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Effect of Adsorbed Protein on Hydroxyapatite Zeta Potential and *Streptococcus mutans* Adherence

ERIC C. REYNOLDS* AND ALBERT WONG

Department of Conservative Dentistry, University of Melbourne, Melbourne 3000, Australia

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The adherence of *Streptococcus mutans* PK1 to hydroxyapatite disks pretreated with various acidic and basic proteins in imidazole buffer was studied. Adsorption of a basic protein onto an hydroxyapatite disk enhanced or had no effect on bacterial adherence, whereas adsorption of an acidic protein reduced adherence. The effect of adsorbed protein on bacterial adherence was of both short and long range. The long-range effect of the acidic proteins in reducing the number of bacteria adhering to hydroxyapatite was related to protein adsorption causing an increase in surface net negative charge, as shown by zeta potential measurement. Basic protein produced a net positive surface charge which facilitated adherence. Within the acidic protein group, the acidic residue percentage of the adsorbed protein was negatively correlated with the number of bacteria adhering, whereas the nonpolar residue percentage was positively correlated with bacterial adherence. Within the basic protein group, the basic residue percentage was correlated with the number of cells adhering. These results indicate the involvement of short-range hydrophobic and ionic interactions in bacterial adherence to protein-coated hydroxyapatite.

Forces involved in the adherence of microorganisms to oral surfaces are of short range, that is, they involve the interaction of molecules on the oral surface with those on the bacterial cell surface. For these interactions to occur, the bacterium must come in close contact with the oral surface. However, the approach of the bacterium will be influenced by long-range interactions, such as electrostatic attraction or repulsion of electrical double layers determined by surface charges. Salivary and dietary proteins adsorbed onto tooth surfaces would be involved in both short- and long-range interactions. Saliva contains both acidic and basic proteins, with isoelectric points ranging from 2 to 11 (7) and capable of adsorbing onto hydroxyapatite (HA) (3). It is possible, therefore, that adsorption of these proteins would produce net negative- and positive-charged tooth surfaces, respectively. The adsorption of a basic protein could enhance the approach of a negatively charged bacterial cell (12), whereas adsorption of an acidic protein could restrict the approach of the bacterium to the surface.

Adsorbed protein could also provide specific binding sites for oral bacteria by the nature of the amino acid groups exposed at the surface. Information on the effect of adsorbed protein (of known chemical composition) on both short- and long-range interactions between oral bacteria and HA is limited. Simonson and Reiher (16) have shown that adsorbed phosvitin (phosphoprotein) and mucin (glycoprotein) inhibited the adherence of *Streptococcus mutans* onto HA and concluded that these proteins competed with the bacteria for HA-binding sites. It is also possible that these compounds inhibited the approach of the bacteria to the HA surface by increasing the electrostatic repulsion between electrical double layers. Rolla (14) has shown that the basic protein group protamine enhanced *S. mutans* adherence, whereas bovine serum albumin (an acidic protein) inhibited adherence. This, he concluded, was due to electrostatic attraction and repulsion, respectively.

In these works, the electrochemical effect of the adsorbed protein on HA was not established, nor were enough proteins of different compositions studied to allow correlation between amino acid composition of the adsorbed protein and the number of bacteria adhering. Therefore, in an effort to determine the influence of adsorbed protein on long- and short-range interactions between HA and bacteria, the effect of a variety of adsorbed proteins and polypeptides of different compositions on the adherence of *S. mutans* was studied. Also, the electrochemical effect of adsorbed proteins and polypeptides on HA particles was determined by measuring zeta potential, and this was correlated with the adherence of *S. mutans* onto HA disks pretreated with each respective protein or polypeptide.

