A statherin and calcium enriched layer at the air interface of human parotid saliva

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Parotid saliva placed in 35-mm-diameter tissue culture dishes developed increasing surface viscoelasticity at the interface with air. A surface layer became visible with time, and was collected and analysed by protein electrophoresis which indicated that a single protein (pI 4.2; molecular mass approx. 6 kDa) predominated. Western blot analysis demonstrated that the major protein band reacted with an antiserum directed against the C-terminal of the calcium-binding salivary protein statherin. Matrix-assisted laser-desorption ionization–time-of-flight MS analysis gave a molecular mass of 5380 Da for the protein, corresponding to the molecular mass of statherin. Staining of film protein in electrophoresis gels was compared with statherin synthesized on a solid phase, and the mean statherin content of film formed from 1 ml of parotid saliva was measured as 7 nmol. The mean calcium content of the surface layer was 250 nmol. Surface rheology was greatly decreased in the presence of EDTA, whereas surface tension of saliva was unaffected by calcium chelation, suggesting that protein accumulated at the surface was unaffected. The results suggest that a layer rich in statherin forms at the interface of saliva and air, and that the surface rheology developed is dependent upon protein interactions mediated by calcium. The surface layer may enhance the function of saliva as a protective layer on the mucosal surfaces and teeth.

Key words: air interface, calcium, saliva, statherin, surface rheology.

INTRODUCTION

Whole saliva is composed of secretions from parotid, submandibular and sublingual glands, and a smaller contribution of saliva from minor salivary glands (e.g. palatal and labial) in the oral submucosa. Saliva is crucial for the maintenance of oral health and performs a number of important functions in the mouth, including lubrication, wetting of the oral mucosa, prevention of tooth demineralization and promotion of tooth remineralization. Saliva also contains antimicrobial peptides and proteins which modify the microbial flora of the mouth [1]. The importance of these qualities is clearly demonstrated by their loss in those individuals with chronic absence of saliva, since they suffer from an increased prevalence of oral infections and caries, and experience problems in eating, speaking and swallowing [2].

Saliva forms a thin mobile layer (8–40 μm thick) over the oral mucosa and teeth [3], and salivary proteins may form a more adherent layer on teeth and apparently also on buccal epithelial cells [4,5]. The rheological properties of saliva determine its flow and retention over the oral mucosal surface and are, to a large extent, dependent upon its content of macromolecular glycoproteins. In particular, oligomeric mucin (MUC 5B), derived from submandibular, sublingual and minor salivary gland secretions, forms an insoluble gel phase in saliva *in vitro* [6,7] and may form a mucoadherent matrix on the oral mucosa surface.

In vitro, saliva has been observed to develop a film up to 100 nm thick at the interface with air. The surface film is independent of the presence of salivary mucins, since it forms on parotid saliva, a fluid that contains little, if any, mucin [8]. Study of the surface rheology of parotid saliva *in vitro* using an oscillating ring surface shear rheometer demonstrated that it is much more viscoelastic than the liquid bulk of saliva underneath [9]. Since saliva forms very thin layers on oral surfaces *in vivo*, it is likely that the interfacial rheology of saliva makes an important contribution to its function. However, relatively little is known about the composition of the surface film, which is the focus of the present study.

EXPERIMENTAL

Collection of saliva

Parotid saliva samples were collected from among the staff, aged between 22 and 45 years, of the Salivary Research Unit, King's College Dental Institute. The protocols used were approved by the local Ethical Review Committee. Parotid saliva was collected using a sterilized Lashley suction cup which was placed over the parotid duct papillae on the buccal surface, and salivary secretion was stimulated by sucking a sugar-free lemon drop of known composition (Simpkins, Sheffield, U.K.). The first five drops of saliva were discarded to clear the dead space in the Lashley cup and tubing, and then saliva was collected into pre-weighed tubes for a period of 10 min. Salivary flow rates were measured gravimetrically (1 ml = 1 g).

Submandibular/sublingual gland saliva was collected as described previously [10] using a suction appliance made from soft polyethylmethacrylate (Eversoft; Myerson, Chicago, IL, U.S.A.) based on the design of Truelove et al. [11]. The suction device was placed on the floor of the mouth over the papillae of the submandibular ducts. A lemon drop was again used as a secretory stimulus. Unstimulated and stimulated whole-mouth salivas were collected for a period of 10 and 5 min respectively. Flow was stimulated by chewing on a tasteless piece of sterile polythene tubing.

