Calcium-dependent Protein Interactions in MUC5B Provide Reversible Cross-links in Salivary Mucus*

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The macromolecular organization within saliva was investigated by tracer diffusion measurements of fluorescent polystyrene microspheres by fluorescence recovery after photobleaching using a confocal microscope (confocal-FRAP). There was a concentration-dependent reduction in microsphere diffusion; this was much greater in the presence of calcium (10 mM) and was reduced by the addition of EGTA (10 mM). These effects on tracer diffusion showed that native saliva contained a macromolecular organization that was sensitive to free calcium concentrations. This was supported by a major increase in the weight average molecular weight of the high molecular weight mucin fraction in saliva (10–62 × 10^6) and an increase in intrinsic viscosity of saliva (753 to 1203 ml/g) both caused by calcium. Analysis of tracer diffusion mass spectrometry in saliva showed a 20-fold increase in the apparent pore size (from 130 nm in 10 mM CaCl₂ to 2600 nm in 10 mM EGTA at physiological concentration). The effect was specific for calcium and was unaffected by up to 2 M NaCl. The calcium binding activity was contained in a high buoyant density fraction of saliva excluded from Sepharose CL-2B. Calcium binding to this fraction gave an approximate K_d of 7 × 10^6 M⁻¹, and the binding was irreversibly destroyed by treatment with 6 M guanidinium chloride and by mild reduction, suggesting it to be to a protein site. This fraction of saliva was shown to contain MUC5B as the single major protein species by positive ion electrospray ionization-tandem mass spectrometry analysis. The results suggested that oligomeric MUC5B in saliva is assembled into much larger linear or branched assemblies through calcium-mediated protein cross-links.

Mucus forms a viscoelastic gel that coats the epithelial surfaces in humans and other vertebrates. The properties of the gel have been interpreted as being predominantly due to entanglement of the long, high molecular weight oligomeric mucins (1, 2). Mucus has also been described as a network weakly cross-linked by non-covalent bonds (3, 4) and suggested mechanisms of interaction have included interchain hydrophobic interactions (5) and carbohydrate-carbohydrate interactions (6–9). Thus the supramolecular organization of the mucin layer is complex, and the molecular basis of this organization remains poorly defined.

A layer of mucus coats the surfaces of the gastrointestinal, reproductive, and respiratory tracts as well as the eyes and oral cavity (14). This layer is the bodies’ first line of defense against chemical, physical, and biological insult and a change in this barrier will compromise health. For example, in the airways overproduction of mucus with aberrant rheological properties is a feature of asthma, cystic fibrosis, and chronic obstructive pulmonary disease. In these situations the change in the physical properties of the barrier leads to a breakdown in mucus clearance from the airways with the attendant problems of poor gas exchange, bacterial colonization, and inflammation (10).

To gain further insight into mucus organization we have recently employed diffusion analysis by fluorescence recovery after photobleaching using a confocal microscope (confocal-FRAP) to study the properties of saliva (15). This technique provides a powerful approach to investigate the properties of macromolecules in concentrated solution (16). It enables molecular mobility to be determined in complex mixtures in the absence of flow and shear forces. In initial work we characterized the concentration dependence of the self-diffusion of the MUC5B mucin, the predominant oligomeric mucin present in saliva. The measurement of lateral translational diffusion of fluorescent microspheres of different size in solutions of purified MUC5B mucin and in native saliva was used to provide a direct estimate of the porosity (15, 17–20). The results showed that purified MUC5B formed concentrated solutions in physiological saline in which there was no evidence of self-association, and the properties at high concentration were as predicted by molecular entanglement of the oligomeric mucins (15). Intertermolecular hydrophobic interactions between mucins were not detected, neither were carbohydrate-carbohydrate interactions (15). However, comparison of native saliva with the solutions of guanidinium chloride (GdmCl) purified MUC5B under similar ionic conditions revealed that saliva had much lower porosity than the purified mucin at comparable concentrations, showing that saliva contained an additional level of organization that was absent from the purified MUC5B mucin (15).

