium bicarbonate gradient (0.05 to 0.5 M). The fractions containing unretarded material (trypsin and neutral and basic PRP-1 tryptic peptides) were readily separated from the amino-terminal tryptic peptide, residues 1 to 30, designated PRP-1(T1). This peptide is highly acidic and is considerably retarded on the DEAE-agarose column. Fractions containing this peptide were lyophilized to give an essentially pure product, the composition of which was confirmed by amino acid analysis.

RESULTS

Adsorption of *A. viscosus* LY7 to experimental pellicles. *A. viscosus* adsorbed in high numbers to experimental pellicles prepared from either human parotid or submandibular saliva (Fig. 2). The saliva could be diluted more than 25-fold without greatly diminishing its adsorption-promoting activity. The organism attached poorly to HA treated with samples of human plasma or serum obtained from the same individual who provided the saliva samples.

To determine the nature of the salivary components responsible for promoting adhesion of the organism, experimental pellicles were prepared from submandibular salivary fractions obtained from Trisacryl GF 2000 columns. Adsorption of *A. viscosus* LY7 cells to HA was promoted by two groups of fractions (Fig. 3). Electrophoresis showed that one group contained the acidic PRPs, among other proteins, whereas the second group contained statherin. Similar results were obtained when parotid saliva was fractionated on Trisacryl GF 2000 columns (data not shown). The two groups of fractions containing adsorption-promoting activity were pooled and rechromatographed on DEAE-agarose columns as described above. Fractions which promoted adsorption of LY7 cells were examined by PAGE and found to contain the PRPs and statherin (Fig. 4).

Adsorption of *A. viscosus* LY7 to pellicles prepared from pure salivary proteins. A family of acidic PRPs has been identified in human (6, 36) and other primate (37) salivas, and their structural relationships have been established (2, 39, 43, 44). Three members of this family have been designated PIF-slow (for parotid isoelectric focusing variant; 2), PRP-1, and PRP-2; all are 150-amino-acid-residue proteins which

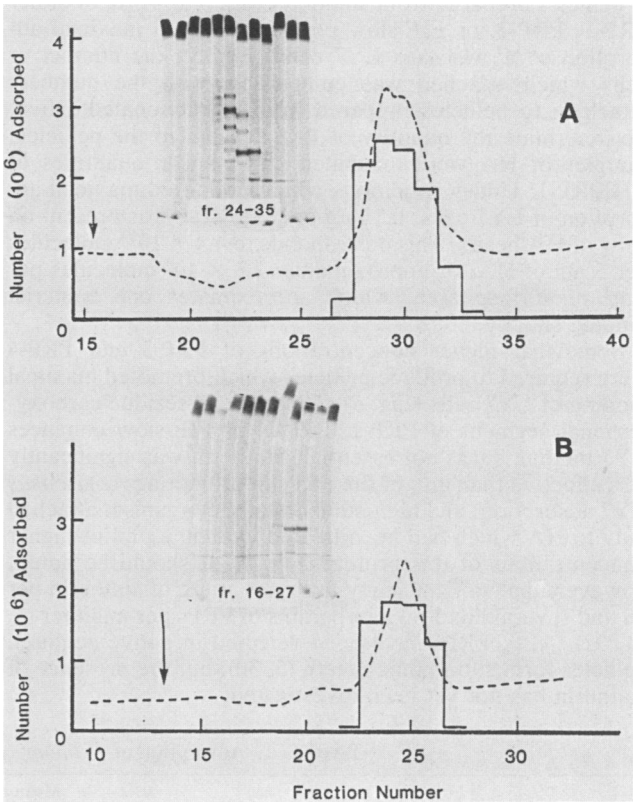

FIG. 4. Attachment of *A. viscosus* LY7 cells to HA treated with subfractions of submandibular saliva chromatographed on DEAE-agarose columns. The A_{220} values of fractions (– – – – –) and the number of *A. viscosus* LY7 cells which attached (——) are indicated. PAGE indicates the presence of PRPs in fractions which promote bacterial attachment (A) and the presence of statherin in fractions which promote bacterial attachment (B). fr., Fractions beyond the arrows were assayed.