Measurement of surface rheology

The surface viscoelasticity of salivas was measured using a surface shear rheometer (CIR-100; Camtel, Royston, Herts., U.K.). This instrument uses an oscillating Du Nouy ring [12]. When the ring is

Abbreviations used: IEF, isoelectric focusing; MALDI–TOF, matrix-assisted laser-desorption ionization–time-of-flight.

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placed at an interface, the frequency and amplitude of the oscillations change, depending on the elasticity and viscosity of the interface. Controlled torque mode was used to measure saliva surface viscoelasticity owing to the extremely high values obtained with the normal measuring protocol. This applies an oscillating sinusoidal torque to the ring, and measures the resultant strain values by means of a proximity detector. The time-dependent stress and strain values are used to calculate the elastic (G′) and viscous (G″) components of the surface modulus. The samples were all measured at a frequency of 0.2 Hz and a torque of 0.02 $\mu N \cdot m^{-1}$. Surface viscoelasticity was also measured in saliva samples to which EDTA, at a final concentration of 2 mM, had been added.

Measurement of surface tension

The surface tension of saliva samples was determined by the pendant drop technique [13]. Parotid saliva samples were dispensed from a syringe, and images of droplets hanging from the end of a flat, Teflon-coated needle were taken by a CCD (charge-coupled device) camera connected to a personal computer. The shape of the droplets is determined by gravity and the surface tension. Droplet shape was analysed to calculate the surface tension. Measurements were made for 15 min at room temperature (22 °C), in the presence or absence of 2 mM EDTA.

Preparation and collection of salivary films for analysis

Using 1 ml aliquots of fresh salivas, films were allowed to form at room temperature in 35-mm-diameter tissue culture dishes for approx. 30 min. Saliva underneath the film (residual saliva) was removed with a pipette, and the film was washed twice by pipetting 1 ml of 20 mM Tris-buffered saline (20 mM Tris/HCl and 0.15 M, pH 7.6) under the film lying on the base of the Petri dish. Finally, the film formed from 1 ml of saliva was solubilized in 1 ml of 0.1 % (v/v) Triton X-100 in 20 mM Tris/HCl (pH 7.6) or in 1 ml of 10 mM EDTA.

Preparation of an anti-statherin antibody

The 14-amino-acid C-terminal sequence of statherin (YPQPYQPQYQQYTF) was synthesized on a solid phase (Harlan Laboratories, Loughborough, U.K.). The peptide was linked to keyhole-limpet haemocyanin, and an antibody was raised in rabbits. The antiserum obtained from the final bleed was affinity-purified on a column with bound peptide antigen (Harlan Laboratories).

Electrophoresis and immunodetection of statherin

Samples of saliva or salivary film dissolved in 10 mM EDTA solution were prepared for electrophoresis by adding 1 vol. of 4× concentration sample buffer (containing 0.08 % lithium dodecyl sulphate and 0.006 % EDTA) (Invitrogen) to 3 vol. of sample. A 1/10 vol. of 0.5 M dithiothreitol (Sigma) was added before electrophoresis using pre-cast 16 % tricine gels (Invitrogen) in a tricine/SDS-containing running buffer according to the manufacturer's instructions using a XCell vertical electrophoresis unit (Invitrogen). Resolved proteins were visualized by staining gels with 0.2 % (w/v) Coomassie Brilliant Blue R250 (Sigma) in 25 % methanol, 10 % ethanoic (acetic) acid at room temperature for 90 min and de-staining in 10 % ethanoic acid without methanol, enabling salivary proline-rich proteins to be visualized as metachromatic pink-staining bands [14]. Relative molecular masses of resolved salivary proteins were determined by comparison with a mixture of standard proteins (Invitrogen).

Alternatively, following completion of electrophoresis, separated proteins were electroblotted on to nitrocellulose membranes (Schleicher and Schuell, Anderman and Co., Kingston-upon-Thames, U.K.) using a wetblot apparatus according to the manufacturer's instructions (Invitrogen), following a method originally described by Towbin et al. [15]. Blotted proteins were stained on nitrocellulose membranes using FITC as described previously [16], then viewed and photographed under UV light ($\lambda = 350$ nm). Blots were probed with the antigen-affinity-purified fraction of rabbit antiserum to human statherin C-terminal peptide. Binding was detected using a biotinylated, affinity-purified, goat anti-rabbit IgG following by horseradish-peroxidase-labelled avidin (Vector Laboratories, Peterborough, U.K.) and then ECL® (enhanced chemiluminescence) detection system (Amersham Biosciences). Chemiluminescence was recorded by film (ECL®).