In the present study, we investigated the basis of the additional organization present in saliva, which reduced tracer diffusion.

**EXPERIMENTAL PROCEDURES**

**Materials—**Trypsin, modified by reductive alkylation to reduce autolysis, was purchased from Promega (Southampton, UK). Acetonitrile (high performance liquid chromatography grade) was purchased from...
Ruthenium Chemicals (Walkerburn, UK). Calcium chloride, urea, Tris, and sodium chloride were from BDH (Poole, UK). Guanidinium chloride (practical grade), EGTA, and iodoacetamide were purchased from Sigma Chemical Co. (Poole, UK). Stock solutions of guanidinium chloride (—8 mM) were treated with charcoal before use. The Pepmap column was from Dionex (Camberley, UK) and radioactive Calcium-45 (1 mCi), Sepharose S-1000, Sepharose CL-2B, and PD-10 size exclusion columns (Phadex G-25) were from Amersham Biosciences (UK). Dithiothreitol (DTT) was from Melford Laboratories (Ipswich, UK), and cesium chloride was from Q-Biogene (Aylesford Ltd., Nottingham, UK).

**Saliva Collection and Determination of MUC5B Concentration**—Fresh whole human saliva was collected from 5–10 healthy donors and centrifuged at 2700 × g for 30 min at 4 °C to remove particulate matter. The supernatant was homogenized with a pH of 6.2–7.0. It was stored at 4 °C and was used within 5 days and showed no significant change in pH or properties over this time. MUC5B mucin concentration was determined by refractive index measurements as described previously (15).

**Viscosity and Molecular Weight Determination**—Three aliquots of saliva (15 ml) were dialyzed separately against: buffer A, 0.1 M NaCl, 20 mM Tris, pH 7.0; buffer B, the same buffer with 10 mM EGTA; or buffer C, the same buffer with 10 mM CaCl2. The dialyze of each sample was kept as control buffer. For molecular weight determination, the dialyzed samples were chromatographed on a Sephacryl S-1000 column kept as control buffer. For molecular weight determination, the dialysate of each sample was centrifugation in 4M GdmCl. This preparation was dialyzed into rele-

**Preparation of Samples for Tracer Diffusion Measurements**—For all tracer diffusion measurements, fluorescent polystyrene microspheres (average diameter 499 nm), preincubated with bovine serum albumin (15), were mixed, 2% (w/v), with the samples, and equilibrated over-

**Viscosity determinations were carried out in an Ostwald capillary viscometer at a constant temperature of 25 °C.** Samples were diluted in their respective buffers (A–C) to give concentration between 29 and 204 μg/ml. For each sample, the flow time in the viscometer was measured five times and averaged. Intrinsic viscosity [η] = lim t→∞ t/c - lim t→0 t/c = τ/c (Eq. 1)

Where t and c are sample and buffer flow times, respectively, and c is the MUC5B mucin concentration. The intrinsic viscosity [η] was measured by plotting the reduced viscosity as a function of mucin concent-

**MUC5B Mucin Preparation from Saliva under Non-denaturing Conditions**—MUC5B mucins were purified from saliva, by established meth-
ods (15). Saliva was initially equilibrated in 6M GdmCl and fraction-
harmed by photobleaching experiments were carried out using a confocal laser microscope (MRC-1000, Bio-Rad, Hemel Hempstead, UK) with an upright epifluorescence microscope (Optiphot 2, Nikon, Japan) (16). For tracer diffusion experiments, 10-μl samples containing 499-nm microspheres were sealed under glass coverslips on a flat microscope slide. The confocal-FRAP technique determines the lateral translational dif-

**Diffusion Measurements by Confo-FRAP—Fluorescence recovery after photobleaching** measurements in the presence or absence of 10 mM CaCl2, the concent-

To determine the effects of cations on tracer diffusion the salts of divalent cations (CaCl2, MgCl2, MnCl2, and ZnCl2) or EGTA were added to give final concentrations up to 4 mM. To assess the reversibility of the effects, after the addition of CaCl2, EGTA was added to a concentration equivalent to the CaCl2 and the confocal-FRAP measurements were repeated. To determine the effect of detergent on tracer diffusion, Trit-