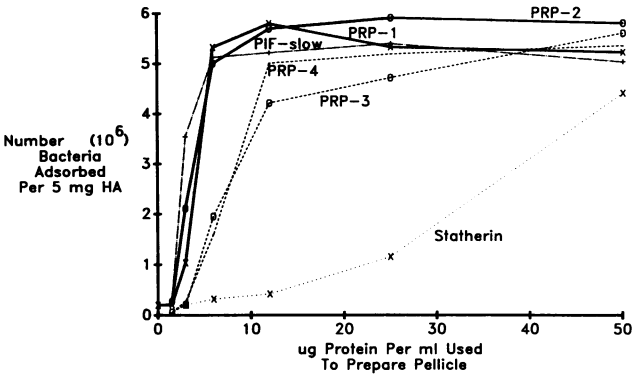

FIG. 5. Attachment of *A. viscosus* LY7 cells to experimental pellicles formed on HA beads from pure salivary PRP-1, PRP-2, PRP-3, PRP-4, PIF-slow, and statherin.

differ in that residues 4 and 50 are asparagine and aspartate (PIF-slow), aspartate and asparagine (PRP-1), or both aspartate (PRP-2). Three other PRPs (PIF-fast, PRP-3, and PRP-4) are 106-amino-acid-residue proteins which are identical to the first 106 residues of the larger proteins from which they are considered to be derived by posttranslational proteolysis (31). The 44-residue protein anticipated from this cleavage, corresponding to residues 107 to 150 of the parent molecules, has been identified in human parotid saliva (25).

Pellicles prepared from solutions with 6 to 12 μg of either PRP-1, PRP-2, or PIF-slow per ml promoted maximal adsorption of *A. viscosus* LY7 cells (Fig. 5); the number of cells which attached was comparable with the numbers attaching to pellicles prepared from unfractionated saliva. To determine the quantity of PRP present in the pellicles, samples of HA were incubated with similar quantities of [^{3}H]PRP-1. Under conditions which promoted maximal adsorption of LY7 cells, 0.12 μg of [^{3}H]PRP-1 was present on 5 mg of HA beads. This corresponds to 4.4×10^{12} molecules per 5 mg of HA, or approximately 1.3×10^4 molecules per μm^2 of surface area, which approximates one bacterial binding site.

Somewhat higher concentrations of PRP-3 and PRP-4 were required to produce pellicles which promoted maximal binding of LY7 cells (Fig. 5). Thus, the 44-residue carboxy-terminal segment of PRP-1, PRP-2, or PIF-slow enhances LY7 binding but is not essential. Statherin was significantly less effective than any of the PRPs in promoting *A. viscosus* LY7 adsorption, and high numbers of the organism attached only to HA which had been treated with 50 μg/ml or higher concentrations of this protein (Fig. 5). It should be noted, however, that saliva usually averages 70 μg of statherin per ml and several hundred micrograms of PRPs per milliliter (3, 24, 32). Also, PRPs have been detected in native acquired pellicles formed on human teeth (5, 30), but the presence of statherin has not yet been investigated.

Adsorption of LY7 cells to pellicles prepared from PRP-1 was not affected by pH over the range of pH 5.0 to 8.0. Adsorption of the organism was also not inhibited by the presence of 50 mM concentrations of each of the 19 amino acids forming PRP-1 (data not shown). Similarly, adsorption of LY7 cells to pellicles prepared from either unfractionated submandibular saliva or pure PRP-1 was not inhibited by 50 mM lactose. This is consistent with the notion that type 1 fimbriae, rather than type 2 fimbriae, are responsible for adhesion of the organisms to salivary pellicles. Cells of *A. naeslundii* ATCC 12104, which possess only type 2 fimbriae (11), did not adsorb effectively to PRP-1 pellicles (Table 1). However, cells of *A. viscosus* T14, *A. odontolyticus* ATCC 17982 and *A. israelii* ATCC 12597 adsorbed almost as well to pellicles prepared from purified PRP-1 as they did to pellicles prepared from unfractionated submandibular saliva (Table 1). Thus, several *Actinomyces* species possess adhesins which recognize adsorbed PRPs.