TABLE 1. Composition of various proteins and polypeptides

No.	Compound	Type	Acidic residues[a]	Basic residues[b]	Nonpolar residues[c]	Neutral polar residues[d]	Reference
1	Poly-L-glutamate	Acidic polypeptide	100				
2	Phosvitin	Acidic phosphoprotein	60.5	15.4	12.5	11.6	17
3	α_{s1}-Casein	Acidic phosphoprotein	20.1	11.3	43.5	25.1	6
4	β-Casein	Acidic phosphoprotein	12.4	8.4	53.1	26.1	6
5	κ-Casein	Acidic phosphoprotein	10.6	9.8	45.3	34.3	6
6	β-Lactoglobulin	Acidic protein	17.9	11.8	46.3	24.0	6
7	α-Lactalbumin	Acidic protein	13.8	11.8	35.0	39.4	6
8	Bovine serum albumin	Acidic protein	17.3	15.7	39.7	27.3	13
9	Histone H1	Basic protein	5.4	33.0	51.9	9.7	11
10	Histone H3	Basic protein	9.6	23.7	45.9	20.8	4
11	Poly-L-lysine	Basic polypeptide		100			

[a] Number of acidic residues per 100 residues (Glu + Asp + SerP).
[b] Number of basic residues per 100 residues (Arg + Lys + His).
[c] Number of nonpolar residues per 100 residues (Gly + Ala + Val + Leu + Ile + Met + Phe + Pro).
[d] Number of neutral polar residues per 100 residues (Asn + Gln + Cys + Ser + Thr + Trp + Tyr).

MATERIALS AND METHODS

Materials. Double-distilled, deionized water was used throughout (>10 MΩ/cm). All chemicals were of analytical reagent grade. The milk proteins α_{s1}-casein, β-casein, and κ-casein were isolated by selective precipitation as described by Zittle and Custer (18). The other proteins and polypeptides were obtained from Sigma Chemical Co., St. Louis, Mo. The compositions of the various proteins and polypeptides used are shown in Table 1. HA Bio-gel HTP was purchased from Bio-Rad Laboratories, Richmond, Calif. Dextran T70 was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden.

Organism. *S. mutans* PK1 (8) was maintained by weekly transfer on agar plates containing 7% defibrinated horse blood (base no. 2; Oxoid, Melbourne, Australia) incubated anaerobically at 37°C for 48 h and then stored at 4°C.

Preparation of cell concentrate. A radioactively labeled cell concentrate was prepared for each experiment by transferring several colonies from an agar plate into Todd-Hewitt broth (Oxoid) containing 5 μCi of [*methyl*-^{3}H]thymidine per ml (25 Ci/mmol; Amersham International Ltd.). Growth of the culture was monitored by measuring turbidity at 650 nm with a Perkin-Elmer spectrophotometer 295E. An exponentially growing culture was centrifuged at 1,000 × *g* for 10 min, and the cell pellet was washed with 0.05 M imidazole buffer (pH 7.0) containing 0.025 M NaCl and then resuspended in the same buffer to give between 5 × 10^8 and 20 × 10^8 cells per ml. Cell enumeration was achieved by diluting the concentrate with 10 mM phosphate buffer (pH 7.0) containing 0.9% (wt/vol) NaCl and 0.4% (wt/vol) Formalin. The cells were stained with methyl violet B in a 5% (vol/vol) phenol solution, heated, and then counted by using a Petroff-Hausser counting chamber (Clay Adams, Div. of Becton, Dickinson and Co., Parsippany, N.J.) at 1,200× magnification.

Adherence of *S. mutans* to pretreated HA disks. HA disks (13 mm in diameter) were prepared by pressing 150 mg of HA under 5 tons of pressure for 5 min in a KBr press, as described by Simonson and Jackola (15). The disks were hydrated with deionized water, placed in small capped vials containing either 2 ml of imidazole buffer (control) or 2 ml of a protein, polypeptide, or dextran solution (1 mg/ml of imidazole buffer). The vials were placed in a 37°C water bath and shaken at 80 rpm for 1 h. The disks were then washed with 5 ml of imidazole buffer in vials shaken at 80 rpm for 20 min at 37°C. After washing, 1.5 ml of a labeled cell concentrate was added to each vial, which was then shaken at 80 rpm for 1 h at 37°C. The disks were again washed with 5 ml of imidazole buffer, allowed to dry, and transferred to scintillation vials containing 5 ml of scintillator (5 g of 2,5-diphenyloxazole per liter of toluene). The radioactivity of each disk was measured by using a liquid scintillation spectrometer (Packard Tricarb). To determine the radioactivity of a known number of bacteria on each disk, a 5-μl sample of the labeled cell concentrate was pipetted onto HA disks pretreated with each protein or polypeptide or dextran. Cell enumeration of the bacterial cell concentrate in equilibrium with the HA disk was determined as described above.

