Phosphoproteins in the Parotid Saliva from the Subhuman Primate *Macaca fascicularis*

ISOlATION AND CHARACTERIZATION OF A PROLINE-RICH PHOSPHOGLYCOPROTEIN AND THE COMPLETE COVALENT STRUCTURE OF A PROLINE-RICH PHOSPHOPEPTIDE*

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Parotid saliva from the cynomolgus monkey (*Macaca fascicularis*) and from pooled human collections displayed the same groups of proteins when fractionated by anion exchange and gel filtration chromatography. We have isolated and characterized a proline-rich phosphoglycoprotein (MPRP) and a proline-rich phosphopeptide (M-statherin) from macaque parotid saliva. MPRP has an apparent molecular weight of 16,900 and displays an unusual chemical composition. It is enriched in proline, glycine, and acidic amino acids, but lacks cysteine, methionine, and tyrosine. MPRP contains 25% (w/w) carbohydrate with 7.6 mol of neutral hexoses, 3.3 mol of galactosamine, 5.9 mol of sialic acid, and 3 mol of phosphorus/mol of protein. M-statherin is a 42-residue phosphopeptide with a high proline, glutamic acid, and tyrosine content, but which lacks threonine, valine, cysteine, methionine, isoleucine, and histidine. The complete covalent structure of M-statherin (Mr = 5,368) is:

\[
\begin{align*}
&\text{NH}_2\text{-Asp-Pse-Pse-Glu-Glu-Lys-Phe-Leu-Arg-Arg-Leu-Arg-Arg-Phe-}\,\text{Asp-Glu-Gly-Arg-Tyr-Gly-Pro-Tyr-Gln-Pro-Phe-Ala-Pro-Gln-Pro-Leu-Tyr-Pro-Gln-Pro-Tyr-Gln-Pro-Gln-Tyr-COOH}
\end{align*}
\]

This is the first complete amino acid sequence of a component in the salivary secretion of a subhuman primate. Phosphoserine occurs at residues 2 and 3. All 13 acidic and basic amino acids are located in the NH\(_2\)-terminal half of the molecule. The carboxyl-terminal half of the molecule is hydrophobic where the tripeptide Tyr-Gln-Pro is repeated three times, the dipeptide Gln-Pro occurs twice, and the tripeptides Tyr-Gly-Pro, Phe-Ala-Pro, and Leu-Tyr-Pro occur once. Evaluation of secondary structure by the Chou-Fasman method predicts an \(
\alpha\ \text{helix in the NH}_2\)-terminal half (residue 4-16) and a \(\beta\ \text{pleated sheet in the carboxyl-terminal half (residues 22-26; 38-42) of the molecule.}

Both MPRP and M-statherin inhibit spontaneous and seeded precipitation from solutions supersaturated with respect to calcium phosphate salts. This suggests that these macaque compounds may function by maintaining saliva supersaturated with respect to calcium phosphate salts, a necessary requirement for stabilization of hydroxyapatite in the surface layers of teeth.

The acidic proline-rich proteins and statherin are among the few human salivary proteins which have been purified to homogeneity and extensively characterized (1-3). These proteins are major constituents of both parotid and submandibular secretions and display unusual amino acid compositions. In the four major proline-rich proteins (I, II, III, and IV), proline, glycine, and glutamic acid account for 60-75% of the amino acid residues (1), while in statherin, proline, glutamic acid, and threonine account for 56% of the amino acid residues.

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The abbreviations used are: PRP, human proline-rich protein; MPRP, macaque proline-rich protein; PTH, phenylthiohydantoin.
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The oral cavity. In order to find an animal model in which to investigate these processes, we have examined the acidic proteins in the parotid saliva of the subhuman primate Macaca fascicularis. This species has been shown to develop a variety of human diseases under experimental conditions, including periodontitis and caries (13).

The present investigation describes the isolation and characterization of a proline-rich phosphoglycoprotein and a proline-rich phosphopeptide from the parotid secretion of M. fascicularis. In addition, the complete amino acid sequence of M-statherin is described as well as the functional characteristics of both MPRP and M-statherin.

EXPERIMENTAL PROCEDURES

Materials

Sodium pentobarbital (Nembutal sodium) was obtained from Abbott. Glucosamine, N-acetylneuraminic acid, and carboxypeptidase A were purchased from Sigma, and glucosamine was obtained from Eastman. Glucose, mannose, galactose, and L-fucose were obtained from Pfaltz & Bauer. Escherichia coli alkaline phosphatase and 1-tosylamido-2-phenylethyl chloromethyl ketone-trypsin were obtained from Worthington. Purified α-actin glycoprotein was kindly provided by Dr. Karl Schmid, Boston University, and cyanogen bromide peptides of α,1(I) chick skin collagen were a gift from Dr. John Higberger of Massachusetts General Hospital. DEAE-Sephadex A-25 and Sephadex (2-75 were purchased from Pharmacia, and Bio-Gel P-2, P-6, A-1.5, and DEAE-Bio-Gel A were from Bio-Rad. Sequencer chemicals and polyethylene were purchased from Pierce. All other reagents were of analytical grade.