Influence of PRP-1 in solution on adsorption of *A. viscosus* LY7 cells to PRP-1 pellicles. Since *A. viscosus* cells bind strongly to PRPs adsorbed onto HA, it was reasonable to expect that PRP molecules in solution would bind to LY7 cells and inhibit their attachment to PRP pellicles. Such inhibition would constitute a test of the hypothesis that the PRPs serve as receptors in pellicles formed from human glandular saliva. However, the presence of PRP-1 or PRP-3 in solution, even at concentrations of 1,000 μg/ml, did not significantly inhibit adhesion of *A. viscosus* LY7 cells to pellicles prepared from either purified PRP-1 or from unfractionated submandibular saliva (Table 2). Similarly, the presence of statherin (100 μg/ml) or of unfractionated submandibular saliva also did not inhibit attachment of the organism. Thus, although adsorbed PRP-1 molecules interact strongly with LY7 cells, PRP molecules in solution do not appear to do so.

The apparent inability of PRP molecules in solution to bind with high affinity to LY7 cells was substantiated with [^{3}H]PRP-1. When 100 μg of pure [^{3}H]PRP-1 was incubated with 2×10^9 *A. viscosus* LY7 cells, less than 0.05 μg of the [^{3}H]PRP-1 became associated with the bacteria. This was approximately the same quantity of [^{3}H]PRP-1 that became associated with cells of *A. naeslundii* ATCC 12104, which do not attach well to PRP-1 pellicles. The small amount of [^{3}H]PRP-1 associated with these organisms evidently represented nonspecific binding.

It was possible that PRP in solution was degraded when incubated with LY7 cells. To investigate this, samples of the [^{3}H]PRP-1 were rechromatographed on DEAE-agarose columns after incubation with LY7 cells. In control preparations of [^{3}H]PRP-1 not incubated with bacteria, 98.0% of the added ^{3}H counts were recovered in the region where intact PRP-1 migrates. Likewise, 97.7% of the ^{3}H counts were recovered in this region from samples of the protein which had been incubated with LY7 cells. In contrast, samples of

TABLE 1. Adsorption of *Actinomyces* strains to experimental pellicles on HA

Pellicle source	Mean no. ± SE (10^6) of bacteria adsorbed/5 mg of HA				
	A. viscosus LY7	*A. viscosus* T14	*A. odontolyticus* ATCC 17982	*A. israelii* ATCC 12597	*A. naeslundii* ATCC 12104
Buffer	0.07 ± 0.01	0.68 ± 0.05	0.06 ± 0.01	0.19 ± 0.01	0.16 ± 0.01
Submandibular saliva	4.35 ± 0.05	3.07 ± 0.38	3.27 ± 0.72	4.18 ± 0.69	2.65 ± 0.05
Parotid saliva	5.65 ± 0.12	3.35 ± 0.24	2.91 ± 0.09	4.33 ± 0.15	ND[a]
PRP-1 (20 μg/ml)	4.67 ± 0.22	4.25 ± 0.28	2.84 ± 0.22	3.87 ± 0.05	0.29 ± 0.11

[a] ND, Not determined.