Zeta potential measurements. Zeta potential of HA particles was obtained from the electrophoretic mobility measurements taken with a Zeta-meter (Zeta-meter Inc., New York, N.Y.). HA (50 mg) was shaken at 80 rpm with 1 ml of imidazole buffer or the various protein, dextran, or polypeptide solutions (5 mg/ml of imidazole buffer) at 37°C for 1 h. The HA was then washed with imidazole buffer and resuspended in 20 ml of deionized water. The time taken for a particle to migrate 1 μm in an electric field was measured with an 8× objective and a 300-V setting. The average of 10 measurements was used. Zeta potential was calculated from the Helmholtz-Smoluchski equation: zeta potential (δ) = $\mu 4\pi\eta/\epsilon$, where μ is the electrophoretic mobility or velocity at unit potential gradient, η is the viscosity of the liquid, and ϵ is the dielectric constant.

Scanning electron microscopy. HA disks pretreated with either imidazole buffer or an α_{s1}-casein solution (1 mg/ml of imidazole buffer) were incubated at 37°C with an *S. mutans* cell concentrate (10^9 cells per ml) for 1 h at 80 rpm. The disks were washed with imidazole buffer and air dried, after which they were gold coated before scanning.

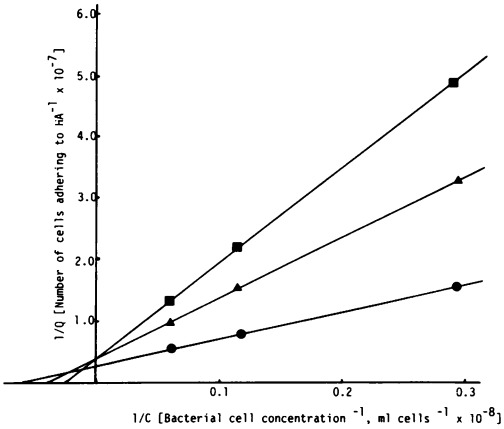

FIG. 1. Langmuir adsorption isotherms (double reciprocal plots) of adherence of *S. mutans* to HA disks. Symbols: ●, control disk; ▲, disk pretreated with bovine serum albumin; ■, disk pretreated with α_{s1}-casein.

RESULTS

The adherence of *S. mutans* PK1 to HA disks followed the Langmuir adsorption model (1), as the experimental results (Fig. 1) satisfied the linearized form of the equation $1/Q = 1/KNC + 1/N$, where C is the concentration of bacteria (cells per milliliter) in equilibrium with the HA disk, Q is the number of cells adhering to the disk, N is the maximum number of cells adhering to the disk, and $1/K$ is the equilibrium concentration corresponding to half maximal binding (i.e., K reflects the affinity of the bacterial cell for HA). The correlation coefficients for the three regressions shown in Fig. 1 are presented in Table 2 together with the affinity constants and maximum number of cells binding to the HA disk. Pretreatment of the HA disks with the two acidic proteins α_{s1}-casein and bovine serum albumin reduced the affinity of the bacterial cell for HA (Table 2). The scanning electron micrographs (Fig. 2) show the effect of α_{s1}-casein on the adherence of *S. mutans* to HA disks.

The effect of the α_{s1}-casein concentration (during HA pretreatment) on the adherence of *S. mutans* to HA disks is shown in Fig. 3. The exponential decrease in bacterial cell adherence with increasing α_{s1}-casein concentration is attributable to the binding of α_{s1}-casein to HA being also of the Langmuir type (unpublished data).