Two-dimensional electrophoresis was performed in a pH 3–10 first dimension using pre-cast IEF (isoelectric focusing) gels (Invitrogen) according to the manufacturer's instructions, followed by a second dimension of SDS/gel electrophoresis in pre-cast 16 % tricine gels (Invitrogen). Following IEF gel strips were prepared and equilibrated with SDS sample medium containing dithiothreitol and placed on to a tricine gel for electrophoresis as described above. The pH gradient of the first dimension was measured by eluting pieces of gel and measuring pH, and by electrophoresis of a mixture of standard proteins (Invitrogen).

Quantification of statherin in films

Digital images of protein bands separated by tricine gel electrophoresis and stained with Coomassie Brilliant Blue R250 were analysed, and total band pixels were determined (Kodak 1D Image Analysis Software; Eastman Kodak Company, Rochester, NY, U.S.A.). Solid-phase-synthesized complete human statherin (gift of Dr Paul Anderson, Centre for Oral Growth and Development, Bart's and The London Queen Mary's School of Medicine and Dentistry, London, U.K.) was loaded on to electrophoresis gels in increasing amounts (typically 0.15–0.6 μg). A standard curve of total number of pixels in bands against protein load was created. Total numbers of pixels in bands of film protein were determined, and statherin content was derived from the standard curve.

MS of film protein

Salivary film dissolved in 0.01 % Triton X-100 was analysed by MALDI–TOF (matrix-assisted laser-desorption ionization–time-of-flight) MS using a Voyager-DE Elite MALDI–TOF mass spectrometer (Applied Biosystems, Warrington, U.K.) equipped with delayed extraction and operated in the linear mode. A matrix of sinapinic acid (3,5-dimethoxy-4-cinnaminic acid; Sigma) was used, and the instrument was calibrated with insulin (molecular mass 5374 Da) (Sigma).

Assay of calcium and inorganic phosphorus

Calcium concentrations of salivas and solubilized films were assayed using *o*-cresolphthalein complexone and 8-hydroxyquinoline (Sigma) according to the manufacturer's instructions. The assay is a modification of that described previously [17]. Inorganic phosphate was assayed using an acid molybdate solution (Sigma) according to the manufacturer's instructions in a modification of an assay described previously [18].

Where appropriate, results are expressed as means ± S.E.M. and were statistically compared using Student's paired *t* test.

RESULTS

Surface viscoelasticity

Surface viscoelasticity of parotid and whole-mouth salivas, measured using the oscillating De Nouy ring surface rheometer,

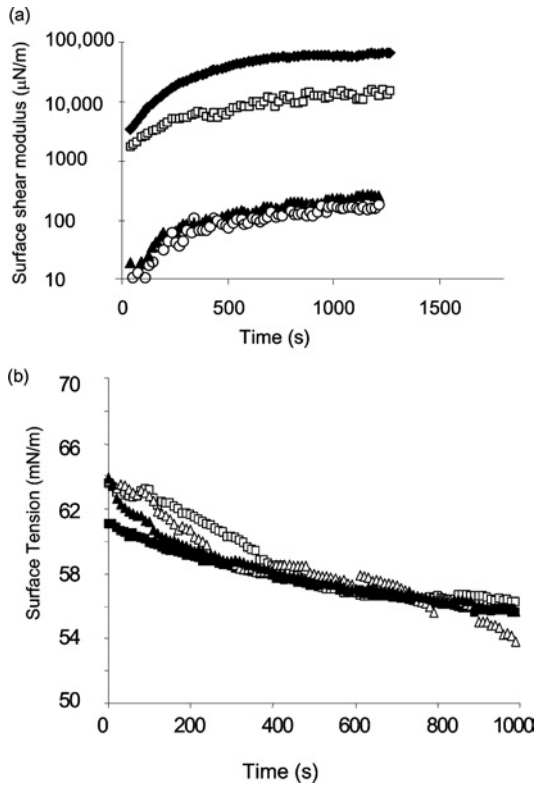

Figure 1 Surface properties of parotid saliva

(**a**) Surface viscoelasticity developed by parotid saliva *in vitro* as determined by an oscillating De Nouy ring surface rheometer (CIR-100). The surface elastic (◆) and viscous (□) moduli increase with time throughout the period of measurement. In the presence of 2 mM EDTA, the magnitude of the elastic (▲) and viscous (○) moduli are greatly reduced. (**b**) Surface tension of two samples of parotid saliva as determined by the pendant drop technique. The surface tension is lowered as protein adsorbs to the interface with time. There is little difference between the two samples in the absence (open symbols) or presence (closed symbols) of EDTA.