**Calcium-dependent Cross-links in Mucus**—Three aliquots of saliva (15 ml) were dialyzed separately against: buffer A, 0.1 M NaCl, 20 mM Tris, pH 7.0; buffer B, the same buffer with 10 mM EGTA; or buffer C, the same buffer with 10 mM CaCl2. The dialyze of each sample was kept as control buffer. For molecular weight determination, the dialyzed samples were chromatographed on a Sephacryl S-1000 column (300 × 10 mm) eluted with their respective buffer (buffers A–C) at a flow rate of 12 ml/h. The average mass molecular mass was determined using an in-line multangle laser light-scattering (MALLS) photometer and a differential refractometer (Dawn DSP, Wyatt Technology, Santa Barbara, CA). For each sample, analysis of the weight average molecular weight was carried out by integrating the major high molecular weight mucin peak between 6 and 15 ml of elution volume with a refractive increment (Δn/dl) of 0.180 mls/ml. The high density fractions containing MUC5B mucin were pooled, dialyzed against 0.1M NaCl, pH 6.5, and part was concentrated against polyethylene glycol (5% w/v) and tested for calcium-

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**Diffusion Measurements by Confo-FRAP—Fluorescence recovery after photobleaching experiments, fluorescent polystyrene microspheres were sealed under glass coverslips on a flat microscope slide. The confocal-FRAP technique determines the lateral translational diffusion coefficients, which were calculated from the time dependence of the plots of the second moment of the radially averaged distribution of bleached fluorophore (16–18, 21). Five replicates were performed for each experiment. In the temperature dependence experiments the temperature was controlled using a cooling and heating system (PE-60, Linkam Instruments, Tadworth, UK) attached to the slide holder on the confocal microscope.

The concentration dependence of translational diffusion coefficients of the tracer in saliva and purified MUC5B was calculated using the scaling equation for tracer diffusion in semi-dilute polymer networks (20) in Equation 2,

\[ D = D_0 \exp(-\beta\xi) \]  

(2)

where D is the measured apparent lateral translational tracer diffusion coefficient, \( D_0 \) is its free diffusion coefficient, and \( \beta \) and \( \nu \) are empirical constants and are related to the average pore size of the network \( \xi \) (Equation 3), which describes the distance between chain entanglement points (22) in Equation 3.

\[ \xi = -d \ln(D/D_0) \]  

(3)

In this equation \( d \) is the diameter of the tracer. This assumes that the polymer forms a regular network of linked strands. For polymers, such as mucin, the network formed by the mucin-coexisting protein and the network formed would be dynamic, \( \xi \) is an average dimension representing a distribution of apparent pore sizes. In semi-dilute solutions of polymers, this analysis of tracer diffusion, which gives an apparent pore size, therefore provides a combined measure of chain
entanglement, together with any additional effect of specific interchain association.

**Calcium Binding**—Calcium binding to the high density MUC5B mucin fraction purified under mild non-denaturing conditions was assessed by size exclusion chromatography. To the mucin fraction (500 μl at 50 μg/ml in 0.1 mM NaCl, pH 6.5) 45CaCl2 (3.256 Ci/mmol) was added to a final concentration of 70 μM and incubated overnight at room temperature. The sample was chromatographed on a column of Sephrose CL-2B (6 × 100 mm) eluted in 0.1 mM NaCl, pH 6.5. Fractions (250 μl) were collected, and the radioactivity was measured by scintillation counting. Calcium binding was also determined as above after treatment of the high density mucin fraction with 6 M GdmCl. A sample of the mucin was dialyzed into 6 M GdmCl (pH 6.2) overnight at 4 °C and then back into 0.1 mM NaCl, pH 6.5, prior to addition of radioactive 45CaCl2. The high density mucin fraction was also treated by adding 10 mM EGTA (open circles) or 10 mM CaCl2 (filled circles) and causing an increase in tracer diffusion. The calculated apparent average pore size (ε) of the saliva was determined from the tracer diffusion measurements using Equation 3. Symbols are defined as in A, C, the tracer diffusion of microspheres (499 nm) in saliva at different concentrations of calcium chloride (filled circles) or EGTA (open circles). D, the calculated apparent average pore size (ε) of saliva at different concentration of calcium chloride or EGTA (symbols as in C) was determined as described above. All measurements were performed by confocal-FRAP in 0.1 mM NaCl, pH 6.2–7.0 at 25 °C, and the error bars show the standard deviation to the calculated mean.

