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Multi-component adsorption model for pellicle formation: The influence of salivary proteins and non-salivary phospho proteins on the binding of histatin 5 onto hydroxyapatite

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Summary The acquired enamel pellicle formed by selective adsorption of proteins in whole saliva is a protective integument on the tooth surface. The purpose of the present study was to investigate the formation of human acquired enamel pellicle using an in vitro hydroxyapatite (HA) model and ^{3}H-histatin 5 to allow accurate measurement of histatin 5 binding in a multi-component experimental system. A binary system was employed by mixing ^{3}H-histatin 5 with one unlabeled protein prior to incubation with HA or by first incubating ^{3}H-histatin 5 with the HA which had been pre-coated with one of a panel of unlabeled proteins (human albumin, salivary amylase, lysozyme, acidic PIFs, statherin, the N-terminal fragment of statherin, and egg yolk phosvitin). A ternary system was employed by mixing ^{3}H-histatin 5 with HA sequentially pre-coated with two different unlabeled proteins, including recombinant histatin 1. The results showed that only salivary statherin and egg yolk phosvitin promote histatin 5 adsorption significantly. The amount of histatin 5 adsorbed was also found to increase as a function of the amount of phosvitin and statherin used to pre-coat HA up to a maximum level that was two- to four-fold greater than that observed on untreated HA. These data suggest that specific protein–protein interactions may play important roles in pellicle formation in vivo.
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Introduction

The acquired enamel pellicle is an organic film on the tooth surface formed by selective adsorption of

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predominantly salivary components.[1] The protein composition of pellicle is important with regard to the function of pellicle, which includes inhibition of calcium phosphate crystallization,[2–4] control of microbial adherence,[5–8] permselective ion transport,[9] and possibly calculus formation. Further exploration of the biological significance of pellicle requires the elucidation of its composition, structure and mechanism of formation. In the past, efforts have been made to gather information on pellicle formation using an in vitro model comprising glandular secretions or whole saliva and hydroxyapatite (HA) powder, beads or discs. The selectivity of the adsorption observed in these studies led to the recognition that specific salivary proteins must be considered pellicle precursor proteins by virtue of their moderate to high affinity for HA.[10–13] Adsorption studies with a single native or recombinant protein provided physical parameters such as binding affinity and number of binding sites for acidic proline-rich proteins,[14,15] statherin,[14] and histatins.[4,16]

Amino acid analysis[17,18] and Western blotting[19,20] of in vivo formed pellicle revealed that this structure contains many proteins and peptides. More recent approaches using a combination of gel electrophoresis and mass spectrometry provided evidence for the presence of more than 100 proteins/peptides of which the major species were albumin, amylase, cystatins, proline-rich proteins, statherin, lysozyme, and histatins.[21,22] The complexity of the protein/peptide constituents of pellicle formed in vivo raises questions concerning the biological relevance of the adsorption data obtained from in vitro studies using a single protein and HA. The analysis of protein interactions with HA should include more than one component to better reflect the multitude and heterogeneity of proteins in saliva. Protein–protein interactions in saliva and/or on HA surfaces must be considered to significantly advance our understanding of the mechanism of pellicle formation. As a first step in this process, we have used the enamel mineral prototype model system established by Moreno et al.[14] to study HA adsorption characteristics of histatin 5 in the presence of one or two additional proteins.

Materials and methods

Proteins and reagents

Synthetic ^{3}H-histatin 5 was synthesized commercially (SynPep Corp., Dublin, CA) using tritiated glycine, the ninth amino acid residue from the N-terminal residue of histatin 5. The specific radioactivity of the protein was 7.2 μCi/mg, and the chemical purity was estimated to be greater than 97% by HPLC analysis. Mass spectral analysis of synthetic ^{3}H-histatin 5 yielded a mass of 3039.0 Da, in agreement with non-tritiated histatin 5 with a mass of 3037.0 Da.

Statherin, PIF-s and amylase were purified from human parotid saliva using published procedures.[13,23] Recombinant histatin 1 (r-histatin 1) was purified from *E. coli* cell extracts as previously described.[24] The recombinant protein lacks two phosphate groups found on serine residues of the native protein. Albumin and lysozyme were obtained from a commercial source (Boehringer-Mannheim, Darmstadt, Germany). Although not of salivary origin, egg yolk phosvitin was also studied, since it is highly phosphorylated and was found (see below) to have a high affinity for HA. Phosvitin was purified using a published procedure[25] and resolved into its two major components by the method of Clark.[26] The fraction eluting first was used in these studies with a molecular weight of 35,500 Da. A peptide containing the N-terminal 10 amino acid residues of statherin (statherin N10) was obtained from American Peptide Company (Sunnyvale, CA). Amino acid analyses were performed as previously described[16,21] to determine the protein concentrations of the stock solutions used in protein adsorption experiments. The concentration of ^{3}H-histatin 5 used in the adsorption experiments was determined by liquid scintillation counting based on the specific radioactivity (7.2 μCi/mg) of the peptide preparations used.

Adsorbents

To study the adsorption of ^{3}H-histatin 5 in the presence of multiple components, ceramic HA beads were used (Bio-Rad Laboratories, Hercules, CA). These beads have a diameter of 80 μm, and have a lysozyme-binding capacity of greater than 12.5 mg/g. For the determination of affinity constants (K) and maximum number of binding sites (N) of ^{3}H-histatin 5 singly or in combination with other proteins, HA powder was used. This HA powder (Reference Material 2910) was obtained from the National Institutes of Standards and Technology (Gaithersburg, MD). Its specific surface area is 18.3 ± 0.3 m^{2}/g with crystal sizes in the range of 0.1–0.5 μm. A complete description of this material is available at http://ts.nist.gov/srm. Sodium chloride was obtained from Sigma–Aldrich (St. Louis, MO).

Adsorption of ^{3}H-histatin 5 onto HA beads

(a) Binary model. Two methods (A and B) were used to investigate histatin 5 adsorption in the binary model. In method A, equal concentrations (15 μM) of an unlabeled protein and ^{3}H-histatin

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