TABLE 2. Effect of PRPs, statherin, and submandibular saliva on adsorption of *A. viscosus* LY7 to experimental pellicles

Pellicle source and additive (concn [μg/ml]) to suspending fluid	Mean no. ± SE (10^6) of bacteria adsorbed/ 5 mg of HA	Relative percent
Buffer (none)	0.08 ± 0.01	
Submandibular saliva		
None	5.90 ± 0.21	100
PRP-1 (1,000)	5.47 ± 0.17	93
PRP-1 (100)	5.77 ± 0.25	98
PRP-3 (1,000)	5.37 ± 0.23	91
PRP-3 (100)	4.18 ± 0.67	71
Statherin (100)	5.52 ± 0.15	94
Submandibular saliva (50%)	5.74 ± 0.27	97
PRP-1 (20)		
None	5.01 ± 0.25	100
PRP-1 (1,000)	5.37 ± 0.03	107
PRP-1 (100)	4.89 ± 0.01	98
PRP-3 (1,000)	4.75 ± 0.22	95
PRP-3 (100)	4.60 ± 0.02	92
Statherin (100)	4.88 ± 0.31	97
Submandibular saliva (50%)	5.77 ± 0.09	115

[^{3}H]PRP-1 incubated with *S. sanguis* C5 cells under comparable conditions were extensively degraded, and only 38% of the added counts per minute were recovered in the PRP-1 fractions. Thus, the inability of [^{3}H]PRP-1 in solution to bind to LY7 cells, or the failure of PRPs in solution to inhibit adhesion, cannot be explained on the basis of degradation of the protein.

It was possible that the radiolabeling procedure may have altered PRP-1 molecules and rendered them unreactive with LY7 cells. To test this possibility, we compared the ability of [^{14}C]thymidine-labeled LY7 cells to attach to pellicles formed from unlabeled and from [^{3}H]labeled PRP-1. However, pellicles prepared from [^{3}H]PRP-1 appeared to be as effective as those prepared from unlabeled PRP-1 in promoting adhesion of the organism (Table 3).

Adsorption of *A. viscosus* LY7 to pellicles prepared from amino-terminal tryptic peptide of PRP-1. The acidic amino-terminal peptide produced by tryptic cleavage of PRP-1 at residue 30, designated PRP-1(T1), is known to be mainly responsible for the high affinity of PRP molecules for HA surfaces (3, 4). However, pellicles prepared by exposing HA beads to up to 1,000 μg of this peptide per ml did not promote *A. viscosus* adsorption (Table 3). Thus, the organism evidently interacts with peptide domains located elsewhere in adsorbed PRP molecules.

DISCUSSION

We have previously proposed (22) that the PRPs play a key role in a protective and reparative system which is important for the integrity of the teeth. The central feature of this system is that human saliva is considerably supersaturated with respect to the calcium phosphate salts which form the dental mineral (23), a state which provides a degree of protection for teeth against demineralization and also provides the potential for repair of demineralized enamel. An untoward consequence of this otherwise advantageous condition would be continual surface-induced deposition of mineral on tooth surfaces. The PRPs, however, are selectively adsorbed from saliva by HA (20), exhibit high affinity for apatitic minerals, and in the adsorbed state, are potent inhibitors of surface-induced mineral deposition (1, 34). This inhibitory activity has been proposed as an important biological function of the acidic PRPs. To fulfill this function, the PRPs would be expected to form part of the acquired pellicle which serves as the outer coating of the dental enamel, and this prediction has been confirmed (5, 30). The present study has now shown that these proteins also serve as a novel class of receptors which promote the adhesion of *A. viscosus* cells to salivary pellicles formed on HA surfaces. These findings provide a molecular explanation for the tropism which *A. viscosus* displays for teeth.