From these preliminary experiments, the effect of other proteins and polypeptides and dextran on the adherence of *S. mutans* to HA was studied by using a bacterial cell concentration of 1.5×10^9 cells per ml and a concentration of HA pretreating solutions of 1.0 mg/ml. The number of cells adhering to HA disks pretreated with various proteins and polypeptides and dextran is shown in Table 3. These results indicate that adsorption of a neutral molecule (dextran) or basic protein or polypeptide onto HA had no effect or enhanced bacterial adherence. However, adsorption of all acidic proteins reduced bacterial cell adherence.

The zeta potential measurements of HA particles treated with the proteins and polypeptides (Table 3) showed that the HA particle reflects the charged environment of the adsorbed molecule. A basic protein or polypeptide (net positively charged at pH 7.0) produced a positive zeta potential, whereas an acidic protein or polypeptide (net negatively charged at pH 7.0) produced a negative zeta potential. The zeta potential measurement was correlated with the number of bacterial cells adhering to the HA previously treated with each respective molecule ($r = 0.6959$; $n = 104$; $P \leq 0.001$) but only for the whole group of proteins and polypeptides studied (1 to 11 in Table 1). Within each protein group (acidic proteins [1 to 8 in Table 1] and basic proteins [9 to 11 in Table 1]), the zeta potential was not correlated with the number of bacteria adhering to HA. The acidic residue percentage of each protein was negatively correlated with the number of bacteria adhering for the total group, as well as within each protein group, acidic and basic (Table 4). A significant correlation between the basic residue percentage of each protein and the number of bacteria adhering was also obtained, but within each protein group, only the correlation within the basic protein group attained significance (Table 4). To minimize the effect of the acidic and basic residues, a group of proteins (3 to 8 in Table 1)

TABLE 2. Effect of α_{s1}-casein and bovine serum albumin on *S. mutans* adherence parameters

Treatment	Correlation coefficient[a]	Affinity constant (K) (ml cell^{-1} x 10^{-10})	Maximum binding (N) (cells x 10^7)
Control	0.9953	7.1	3.2
Bovine serum albumin	0.9957	2.8	3.1
α_{s1}-Casein	0.9968	2.3	2.8

[a] $n = 9$; $P \ll 0.001$.