Collection of Saliva

To obtain parotid saliva from the subhuman primate M. fascicularis, a Cadron-Crittenden device (14), half the size as described for the collection of human parotid secretion, was constructed from Delrin. Under sodium pentobarbital anesthesia (23 mg/kg), secretion was stimulated with pilocarpine (1.5 mg/kg) and collected from both parotid glands simultaneously into ice-chilled graduated cylinders. The weekly collections of parotid saliva samples ranged between 20 and 200 ml, and finally increased for 10.8 h to reach 1.5 M and kept constant at this concentration for the last 4.8 h. Fractions were collected at 20-min intervals and those containing MPRP were pooled, dialyzed against water at 4°C, lyophilized, and stored at -16°C.

Isolation of Macaque Statherin

M-statherin was isolated by two different methods.

Method 1—Chromatography of parotid saliva on a DEAE-Sephadex A-25 column (as described for the isolation of MPRP) yielded crude M-statherin (see Fig. 1B). This material was further purified by chromatography on a Sephadex G-75 column (as described for the isolation of MPRP). M-statherin eluting from Sephadex G-75 was desalted on a Bio-Gel P-2 column in 0.05 M NH₄HCO₃, pH 8.0, lyophilized, and stored at -16°C until used.

Method 2—Undialyzed, lyophilized parotid saliva (300 mg) was dissolved in 5% formic acid, clarified by centrifugation, and subjected to gel filtration on a Bio-Gel P-6 column (2.6 x 60 cm) at a flow rate of 19.2 ml/h in 3% formic acid. The column was monitored at 280 nm. Fractions containing M-statherin were subjected to anion exchange chromatography on Bio-Gel DEAE-agarose in 0.05 M Tris-HCl, pH 8.0, containing 0.025 M NaCl and 0.5% chloroform. The column (1.6 x 34 cm) was eluted using chromatography buffer with a linear gradient from 0.025-0.8 M NaCl and a flow rate of 15 ml/hr. Desalting was achieved by gel filtration on a Bio-Gel P-2 column in 0.05 M NH₄HCO₃, pH 8.0, as above.

Polycrylamide Gel Electrophoresis

Electrophoresis in 7.5% polyacrylamide gel was performed as described by Davis (15), which includes the use of a stacking gel. Lyophilized protein samples were dissolved in a solution of water, stacking gel buffer, and 40% sucrose in a ratio of 3:1:4, allowing 0.2 ml of this mixture to be layered in the presence of electrophoresis buffer on top of the stacking gel. For the electrophoretic analysis of proteins during the chromatographic fractionations, aliquots of 0.3 ml of each fraction were combined with 0.1 ml of 40% sucrose and 0.2 ml of this mixture was applied per gel column. Electrophoresis was carried out at 4 mA/tube and terminated when the bromphenol blue band reached a position 3 mm from the anodic end. The gels were fixed and stained in a solution of 0.25% amido black in 7% acetic acid. Destaining was accomplished by diffusion in 7% acetic acid for 18 to 24 h.

For an initial survey of carbohydrate content, gel electrophoretograms of pure MPRP and M-statherin were fixed and stained using the periodic acid-Schiff procedure as described by Segrest and Jackson (16). As controls bovine serum albumin and plasma α-acid glycoprotein were used.

Amino Acid Analysis

Protein samples of 0.1 to 0.2 mg were hydrolyzed in 1 ml of constant boiling HCl at 108°C for 24, 48, and 72 h in evacuated tubes. Quantitative analyses were carried out on a Beckman 119CL amino acid analyzer using a one-column system or on a Jelco 6AH amino acid analyzer using a two-column system. The values for threonine and serine were determined by extrapolation to zero time hydrolysis.

Carbohydrate Analysis

Neutral sugar was determined by the anthrone procedure as described by Shields and Burnett (17) using an equimolar mixture of mannose, galactose, and glucose as standard. Fucose was measured by the Dische-Shettles cysteine-sulfuric acid method as described by Sprio (18). Sialic acid was determined by the method of Warren (19) with N-acetylneuraminic acid as standard. Hexosamines were determined by hydrolyzing samples in 4 N HCl for 8 h at 100°C. Quantitative analyses were performed on the amino acid analyzer by a single-column elution program using as standards glucosamine and galactosamine in a ratio of 2:1.