increased with time. Figure 1(a) shows a trace, typical of many obtained with parotid salivas from different individuals ($n > 5$), of surface viscous and elastic moduli. Surface viscoelasticity of parotid saliva and its development over time are greatly reduced in the presence of 2 mM EDTA. The development of surface viscoelasticity is accompanied by the formation of a crystalline-like surface layer that is visible by eye at approx. 15–30 min. In the presence of EDTA, a visible film is not formed, and, at 15 min, the elastic modulus (G′) was decreased over 300-fold (189 compared with 60 800 $\mu N \cdot m^{-1}$) (Figure 1a), and the viscous modulus (G″) was decreased over 100-fold (119 compared with 12 900 $\mu N \cdot m^{-1}$) (Figure 1a).

Surface tension

Surface tension of parotid saliva decreases with time as surface-active material adsorbs to the interface as shown in Figure 1(b). Unlike the surface viscoelasticity, there is little effect of EDTA on the surface tension values.

Electrophoresis of salivary proteins

SDS/gel electrophoresis of surface films formed on parotid, submandibular/sublingual and whole-mouth salivas revealed that films are largely composed of a single protein species with a molecular mass of approx. 6 kDa, as compared with protein standards (Figure 2a). Other minor proteins were also present in surface films. Some of the latter appeared to represent contamination by more abundant salivary proteins, although a doublet of proteins present in electrophoresis gels of parotid films (see arrow in Figure 2c) is not abundant in parotid saliva. Consecutive washing of parotid films with Tris-buffered saline largely removed all of these proteins (Figure 2c). Electroblotting of electrophoretically separated proteins on to nitrocellulose and probing of membranes with an antibody raised against the C-terminal peptide sequence of statherin indicated that the major low-molecular-mass protein band contained statherin (Figure 2b). Two-dimensional electrophoresis of solubilized film protein resolved the statherin-containing low-molecular-mass band (6 kDa) as a single major spot (pI 4.2) when stained by dye (Figure 2d) or silver (results not shown).

MS of film protein

In order to provide further evidence of the identity of the major protein present in salivary films, MS was performed. The trace shown in Figure 3 is typical of several determinations carried out by MALDI–TOF MS analysis following calibration with an insulin standard (molecular mass 5374 Da). The mean molecular mass for the major film protein, determined from four analyses was 5379.59 ± 0.66 Da.

Assay of statherin in films

Synthetic full-length statherin was resolved as a single major species in SDS/gel electrophoresis (Figure 2e). Digital images of gels were analysed, and the relationship between Coomassie Brilliant Blue staining intensity (total pixel number in band) and gel loading was linear over the range used (0.15–0.6 μg of statherin). Film protein was loaded on to gels, typically in 10–20 μl volumes, to give similar band intensities as that of standard statherin (Figure 2e). The statherin content of bands electrophoresed in film samples was determined using a synthetic statherin standard curve. The mean statherin content of films formed from 1 ml aliquots of parotid saliva samples from four subjects was 37.7 ± 8.5 μg or 7 ± 1.5 nmol.

Assay of calcium and inorganic phosphate

Visual inspection of the salivary surface films formed *in vitro* after approx. 30 min of development suggested that they have a slightly crystalline appearance that becomes more obvious when films disintegrate with vigorous vortex mixing, with the appearance of needle-like crystals of tightly folded film. Since EDTA was also found to prevent the formation of films, calcium assays of collected films were performed, results were compared with those for saliva, and residual saliva was collected from underformed films. The mean calcium concentration of samples of fresh parotid saliva from five subjects determined using the *o*-cresolphthalein assay was 0.69 ± 0.11 μmol/ml. Figure 4 shows the total amount of calcium present in 1 ml aliquots of saliva, films solubilized in 1 ml of 0.1 % Triton X-100 and 1 ml aliquots of residual salivas formed from 1 ml of saliva. The mean calcium content of films was 0.25 ± 0.07 μmol. Thus 38 ± 10 % of the total salivary content of calcium is present in the films formed on the surface of salivas.