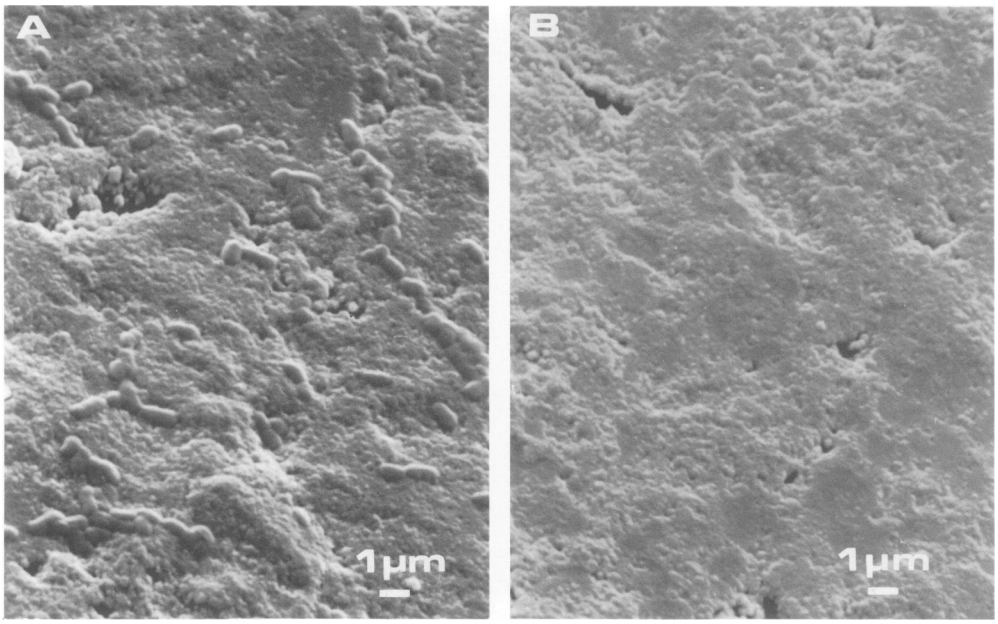

FIG. 2. Representative scanning electron micrographs showing the effect of adsorbed α_{s1}-casein on the adherence of *S. mutans*. (A) HA disk pretreated with imidazole buffer and then incubated with *S. mutans* cell concentrate. (B) Disk pretreated with α_{s1}-casein (1 mg/ml) in imidazole buffer before incubation with *S. mutans*.

with similar percentages of acidic and basic residues was used to correlate the nonpolar residue percentage and neutral polar residue percentage with the number of bacteria adhering to HA. The correlation between the nonpolar residue percentage and the number of cells adhering attained significance (Table 4).

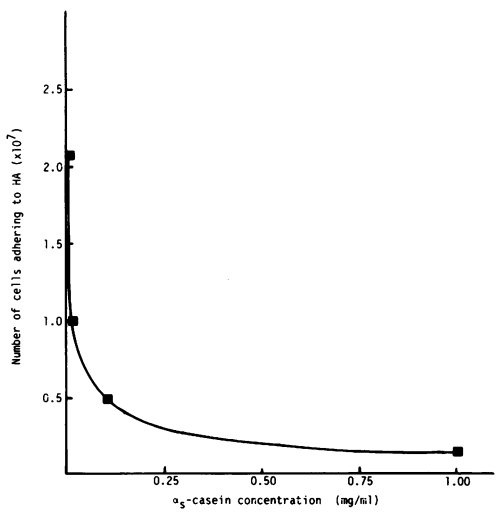

FIG. 3. Effect of α_{s1}-casein concentration on adherence of *S. mutans* to HA disks. The concentration of α_{s1}-casein in the pretreating solution was varied between 0.01 and 1.00 mg/ml in imidazole buffer.

DISCUSSION

The adherence of *S. mutans* to HA disks followed the Langmuir adsorption model as shown by Clark et al. (5) for *S. mutans* adherence onto HA beads. However, in contrast to studies with HA beads (2, 5, 9), the affinity constants reported here are an order of magnitude lower. This could reflect the different bacterial strain or method used. Beads are separat-

TABLE 3. Effect of various compounds on zeta potential and bacterial adherence to HA

Compound adsorbed onto HA	Zeta potential[a] (mV)	No. of bacterial cells adhering to HA disk[b] ($\times 10^7$)
Poly-L-glutamate	−25.4 ± 3.7	0.57 ± 0.12
α_{s1}-Casein	−24.5 ± 3.0	0.59 ± 0.32
α-Lactalbumin	−21.0 ± 1.8	1.06 ± 0.41
Phosvitin	−19.7 ± 2.8	0.53 ± 0.19
β-Casein	−19.7 ± 2.2	1.26 ± 0.43
κ-Casein	−18.5 ± 2.8	0.62 ± 0.19
β-Lactoglobulin	−13.6 ± 1.9	0.94 ± 0.49
Bovine serum albumin	−13.0 ± 2.6	0.61 ± 0.19
Dextran	−10.7 ± 1.5	2.29 ± 0.86
None	− 9.1 ± 1.2	1.90 ± 0.71
Poly-L-lysine	+20.2 ± 3.7	3.72 ± 1.12
Histone H1	+29.6 ± 3.5	2.26 ± 1.01
Histone H3	+32.7 ± 7.6	2.65 ± 1.02

[a] Mean ± standard deviation; $n = 10$.
[b] Mean ± standard deviation; $n = 8$.