**RESULTS**

Previous work measuring tracer diffusion of fluorescent polystyrene microspheres by confocal-FRAP showed that GdmCl-purified salivary MUC5B mucin was much more permeable to tracer diffusion than saliva at similar concentrations of MUC5B mucin (15). We therefore investigated the properties of saliva further to discover the basis for this difference in molecular organization. Tracer diffusion in saliva and GdmCl-purified MUC5B solution showed a concentration-dependent reduction in diffusion of microspheres (499-nm diameter) (Fig. 1A). This followed the general form predicted for entanglement of high molecular weight polymers (see Equation 2) (20). From the results of tracer diffusion the change in apparent pore size with concentration was calculated (see “Experimental Procedures,” Equation 3) (Fig. 1B). This showed that the apparent pore size in GdmCl-purified MUC5B solution was four times larger than in saliva, when extrapolated to similar MUC5B concentration (100 μg/ml) close to that found physiologically. The difference was not due to ionic strength effects, because both preparations were analyzed in 0.1 mM NaCl. To understand the basis for the difference in apparent pore size, we carried out further tracer diffusion experiments on saliva at concentrations of MUC5B in the mid-range of the concentration-dependence curve (45–55 μg/ml, Fig. 1A). At this concentration any change in tracer diffusion therefore reflects changes in the organization of the macromolecules in saliva that determine the rate of diffusion.

**Effect of Calcium and EGTA on Saliva**—The addition of CaCl2, up to 4 mM to saliva in 0.1 mM NaCl, caused a major decrease in the diffusion of the microspheres, which fell from 5.6 × 10−9 to 1.8 × 10−9 cm2.s−1 (Fig. 1C). From these diffusion measurements, the apparent pore size in saliva (calculated from Equation 3, see “Experimental Procedures”) decreased from ~1200 to 300 nm after addition of 4 mM CaCl2 (Fig. 1D). In a similar experiment at the same saliva concentration, adding EGTA increased tracer diffusion (Fig. 1C), and at over 3 mM EGTA the diffusion became equal to the free diffusion. The result suggested that EGTA was chelating calcium in saliva and causing an increase in tracer diffusion. The calculated apparent pore size increased from ~1200 to 7000 nm in the presence of 2.5 mM EGTA (Fig. 1D). The apparent pore size of saliva from tracer diffusion measurements was thus increased six times by EGTA. These major changes in tracer diffusion at a constant concentration of the saliva suggested that calcium has a major effect on the organization of the macromolecules in saliva. The reversibility of this effect was shown by confocal-FRAP analysis of a sample treated with EGTA and then re-equilibrated with calcium and re-tested. The tracer diffusion increased by EGTA returned to a low rate in the presence of...
The tracer diffusion values were fitted using a non-linear least square analysis to the scaling equation for tracer diffusion in a semi-dilute polymer network (Equation 2). Critical concentration was estimated from the concentration at which diffusion became concentration dependent for each tracer. The apparent average pore size of MUC5B at physiological concentration (100 μg/ml) was estimated from Equation 3.