Previous studies have demonstrated that the adhesion of *A. viscosus* cells to experimental pellicles is dependent upon the presence of type 1 fimbriae (8–11). Thus, fimbriae-deficient mutants do not attach well to pellicles, and treatment of *A. viscosus* cells with immunoglobulin G antibodies or Fab fragments against type 1 fimbriae inhibits adhesion. It seems likely that the type 1 fimbriae of *A. viscosus* LY7 cells also function as the adhesins involved in binding to PRP or statherin molecules on HA surfaces. Adhesion of this strain to experimental pellicles is inhibited by anti-type 1, but not by anti-type 2, Fab antibody fragments (Clark, personal communication). In addition, adhesion of the organism to pellicles prepared from submandibular saliva or purified PRPs was not inhibited by lactose. This indicates that type 2 fimbriae were not involved, since they are galactosyl-binding lectins (8). Also, PRP molecules and statherin are not glycosylated (4, 21), and therefore, they would not be expected to interact with galactosyl-binding type 2 fimbriae. In fact, *A. naeslundii* ATCC 12104, which possesses only type 2 fimbriae (9–11), failed to bind effectively to PRP pellicles.

One of the remarkable findings in the present study was that although PRP molecules adsorbed on HA surfaces interact strongly with *A. viscosus* cells, the same proteins in solution do not appear to bind to cells of the organism, nor do they affect its attachment to pellicles. A possible explanation for this unexpected behavior is that hidden molecular segments of PRP became exposed as a result of conformational changes in the protein when it adsorbed to HA which could react with the adhesins of LY7 cells.

That a major conformational change does occur when PRP molecules adsorb to HA surfaces has been previously suggested by studies of their calcium ion-binding properties and

TABLE 3. Adsorption of *A. viscosus* LY7 to pellicles prepared from PRP-1, [^{3}H]PRP-1, and the acidic amino-terminal peptide derived from PRP-1

Pellicle source (concn [μg/ml])	Mean no. ± SE (10^6) of bacteria adsorbed/ 5 mg of HA	Relative percent
Buffer	0.04 ± 0.01[a]	1
Submandibular saliva	4.27 ± 0.19[a]	100
PRP-1 (100)	4.26 ± 0.26[a]	100
PRP-1 (10)	3.49 ± 0.04[a]	82
[^{3}H]PRP-1 (100)	4.17 ± 0.09[a]	98
[^{3}H]PRP-1 (10)	2.96 ± 0.03[a]	69
PRP-1(T1)[b] (1,000)	0.03 ± 0.01	1
PRP-1(T1) (100)	0.05 ± 0.01	1

[a] Bacteria labeled with [^{14}C]thymidine.
[b] Amino-terminal tryptic peptide, residues 1 to 30, from PRP-1.

by the thermodynamics of their adsorption onto HA surfaces. PRP molecules are characterized by a high degree of charge and structural asymmetry. The 30-residue highly negatively charged amino-terminal segment of PRP-1 contains 2 phosphoserine residues, in addition to 13 of the 15 negatively charged residues present in the molecule, and this segment has been shown to mediate adsorption of the protein to HA (3, 4). It is significant that this 30-residue amino-terminal segment does not promote adsorption of LY7 cells to HA surfaces, and therefore it is clear that *A. viscosus* cells recognize peptide domains located elsewhere in the molecule.

The composition of this 30-residue segment is remarkably different from the carboxy-terminal segment (residues 31 to 150), which is rich in proline, glycine, and glutamine, and contains 10 of the 11 positive charges present in PRP-1. Examination of the structures of the PRPs suggests that, in their native configuration, ionic interactions are likely to occur between these negatively and positively charged segments. Such a configuration is supported by studies of the calcium-binding properties of the molecule. Bennick and co-workers (7) showed that the amino-terminal peptide obtained by tryptic cleavage at residue 30 binds more calcium than does the native protein. This strongly suggests that blocking of acidic calcium-binding sites occurs in the native protein by intermolecular interactions. The breaking of these ion-pair bonds with a resulting conformational change would be expected to occur when the acidic amino-terminal segment binds to HA.

Strong support for a conformational change is also derived from thermodynamic studies. Moreno and co-workers (33) have shown that the adsorption of PRPs to HA is an endothermic process driven by an increase in entropy. Several effects are responsible for this increase, including a significant contribution attributable to changes in molecular conformation as the protein adsorbs onto the HA surfaces.