The mean inorganic phosphate concentration of parotid salivas from five subjects was 4.5 ± 0.4 μmol/ml. Films formed from aliquots of the same samples were solubilized in 1 ml of 0.1 % Triton X-100 and contained 0.31 ± 0.03 μmol/ml inorganic phosphate. The mean ratio of calcium/inorganic phosphate in films from parotid salivas of five subjects was 1.16 ± 0.19 compared with a mean ratio of 0.18 ± 0.02 for fresh parotid salivas from the same subjects.

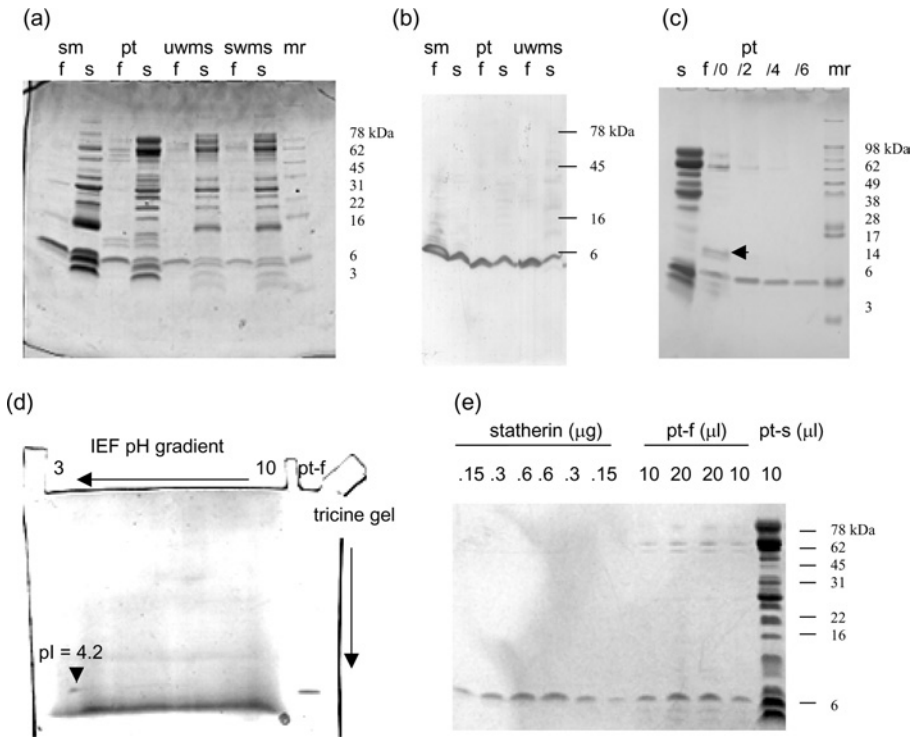

Figure 2 Electrophoresis of proteins present in the film formed at the air interface with saliva after 30 min

(**a**) Dye-stained tricine electrophoresis gel comparing proteins present in films (f) formed from salivas (s). Submandibular (sm), parotid (pt), unstimulated whole-mouth saliva (uwms) and stimulated whole-mouth saliva (swms) were electrophoresed under non-reducing conditions. Electrophoresis of a mixture of molecular-mass-standard marker proteins (78, 62, 45, 31, 22, 16, 6 and 3 kDa) is also shown (mr). (**b**) The presence of statherin as the major film protein was confirmed by Western blotting a tricine gel using a polyclonal antibody raised against a synthetic C-terminal peptide of statherin. Films (f) and salivas (s). (**c**) Dye-stained tricine electrophoresis gel comparing proteins present in parotid (pt) saliva (s) and films (f) formed from parotid saliva and washed 0, 2, 4 and 6 times with Tris-buffered saline (pH 7.6). A number of minor protein bands are removed by washing, suggesting that they are not required for forming or maintaining a film. Some of the minor bands appear to be contaminating major salivary proteins, although a doublet (arrow) appears to be concentrated in the film. Electrophoresis of a mixture of molecular-mass-standard marker proteins (98, 62, 49, 38, 28, 17, 14, 6 and 3 kDa) is also shown (mr). (**d**) Two-dimensional electrophoresis of protein present in a film formed on parotid saliva sample (pt-f). The first dimension was a pH 3–10 IEF gel, and the second dimension was in a tricine gel. The pI of the major film protein was determined as 4.2 by measuring the pH gradient of IEF strips and by comparison with the electrophoretic mobility of standard proteins of known pI which were run simultaneously. For comparison, a sample of film protein was electrophoresed in a tricine gel alone, in a lane adjacent to the IEF gel. (**e**) Dye-stained SDS gel comparing synthetic statherin, 0.15, 0.3, 0.6, 0.6, 0.3 and 0.15 μg loads (lanes 1–6), with proteins present in films: 10, 20, 20 and 10 μl loads formed from parotid saliva (lanes 7–10). Proteins present in 10 μl of parotid saliva (pt-s) (lanes 11) are shown for comparison. The positions (sizes in kDa) of molecular-mass-marker proteins are also shown.