### Table I

**Analysis of tracer diffusion**

The tracer diffusion values were fitted using a non-linear least square analysis to the scaling equation for tracer diffusion in a semi-dilute polymer network (Equation 2). Critical concentration was estimated from the concentration at which diffusion became concentration dependent for each tracer. The apparent average pore size of MUC5B at physiological concentration (100 μg/ml) was estimated from Equation 3.

| Critical concentration (μg/ml) | Lateral diffusion D₀ × 10⁻⁹ cm² s⁻¹ | Estimated size (ℓ) at 100 μg/ml of MUC5B nm |
|-----------------------------|--------------------------|------------------|
| Saliva                      | 36.9                     | 10.9             |
| Saliva plus 10 mM CaCl₂     | 5.5                      | 8.6              |
| Saliva plus 10 mM EGTA      | 240                      | 12.9             |
| GdmCl MUC5B                 | 324                      | 12.9             |
| GdmCl MUC5B plus 10 mM CaCl₂ | 320                     | 13.2             |

### Table II

**Viscosity and molecular weight distribution**

The intrinsic viscosity [η] of saliva, saliva with CaCl₂, and saliva with EGTA was calculated by extrapolation of the fitted linear function at zero concentration (Fig. 2). Weight average molecular weight determinations were from MALLS analysis of the samples (as above) by integrating data from the high molecular weight fraction eluting between 6 and 15 ml (Fig. 2).

| [η] (mΠ g⁻¹) | Weight average molecular weight (× 10⁶) |
|--------------|--------------------------------------|
| Saliva       | 1033                                  |
| Saliva plus 10 mM CaCl₂ | 1203                               |
| Saliva plus 10 mM EGTA     | 733                                   |

Cation (results not shown). The calcium effect was independent of pH in the neutral range pH 6–8 (results not shown). The experiments suggested that calcium was easily bound and removed from sites of interaction and that the change in macromolecular organization in saliva was freely reversible.

A more complete assessment of the effects of calcium on tracer diffusion over the full range of saliva concentration was obtained (Fig. 1A). There was a major reduction of tracer diffusion throughout the concentration range compared with untreated saliva, with the effect detected at very low concentrations of MUC5B (5.5 μg/ml). In contrast, in the presence of EGTA, the saliva was much more permeable to tracer diffusion, and effects on microsphere diffusion were only detected above 240 μg/ml (Fig. 1A). From these results the apparent pore size showed approximately a 20-fold difference between EGTA- and CaCl₂-treated saliva (Table I) when compared at a physiological concentration. Interestingly, the tracer diffusion of the microspheres in GdmCl-purified MUC5B mucin solution showed a similar concentration dependence as in saliva treated with EGTA (Fig. 1A).

These initial observations of calcium effects on tracer diffusion were investigated further by measuring two other independent properties. The viscosity of saliva was similar to that previously determined by others under similar ionic conditions (23, 24). However, with the addition of 10 mM CaCl₂, there was a large increase in intrinsic viscosity (1033 to 1233 mΠ g⁻¹), and with 10 mM EGTA there was a large decrease in intrinsic viscosity (1033 to 733 mΠ g⁻¹) (Fig. 2A and Table II). The presence of calcium thus caused the saliva to be more viscous. Analysis of native saliva by gel filtration on S-1000 combined with MALLS analysis showed it to contain a high molecular weight fraction with an approximate weight average molecular weight of 28 × 10⁶ in the presence of EGTA (10 mM) (Fig. 2B and Table II). Calcium thus caused a major increase in (a) the viscosity of saliva and (b) the average molecular weight of the high molecular weight components in saliva.

**Effect of Solvent Ionic Strength and Divalent Cations (Mg²⁺, Mn²⁺, and Zn²⁺) on Tracer Diffusion in Saliva**—The effect of calcium on tracer diffusion in saliva was unaffected by NaCl concentration 0.1 to 2 mM (Fig. 3A). The calcium effect was thus unlikely to be due to simple electrostatic interaction among the macromolecules within saliva. The effects of other divalent ions...
were investigated. With addition of MgCl₂, MnCl₂, and ZnCl₂ up to 4 mM there was no change in tracer diffusion (Fig. 3B). The results showed that other common divalent metal ions cannot substitute for calcium ions and therefore that calcium has a specific effect on macromolecular organization in saliva.