Collectively, the data obtained indicate that the acidic PRPs and statherin function as the major salivary receptors which promote the adhesion of *A. viscosus* cells to apatitic surfaces similar to those of teeth. Since these proteins appear to be unique to saliva, their presence in the adsorbed state on tooth surfaces explains why *A. viscosus* cells appear to require the presence of teeth for their establishment in the oral cavity. The PRPs and statherin are carbohydrate-free proteins (4, 21), and their apparent activity as receptors for bacterial attachment appears to be the first clear example of a pure protein serving in this capacity on an oral surface.

An important principle emerges from these studies which seems likely to prove applicable to other host-parasite interactions. The PRPs form up to 40% of the protein in saliva (3, 21), and if *A. viscosus* cells could interact with these molecules in solution, there would be little likelihood that many organisms would be able to attach to the pellicle coating on tooth surfaces. However, the apparent ability of *A. viscosus* cells to recognize molecular segments of PRPs which are exposed only when the molecules are adsorbed to mineral surfaces provides a novel and highly efficient mechanism for the organism to attach selectively to teeth. This enables *A. viscosus* cells to evade the cleansing functions ascribed to salivary secretions; indeed, addition of saliva to the bacterial suspending fluid did not affect attachment (Table 2). It is easy to envision that there would be a strong selection pressure favoring the evolution of such a mechanism in bacteria which regularly colonize tissue surfaces. In view of this, we anticipate that other examples of bacteria which recognize determinants which are exposed in surface-associated molecules but which are hidden in similar molecules in solution will soon be discovered.

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LITERATURE CITED

1. **Aoba, T., E. C. Moreno, and D. I. Hay.** 1984. Inhibition of apatite crystal growth by the amino-terminal segment of human salivary acidic proline-rich proteins. Calcif. Tissue Int. **36**:651–658.
2. **Azen, E. A., and C. Denniston.** 1981. Genetic polymorphism of PIF (parotid isoelectric focusing variant) proteins with linkage to the PPP (parotid proline-rich protein) gene complex. Biochem. Genet. **19**:475–485.
3. **Bennick, A.** 1987. Structural and genetic aspects of proline-rich proteins. J. Dent. Res. **66**:457–461.
4. **Bennick, A., M. Cannon, and G. Madapallimattam.** 1979. The nature of the hydroxyapatite-binding site in salivary acidic proline-rich proteins. Biochem. J. **183**:115–126.
5. **Bennick, A., G. Chau, R. Goodlin, S. Abrams, D. Tustian, and G. Madapallimattam.** 1983. The role of human salivary acidic proline-rich proteins in the formation of acquired dental pellicle *in vivo* and their fate after adsorption to the human enamel surface. Arch. Oral Biol. **28**:19–27.
6. **Bennick, A., and G. E. Connell.** 1971. Purification and partial characterization of four proteins from human parotid saliva. Biochem. J. **123**:455–464.
7. **Bennick, A., A. C. McLaughlin, A. A. Grey, and G. Madapallimattam.** 1981. The location and nature of calcium-binding sites in salivary acidic proline-rich phosphoproteins. J. Biol. Chem. **256**:4741–4746.
8. **Cisar, J. O., A. L. Sandberg, and S. E. Mergenhagen.** 1984. The function and distribution of different fimbriae on strains of *Actinomyces viscosus* and *Actinomyces naeslundii*. J. Dent. Res. **63**:393–396.
9. **Clark, W. B.** 1985. *Actinomyces* fimbriae and adherence to hydroxyapatite, p. 103–108. *In* S. E. Mergenhagen and B. Rosan (ed.), Molecular basis for oral microbial adhesion. American Society for Microbiology, Washington, D.C.
10. **Clark, W. B., T. T. Wheeler, and J. O. Cisar.** 1984. Specific inhibition of adsorption of *Actinomyces viscosus* T14V to saliva-treated hydroxyapatite by antibody against type 1 fimbriae. Infect. Immun. **43**:497–501.
11. **Clark, W. B., T. T. Wheeler, D. D. Lane, and J. O. Cisar.** 1986. *Actinomyces* adsorption mediated by type-1 fimbriae. J. Dent. Res. **65**:1166–1168.
12. **Curby, W. A.** 1953. Device for collection of human parotid saliva. J. Lab. Clin. Med. **41**:493–496.
13. **Davis, B. J.** 1964. Disc electrophoresis. II. Method and application to human serum proteins. Ann. N.Y. Acad. Sci. **121**:404–427.
14. **Ellen, R. P.** 1976. Establishment and distribution of *Actinomyces viscosus* and *Actinomyces naeslundii* in the human oral cavity. Infect. Immun. **14**:1119–1124.
15. **Ellen, R. P., D. W. Banting, and E. D. Fillery.** 1985. Longitudinal microbiological investigation of a hospitalized population of older adults with a high root surface caries risk. J. Dent. Res. **64**:1377–1381.
16. **Ellen, R. P., E. D. Fillery, K. H. Chan, and D. A. Grove.** 1980. Sialidase-enhanced lectin-like mechanism for *Actinomyces viscosus* and *Actinomyces naeslundii* hemagglutination. Infect. Immun. **27**:335–343.
17. **Ellen, R. P., D. N. Segel, and D. A. Grove.** 1978. Relative proportions of *Actinomyces viscosus* and *Actinomyces naeslundii* in dental plaques collected from single sites. J. Dent. Res. **57**:550.
18. **Gibbons, R. J., and I. Etherden.** 1982. Enzymatic modification