TABLE 4. Correlation between number of bacteria adhering to HA and composition of adsorbed protein

Correlation	*n*	Correlation coefficient
Acidic residue percentage to cells adhering		
Proteins 1 to 11[a]	88	−0.4349[b]
Proteins 1 to 8	64	−0.3161[c]
Proteins 9 to 11	24	−0.4045[d]
Basic residue percentage to cells adhering		
Proteins 1 to 11	88	0.7497[b]
Proteins 1 to 8	64	−0.0254[e]
Proteins 9 to 11	24	0.4963[c]
Nonpolar residue percentage to cells adhering		
Proteins 3 to 8	48	0.2962[d]
Neutral polar residue percentage to cells adhering		
Proteins 3 to 8	48	0.0499[e]

[a] These numbers refer to those in Table 1.
[b] $P < 0.001$.
[c] $P < 0.01$.
[d] $P < 0.05$.
[e] Not significant.

ed from unadsorbed bacteria by allowing the former to settle; hence, error could result from sedimentation of unadsorbed bacteria.

The effect of the acidic proteins in reducing the affinity of the bacteria for the HA disk appears to be related to the adsorption of the protein onto HA causing an increase in surface net negative charge, thereby influencing the long-range interaction between the bacterium and the surface. The increase in surface net negative charge would cause an increase in repulsion of electrical double layers surrounding the bacterium and HA (10), thereby increasing the potential energy barrier which hinders the approach of the bacterium and tends to prevent attachment. Adsorption of basic proteins created a net positive surface charge which, together with van der Waals attraction, would enhance the approach of the bacterium to the surface and facilitate adherence.

The lack of correlation between bacterial adherence and zeta potential within the acidic and basic protein groups indicates that within each group, short-range interactions are overriding simple repulsion and attraction of electrical double layers. The positive correlation within the basic protein group between bacterial adherence and the basic residue percentage suggests the involvement of ionic interactions between the negatively charged groups on the bacterial surface and the positively charged groups on the adsorbed protein (Fig. 4). It seems unlikely that the adherence of bacteria to HA surfaces coated with acidic proteins is mediated by calcium

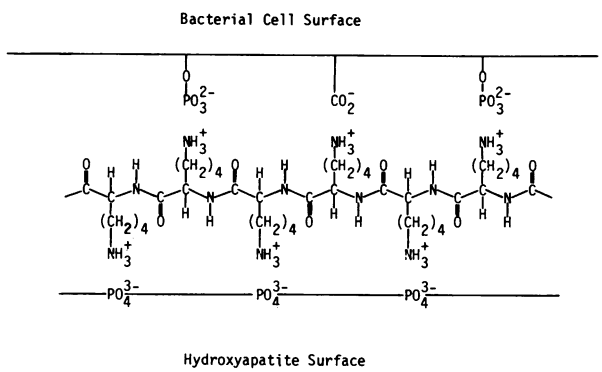

FIG. 4. Schematic representation of the interaction of an adsorbed polylysine sequence and a bacterial cell surface.

bridges between the negatively charged groups on both surfaces, as proposed by Rolla (14), due to the negative correlation between the acidic residue percentage and bacterial adherence within both the acidic and basic protein groups (Table 4). As the acidic residues of these proteins are involved in the adsorption of the protein onto HA (13a), these results are consistent with the proposition of Simonson and Reiher (16) that the acidic proteins compete with the bacteria for HA-binding sites. The positive correlation between nonpolar residue percentage and bacterial adherence within the acidic protein group suggests that hydrophobic interactions occur between nonpolar residues on the bacterial surface and the adsorbed protein.

In conclusion, the work presented here shows that protein adsorbed onto the HA surface is involved in both long- and short-range interactions between bacteria and the HA surface. Acidic proteins reduced the number of bacteria adhering by increasing the repulsion between electrical double layers surrounding the bacterium and the HA. This effect was further modified by the density of nonpolar, acidic and basic amino acids of the adsorbed protein, indicating the involvement of short-range hydrophobic and ionic interactions between adsorbed protein and bacteria.

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