Effect of Temperature on Tracer Diffusion in Saliva—Tracer diffusion was determined on saliva equilibrated at different temperature (15–52.5 °C) (Fig. 3C). As the temperature increased the tracer diffusion of the microspheres increased by 20% over the full range of temperature, when corrected for the effect of viscosity changes of the solvent on tracer diffusion. The effect was reversible, because on cooling the diffusion of the microspheres decreased and returned to the initial value. From these data there was an increase in the apparent pore size with temperature from ~850 nm at 15 °C to 1360 nm at 52.5 °C (Equation 3). These results suggested that the porosity of saliva to tracer diffusion was dependent on a thermodynamic equilibrium of calcium binding that was reversibly affected by temperature with partial dissociation at 52.5 °C compared with 15 °C.

Effect of Triton X-100 on Tracer Diffusion in Saliva—To investigate the characteristics of the calcium-dependent interactions that maintained saliva properties, we investigated the effect of the non-ionic detergent, Triton X-100 (Fig. 3D). Microsphere tracer diffusion was not influenced by the presence of Triton up to 2% (v/v). This gave no evidence that lipid components, which might be associated with the mucin, had any role in the calcium-dependent effect on saliva.

Calcium Binding to Salivary Mucins—To test for calcium binding to the macromolecules in saliva, fractionation of the saliva was carried out by CsCl equilibrium density gradient centrifugation under mild non-denaturing conditions to separate the high density fractions containing MUC5B mucin from other non-associated proteins. Positive ion ESI-MS-MS analysis of tryptic peptides derived from this high density fraction indicated that it was enriched in MUC5B (27 peptides) but also contained lesser amounts of MUC7 (one peptide), cystatin (two peptides), amylase (two peptides), and Gp-340 (one peptide). Chromatography of this fraction on Sepharose CL-2B after incubation with radioactive⁴⁵CaCl₂ showed there was calcium binding to a high molecular weight fraction eluting in the void of the column (Fig. 4). This showed that the calcium binding remained with the MUC5B fraction. The high density MUC5B mucin fraction was also purified further by size exclusion chromatography on Sepharose CL-2B in the presence of 10 mM EGTA. Because of the different size exclusion limits of S-1000 (Fig 2B) and Sepharose CL-2B (see Fig. 4), the major high molecular weight fraction in the presence of EGTA still eluted close to the void volume of the column and this fraction retained the calcium-dependent effects on tracer diffusion. It showed a decrease in tracer diffusion coefficient of 35% in the presence of 10 mM CaCl₂ (8.68 ± 0.30 × 10⁻⁹ to 5.64 ± 0.18 10⁻⁹ cm² s⁻¹), which showed that the effect was not dependent on a low molecular weight MUC5B-associated protein released by EGTA.

The high molecular weight MUC5B fraction recovered from the void volume after Sepharose CL-2B chromatography in the presence of CaCl₂ (10 mM) was analyzed after trypsin digestion by positive ion ESI-MS-MS. The results showed that it was more purified and lacked the trace components present in the high density mucin fraction. MUC5B was the only protein detected, and of the 19 MUC5B peptides identified 18 were unique sequences and only one was from a repeated sequence (Table III). Even though MUC5B has a very high molecular weight, this technique would reveal other proteins (however small), if they were present at comparable molar amounts.
Density MUC5B mucin fraction was determined by incubation with 45CaCl2. The high density MUC5B mucin fraction, after mild reduction with DTT (1 h at 37°C), and after DTT treatment (open triangles) and after DTT treatment (plain triangles), were collected and their radioactivity was determined by scintillation counting.

Calcium-dependent Cross-links in Mucus

Previously we showed by confocal-FRAP measurements of tracer diffusion that concentrated solutions of GdmCl-purified MUC5B did not replicate the properties of saliva (15). It was therefore clear that additional factors must play an important role in the organization of the mucins in saliva, and we present evidence here that calcium-dependent interactions were responsible for this difference in properties. Calcium had a major role in the permeability of saliva to tracer diffusion causing a 20-fold change in the apparent pore size of saliva from a calcium-depleted to a calcium-saturated state. The effects of calcium on the tracer diffusion in saliva were reversible after treatment by EGTA and were specific to calcium. Furthermore, the addition of calcium to saliva resulted in the formation of a high molecular weight macromolecular fraction with a 6-fold increase in weight average molecular weight and a large increase in intrinsic viscosity.