of bacterial receptors on saliva-treated hydroxyapatite surfaces. Infect. Immun. **36**:52–58.
19. **Gibbons, R. J., and I. Etherden.** 1985. Albumin as a blocking agent in studies of streptococcal adsorption to experimental salivary pellicles. Infect. Immun. **50**:592–594.
20. **Hay, D. I.** 1973. The interaction of human parotid salivary proteins with hydroxyapatite. Arch. Oral Biol. **20**:1517–1529.
21. **Hay, D. I.** 1983. Human glandular salivary proteins, p. 319–335. *In* E. P. Lazzari (ed.), CRC handbook of experimental aspects of oral biochemistry. CRC Press, Inc., Boca Raton, Fla.
22. **Hay, D. I., E. C. Moreno, and D. H. Schlesinger.** 1979. Phosphoprotein inhibitors of calcium phosphate precipitation from salivary secretions. Inorg. Perspect. Biol. Med. **2**:271–285.
23. **Hay, D. I., S. K. Schluckebier, and E. C. Moreno.** 1982. Equilibrium dialysis and ultrafiltration studies of calcium and phosphate binding by human salivary proteins. Implications for salivary supersaturation with respect to calcium phosphate salts. Calcif. Tiss. Int. **34**:531–538.
24. **Hay, D. I., D. J. Smith, S. K. Schluckebier, and E. C. Moreno.** 1984. Relationship between concentration of human salivary statherin and inhibition of calcium phosphate precipitation in stimulated parotid saliva. J. Dent. Res. **63**:857–863.
25. **Isemura, S., E. Saitoh, and K. Sanada.** 1980. The amino-acid sequence of a salivary proline-rich peptide, P-C, and its relation to a salivary proline-rich phosphoprotein, protein C. J. Biochem. **87**:1071–1077.
26. **Jentoft, N., and D. G. Dearborn.** 1979. Labeling of proteins by reductive methylation using sodium cyanoborohydride. J. Biol. Chem. **254**:4359–4365.
27. **Jordan, H. V., and P. H. Keyes.** 1964. Aerobic gram-positive filamentous bacteria as etiologic agents of experimental periodontal disease in hamsters. Arch. Oral Biol. **9**:401–414.
28. **Lashley, K. S.** 1916. Reflex stimulation of the human parotid gland. J. Exp. Psychol. **1**:461–492.
29. **Loesche, W. J., and S. A. Syed.** 1978. Bacteriology of human experimental gingivitis: effect of plaque and gingivitis score. Infect. Immun. **21**:830–839.
30. **Kousvelari, E. E., R. S. Baratz, B. Burke, and F. G. Oppenheim.** 1980. Immunochemical identification and determination of proline-rich proteins in salivary secretions, enamel pellicle and glandular tissue specimens. J. Dent. Res. **59**:1430–1438.
31. **Maeda, N., H. S. Kim, E. A. Azen, and O. Smithies.** 1985. Differential RNA splicing and post-translational cleavages in the human salivary proline-rich protein gene system. J. Biol. Chem. **260**:11123–11130.