Phosphate Determination

Protein samples were ashed using magnesium nitrate according to the method of Ames and Dubin (20). Aliquots of protein and stand-
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ards, with or without hydrolysis, were subjected to phosphate determination as described by Chen et al. (21). Anhydrous Na₂HPO₄ served as a standard and lysozyme and pepsin as controls. Absorbance measurements were carried out on a Beckman Model 25 Spectrophotometer at 660 nm.

Molecular Weight

The molecular weight of macaque proline-rich protein was determined by gel filtration according to the method of Rao and Adams (22). Protein samples were dissolved in 1 ml of buffer, 0.05 M Tris-HCl, pH 7.5, containing 1.0 M CaCl₂, and subjected to chromatography on a Bio-Gel A-1.5 column (1.6 × 92.5 cm). The column was equilibrated with the same buffer and eluted in upward flow at 10.8 ml/h at room temperature. Calibration was carried out employing blue dextran 2000 and the addition of 10 μl of tritiated water to the protein samples. The column effluent was monitored continuously at 230 nm as described earlier. The cyanogen bromide peptides CB6, CB3, CB7, and CB6B derived from the α₁(I) chain of chick skin collagen and PRP III served as standards.

Automated Sequential Degradation

Automated Edman degradation (23) was carried out on a Beckman 890 C Sequencer using Program No. 121078 with 0.25 M Quadrol (N,N,N',N'-tetrakis(2-hydroxypropyl)ethylenediamine) and a combined SI and S₆ wash. Samples were placed in the cup with Beckman Sample Application Program No. 02772. Polybrene (3 mg) was placed in the cup and one complete cycle was run followed by application of the sample, which in each case was subjected to double coupling. The phenylthiohydantoin of norleucine (33 nmol) was added to each tube.

**FIG. 1.** Anion exchange chromatography on DEAE-Sephadex A-25 of 1 g of human (A) and 1 g of M. fascicularis (B) parotid saliva protein. Procedural details are described under "Experimental Procedures." The dashed line shows the linear NaCl gradient monitored by continuous conductivity measurements. HIS, histidine-rich peptide; STAT, statherin; MSTAT, macaque statherin.
in the fraction collector prior to sequencing to serve as an internal standard. Conversion to the PTH-derivatives was performed as described (24). PTH-derivatives were identified by high pressure liquid chromatography (25), gas liquid chromatography (26), thin layer chromatography (27), or back hydrolysis (28).

Escherichia coli Alkaline Phosphatase Digestion

Approximately 24 nmol of M-statherin were dissolved in 1.0 ml of distilled water, and 2.0 ml of 1.5 M Tris-HCl, pH 8.0, and 7.5 μl of enzyme (13.4 mg/ml; 40 units/mg) were added. The reaction mixture

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**FIG. 2. Purification of MPRP.** Procedural details are described under “Experimental Procedures” A, elution profile of crude MPRP (fractions 35-41, Fig. 1B) from a Sephadex G-75 column. B, fractions 26-34 from A were pooled and the recovered protein was chromatographed on a DEAE-Sephadex A-25 column. Electrophoretically pure MPRP was recovered in fractions 97-113. The dashed line shows the complex NaCl gradient monitored by continuous conductivity measurements.
was incubated at 25°C for 90 min. After incubation the reaction mixture was dialyzed in Spectrapor 3 dialysis membrane against distilled water and lyophilized. The residue was dissolved in 0.3 ml of 15% acetic acid and subjected to automated sequential degradation (4 steps). In the control experiment, approximately 24 nmol of protein were dissolved in 0.3 ml of 15% acetic acid and subjected to automated sequential degradation as described above.

**Tryptic Digestion of M-statherin**

Trypsin digestion of M-statherin was carried out at an enzyme to substrate ratio of 1:5 in 0.05 M Tris-HCl, pH 8.0, containing 0.001 M CaCl₂ and 0.5% chloroform for 6 h at 37°C. Tryptic peptides were recovered by lyophilization and fractionated on a Bio-Gel P-2 column (1.6 x 85.5 cm) equilibrated in and developed with 0.05 M NH₄HCO₃, pH 8.0, at a flow rate of 9.9 ml/h.