The calcium-mediated effect on tracer diffusion in saliva was maintained at ionic strength from 0.1 M up to 2.0 M NaCl. This distinguishes the present results from previous reports (3, 26) describing effects on mucin viscosity and rheological properties with increasing NaCl concentrations. These may have resulted from electrostatic interactions between mucins at ionic strengths, below those used in this study. The results in 2% Triton X-100 also showed no evidence for interactions due to lipid, as has been reported with high concentration of purified mucin (40 mg/ml) (27).

**TABLE III**

Peptides identified by ESI-MS-MS analysis

The sample analyzed was a trypptic digest of the MUC5B mucin fraction purified in two stages. First, under non-denaturing conditions by CsCl density gradient centrifugation, and second, by size exclusion chromatography (in the presence of calcium) on Sepharose CL-2B (see text). The parent and fragment y' ion masses were within 50 mDa of the theoretical values.

| Sequence         | Position in MUC5B polypeptide |
|------------------|------------------------------|
| TFDGDVFR         | 87–94                        |
| AAYEDFVNLQR      | 109–119                      |
| EELPSR           | 155–161                      |
| TGLLVEQSGDYIK    | 162–174                      |
| LTPQPGNQLQK      | 226–236                      |
| CPELPFR          | 321–327                      |
| AVTSLDDDGTAIR    | 481–494                      |
| LTDPNSAFSR       | 626–635                      |
| GVQLSDWR         | 678–685                      |
| GPGQDPKY         | 978–986                      |
| TSVFIR           | 1009–1014                    |
| KTSVFIR          | 1008–1014                    |
| LSPSCDALARPK     | 1058–1069                    |
| DPCANTFR         | 1070–1078                    |
| DGNYYDVGAR       | 1225–1234                    |
| SEQLGDVESYDK     | 1521–1533                    |
| NWEQGQVFK        | 1579–1587                    |
| AQAAQGVQPLR      | 2362–2371, 2891–2900, 3589–3598 |
| TWLVPDSR         | 5190–5197                    |

These results suggest that the calcium binding is most likely a property of MUC5B mucin.

Treatment of the high density mucin fraction with 6 M GdmCl overnight, followed by equilibration in 0.1 M NaCl prior to Sepharose CL-2B chromatography resulted in 90% loss of calcium binding (Fig. 4). This important result suggested that GdmCl treatment, frequently used during MUC5B mucin purification, caused the irreversible loss of calcium-dependent properties. Measurements of tracer diffusion in GdmCl-purified mucin confirmed that there was no effect of calcium (Fig. 1, A and B). Calcium binding was also largely lost from the MUC5B fraction after mild reduction with DTT (1 h at 37°C) (Fig. 4).

The affinity of calcium binding to a sample of the high density MUC5B mucin fraction was determined by incubation with different concentrations of radioactive 45CaCl2 (0.2 to 5 μM) and chromatography on PD-10 columns. Scatchard analysis of the calcium bound at different concentrations gave an estimate of the dissociation constant (Kd) of calcium binding to the mucin fraction of ~7 x 10^-6 M (Fig. 5). The approximate concentration of MUC5B in this fraction was 7 x 10^-6 M (using 2.1 x 10^6 as the MUC5B mucin subunit molecular weight (15)), and from this concentration the number of calcium binding sites was calculated to be 0.8 binding sites per mole of MUC5B subunit. These results suggested that MUC5B contained a protein site that was responsible for calcium binding and was sensitive to mild chemical reduction and to denaturation by GdmCl.