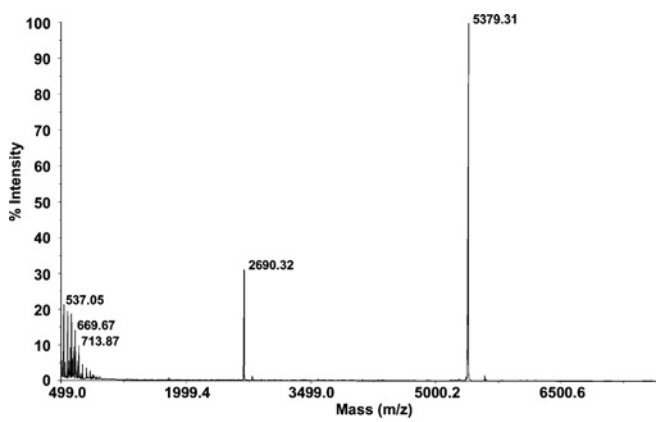

Figure 3 MS of film protein

A major peak was obtained in MALDI–TOF MS analysis of film protein using a sinapinic acid matrix. The peak molecular mass of 5379 Da corresponds to diphosphorylated statherin. The instrument was calibrated using insulin (molecular mass 5734 Da). The trace shown is typical of those obtained for parotid salivary films. A secondary peak was seen at 2690 Da, exactly half of the molecular mass of statherin. A similar peak was seen in the trace for standard insulin, at exactly half of the molecular mass for insulin.

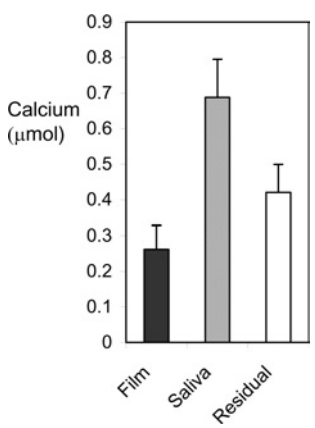

Figure 4 Calcium content of salivary surface films and saliva

The total calcium content of 1 ml of saliva, surface film resuspended in 1 ml of Tris/HCl containing Triton X-100 and 1 ml of residual saliva following film formation for 30 min. Calcium was assayed using *o*-cresolphthalein complexone and 8-hydroxyquinoline. A substantial proportion (38 %) of total salivary calcium was present in the film, resulting in a statistically significant decrease ($P < 0.001$) in the calcium content of residual parotid saliva following film formation compared with parotid saliva. $n = 5$.

DISCUSSION

Saliva is supersaturated with calcium phosphate salts, a property that is crucial to the maintenance of the apatite content of teeth, lowering rates of acid-induced demineralization and enabling remineralization of the carious lesions [19]. Statherin is a small salivary protein that has been demonstrated to be crucial to the maintenance of teeth, since it inhibits primary precipitation of hydroxyapatite and other calcium phosphate salts from saliva [20]. In combination with acidic proline-rich proteins, statherin also appears to inhibit unwanted secondary precipitation of hydroxyapatite on to the tooth surface [19]. Inhibition of calcium phosphate precipitation is considered to be the main function of statherin and depends to a large extent upon the amphipathic structure of the statherin molecule, which has a hydrophilic phosphorylated head and a hydrophobic C-terminal tail [19].