32. **Mandel, L. D., and A. Bennick.** 1983. Quantitation of human salivary acidic proline-rich proteins in oral diseases. J. Dent. Res. **62**:943–945.
33. **Moreno, E. C., M. Kresak, and D. I. Hay.** 1982. Adsorption thermodynamics of acidic proline-rich salivary proteins onto calcium apatites. J. Biol. Chem. **257**:2981–2989.
34. **Moreno, E. C., K. Varughese, and D. I. Hay.** 1979. Effect of human salivary proteins on the precipitation kinetics of calcium phosphate. Calcif. Tiss. Int. **28**:7–16.
35. **Oppenheim, F. G.** 1970. Preliminary observations on the presence and origin of serum albumin in human saliva. Helv. Odontol. Acta **14**:10–17.
36. **Oppenheim, F. G., D. I. Hay, and C. Franzblau.** 1971. Proline-rich proteins from human parotid saliva. Isolation and partial characterization. Biochemistry **10**:4233–4238.
37. **Oppenheim, F. G., G. D. Offner, and R. F. Troxler.** 1985. Amino acid sequence of a proline-rich phosphoglycoprotein from parotid secretion of the subhuman primate *Macaca fascicularis*. J. Biol. Chem. **260**:10671–10679.
38. **Qureshi, J. V., and R. J. Gibbons.** 1981. Differences in the adsorptive behavior of human strains of *Actinomyces viscosus* and *Actinomyces naeslundii* to saliva-treated hydroxyapatite surfaces. Infect. Immun. **31**:261–266.
39. **Schlesinger, D. H., and D. I. Hay.** 1979. Complete primary structure of a proline-rich phosphoprotein (PRP-4), a potent inhibitor of calcium phosphate precipitation in human parotid saliva, p. 133–135. *In* G. Gross and J. Meienhofer (ed.), Peptides, structure and biological function. Proceedings of the 6th American Peptide Symposium. Pierce Chemical Co., Rockford, Ill.
40. **Schlesinger, D. H., and D. I. Hay.** 1986. Complete covalent structure of a proline-rich phosphoprotein (PRP-2), an inhibitor of calcium phosphate crystal growth from human parotid saliva. Int. J. Peptide Protein Res. **27**:373–379.
41. **Summey, D. L., and H. V. Jordan.** 1974. Characterization of bacteria isolated from human root surface carious lesions. J. Dent. Res. **53**:343–351.
42. **Syed, S. A., and W. J. Loesche.** 1978. Bacteriology of human experimental gingivitis: effect of plaque age. Infect. Immun. **21**:821–829.
43. **Wong, R. S., and A. Bennick.** 1980. The primary structure of a salivary calcium-binding proline-rich phosphoprotein (protein C), a possible precursor of a related salivary protein A. J. Biol. Chem. **255**:5943–5948.
44. **Wong, R. S., T. Hofmann, and A. Bennick.** 1979. The complete primary structure of a proline-rich phosphoprotein from human saliva. J. Biol. Chem. **254**:4800–4808.