**DISCUSSION**
The reversibility of the calcium effect on the saliva properties suggested it to be governed by an equilibrium, dependent on free calcium concentration. Removal of calcium in saliva by EGTA increased its porosity to tracer diffusion to become similar to that of GdmCl-purified MUC5B mucin. Furthermore, this also caused a decrease in the intrinsic viscosity (733 ml/g), compared with “native” (1033 ml/g) and calcium-supplemented (1203 ml/g) saliva. Further experiments confirmed that GdmCl-purified MUC5B from saliva lacked any calcium effect on tracer diffusion. This appeared to be due to the loss of calcium binding, because treatment with 6 M GdmCl destroyed the calcium binding of MUC5B mucins prepared in the absence of chaotropics. Because GdmCl treatment destroys calcium binding, it accounts for the good agreement between the intrinsic viscosity of GdmCl-purified cervical mucins (28), which have also been shown to be largely MUC5B (29), and that found for saliva in EGTA (635 and 733 ml/g, respectively).

The results from tracer diffusion, molecular weight analysis, and intrinsic viscosity showed that calcium-dependent interactions had a major effect on saliva properties. The calcium-dependent properties initially detected in saliva were shown to be retained in MUC5B mucin purified by equilibrium density gradient centrifugation and by size exclusion chromatography. Furthermore, calcium binding was present in the high density MUC5B fraction and was retained in the high molecular weight size exclusion fraction derived from it. Analysis of these fractions by mass spectrometry showed MUC5B to be the only protein detected in the most pure fraction. From these results it appeared that calcium binding was a property of MUC5B mucin and that calcium mediated the formation of intermolecular cross-links between the oligomeric MUC5B mucins. This would be compatible with the observed calcium effects on saliva, including an increase in average molecular weight, an increase in intrinsic viscosity, and a decrease in tracer diffusion. Intermolecular cross-links between oligomeric MUC5B mucin may result in the formation of branched structures, but the present results cannot rule out the formation of much larger structures (unbranched) oligomeric MUC5B assemblies. Further experiments are required to discriminate between these models.

The involvement of calcium in the structure of mucus has been postulated by other workers. Energy dispersive x-ray analysis of samples of saliva has previously revealed increased calcium in the structural areas of the visualized polymer network (11). In addition, it has also been reported that an increase in the concentration of calcium from 2 to 4 mM produced a 4-fold decrease in the diffusion of polymer mucin in mucus (2), and cross-linking between mucins has been proposed as a likely explanation. Although some changes in mucus viscosity in the presence of calcium have previously been noted in addition to increased solubility and dissociation of mucin complexes in the presence of EDTA (4, 30—32), there has been no clear recognition of the role of calcium as described in the present study. Previous studies associating calcium with mucus secretion have not resulted in any characterization of calcium binding in saliva or in purified mucins with the features identified in this study. The calcium binding identified here has the properties of interaction with a specific protein site, because it is irreversibly denatured in 6 M GdmCl and by the mild reduction, such as for disulfide bonds. The binding had a $K_d$ of about $7 \times 10^{-6}$ M, and it was specific for calcium.

Previous studies have reported calcium binding on mucins and mucus (4, 30, 31, 33—36), but the properties have generally lacked specificity and have been determined after exposure to denaturing conditions. Effects on purified mucins (3, 33—35) have shown the characteristics of electrostatic interactions involving carbohydrate structures such as sialic acids (3, 33, 35) and have not been specific for calcium (3, 31). The standard methods developed for mucin purification (5, 37, 38) have routinely used 6 M GdmCl as a denaturing solvent to ensure the removal of associated proteins. Present results show that such treatment destroys the calcium effects in MUC5B preparations, but as the oligosaccharides remain intact this rules out their participation in the calcium-dependent effects on saliva. The present results thus suggest that calcium binding to protein sites in MUC5B mucin causes the assembly of MUC5B into larger structures. This is an organization in addition to the characterized covalent disulfide-bonded oligomeric structures of MUC5B. There are candidate calcium-binding domains in the MUC5B protein structure, including sequences related to epidermal growth factor-like domains and a C-lectin-like domain, which in other proteins have been shown to bind calcium (39).

The present results thus show that the saliva properties are strongly influenced by non-covalently calcium-dependent interactions under physiological conditions. Because the concentration of calcium in saliva is between 1 and 2 mM, the regulation of calcium concentration provides a mechanism that may control its physical properties. This calcium-mediated mechanism identified here may apply to other related sources of mucus containing the MUC5B mucin, such as respiratory and cervical mucus.

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