In the present study, it has been demonstrated that diphosphorylated statherin (molecular mass 5380 Da; [19]) is the major protein component of a viscoelastic layer that forms at the interface of parotid salivas, submandibular/sublingual and whole-mouth salivas with air. The identification of statherin as the major film protein was made using SDS/gel electrophoresis and Western blotting, two-dimensional electrophoresis and MS using a MALDI–TOF system. Given the amphipathic structure of statherin, it might be expected to be a very surface-active protein moving rapidly to the air interface, with the hydrophobic tail directed towards air and the hydrophilic head directed towards the aqueous phase. Experimentally purified statherin shows a high affinity for solid hydrophobic surfaces, and the degree of binding increases with the addition of calcium, possibly with the formation of multilayers [21]. β-Casein is a similarly amphipathic calcium-binding protein that shows viscoelasticity at the air/water interface [22,23] and strongly adsorbs to hydrophobic surfaces in increasing amounts in the presence of calcium [24].

Statherin is a small molecule with little secondary structure in aqueous solution [25] and thus is not typical of the globular proteins that form elastic interfaces [26]. In fact, the values for surface viscoelasticity of parotid saliva, obtained in the present study, are unlikely to be caused by a monolayer of unstructured 6 kDa protein. This view is supported by the surface tension results shown in Figure 1(b). Here there is little difference in the surface tension in the presence or absence of EDTA. Surface tension is sensitive to the protein concentration and composition of the interfacial layer. These results suggest that the composition of the interface is very similar in the presence or absence of EDTA. That is, the primary adsorbed layer in both cases is probably composed of statherin, and, in the presence of EDTA, the statherin forms a relatively weak interfacial film. However, in the absence of EDTA, when calcium is free to interact with proteins, a much stronger interfacial film is formed, perhaps even multiple layers of protein/calcium may be formed, which may explain the extremely robust interfacial films formed by saliva. The surface films contain very high concentrations of calcium, since almost 40 % of total salivary calcium was concentrated into the surface layer. The presence of calcium ions has also been observed to increase surface viscoelasticity in other proteins [27]. Surface shear rheology is very sensitive to intermolecular interactions, therefore it seems likely that calcium is responsible for inducing strong interactions between statherin molecules at the interface. This may also promote the formation of multimers and even micelles that, in effect, increase the size and viscoelasticity of statherin.

Previous estimates of the thickness of the salivary surface using elipsometry indicate that it is relatively thick (100 nm; [8]) compared with films formed by globular proteins, such as BSA (4 nm; [26]), an observation that also suggests the formation of multilayered complexes. Using the published value for the thickness of the salivary film as a guide, it can be calculated that 0.25 μmol of calcium is present in 0.001 ml of film, suggesting that the concentration of calcium in films is equivalent to approx. 250 mM, or 500 times greater than that present in saliva. The amount of statherin in the film can be similarly estimated as 7 nmol in 0.001 ml, or equivalent to a concentration of 7 mM. The concentration of statherin in parotid saliva has been determined previously as 0.013 mM [19].

Protein electrophoresis of salivas from different individuals indicated that other salivary proteins are also present in relatively small amounts in the salivary films. The presence of these minor proteins varied between individuals and between the types of saliva, and they were largely removed by washing. Overall, this suggests that the presence of the minor proteins is not essential for the formation and maintenance of the film.

At present, the function of a salivary film *in vivo* is uncertain. Previous studies have measured and calculated the thickness of the salivary layer covering the oral mucosa, and values in the range 8–40 μm have been published [3]. The presence of a protein/calcium-enriched viscoelastic film at the air interface of this thin layer of saliva may increase its retention on surfaces. Such retention would facilitate two important functions of saliva: preservation of a moist oral mucosa and lubrication between surfaces, lessening abrasive damage. In fact, an earlier study provided evidence that statherin functions as an important boundary lubricant in saliva [28]. The presence of a statherin/calcium-enriched layer on the surface of teeth may also serve to provide a zone of particularly high calcium concentration that facilitates the mineralization of teeth, without unwanted secondary precipitation on to the tooth surface.

We gratefully acknowledge the assistance of Ted Terrelli with MALDI–TOF analyses and funding from Guy's and St Thomas' Charitable Foundation.

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Received 6 December 2004/2 March 2005; accepted 16 March 2005
Published as BJ Immediate Publication 16 March 2005, DOI 10.1042/BJ20042012