**Carboxypeptidase A**

Carboxypeptidase A digestion of M-statherin was performed in 0.2 M N-ethylmorpholine acetate buffer, pH 8.5, at an enzyme to substrate ratio of 1:50. The concentration of M-statherin was 0.2 mg/ml. The 1.5-m1 reaction mixture was allowed to stand at room temperature and 0.2-ml aliquots were removed at each of 4 time periods (0.5, 30, 60 min). The pH of each aliquot was immediately adjusted to 2.0 with 1.0 N HCl. Following lyophilization, the residue was suspended in 0.01 M HCl, centrifuged, and free amino acids in the supernatant were detected by amino acid analysis on a Beckman 119 CI amino acid analyzer using a one-column system with lithium buffers (29).

**Inhibition of Calcium Phosphate Precipitation**

Both MPRP and M-statherin were tested for their ability to inhibit spontaneous precipitation from a solution saturated with respect to calcium phosphate salts, and to inhibit crystal growth in seeded systems. Both assays were performed essentially as described by Hay et al. (12), except that the former assay was carried out at 37°C instead of room temperature, and the calcium concentration used was 3.4 mM instead of 4.0 mM. Relative values of specific inhibitory activity were obtained by first testing a wide and then a narrow range of protein concentrations. From the data obtained values for 50% inhibition were calculated.

**RESULTS**

**Fractionation of Human and Macaque Proteins**

The elution profiles of 1 g of human and 1 g of macaque parotid saliva protein from a DEAE-Sephadex A-25 column carried out under identical chromatographic conditions are shown in Fig. 1. Overall, a remarkable similarity between the major protein groups obtained from the two species is evident. Careful comparison of the NaCl gradient with the elution pattern, and with the electrophoretograms of proteins contained in individual fractions, indicate that the major macaque parotid proteins display a net charge and molecular size or shape similar to those found in man.

For human parotid saliva (Fig. 1A), it has been established that fractions 9-15, representing the nonadsorbed proteins, contain α-amylase isoenzymes, lysozyme, partially characterized glycoproteins, and smaller basic proteins (30, 31, 33). In the very early phase of the salt gradient, IgA elutes in fractions 26-30 visible at the top of the disc gels. The dominant components eluting in fractions 31-37 are the 4 major proline-rich proteins (I-IV) and several minor proline-rich proteins (1, 32). The last major group of proteins eluting in this system is present in fractions 43-50, containing two partially overlapping proteins. These are known to be of low molecular weight and have been identified as the histidine-rich peptide (33) and statherin (3).

As noted above the elution profile of the major macaque parotid proteins (Fig. 1B) is remarkably similar to that observed for the human parotid proteins (Fig. 1A). The alignment and comparison of human and macaque elution profiles including their linear salt gradients indicate that macaque parotid secretion contains a single major protein (fractions 35-41) eluting in the same gradient range as the PRPs and a major component (fractions 44-48) eluting in the same gradient range as statherin. The chromatographic and electrophoretic behavior of these macaque proteins indicated that their molecular size and charge are very similar to those of

### Table I

| Amino acid | MPRP | PRP I | PRP III |
|------------|------|-------|---------|
| Aspartic acid | 12.1 | 10.9 (11) | 10.5 (11) |
| Threonine | 3.3 | 0 (0) | 0 (0) |
| Serine | 11.9 | 4.8 (6) | 4.0 (5) |
| Glutamic acid | 20.9 | 41.2 (40) | 30.6 (29) |
| Proline | 29.1 | 43.1 (41) | 25.9 (24) |
| Glycine | 16.0 | 32.3 (31) | 20.0 (20) |
| Alanine | 2.8 | 1.0 (1) | 1.1 (1) |
| Valine | 2.1 | 2.3 (3) | 2.6 (3) |
| Isoleucine | 1.8 | 1.5 (2) | 1.7 (2) |
| Leucine | 2.4 | 2.7 (3) | 3.3 (3) |
| Cysteine | 0 | 0 (0) | 0 (0) |
| Methionine | 0 | 0 (0) | 0 (0) |
| Phenylalanine | 1.9 | 1.0 (1) | 1.0 (1) |
| Tyrosine | 0 | 0 (0) | 0 (0) |
| Lysine | 6.4 | 2.4 (2) | 1.2 (1) |
| Histidine | 11.3 | 3.2 (3) | 2.1 (2) |
| Arginine | 4.1 | 6.2 (6) | 4.1 (4) |

### Table II

| Carbohydrate and phosphate composition of MPRP |
| Residues/1000 amino acids | % w/w | Residues/mol of protein* |
|--------------------------|-------|-------------------------|
| Neutral hexoses | 58.6 | 7.40 | 6.95 |
| Galactosamine | 45.3 | 6.84 | 5.34 |
| Glucosamine | 1.7 | 0.26 | 0.20 |
| L-Fucose | 1.4 | 0.15 | 0.16 |
| Sialic acid | 40.8 | 10.80 | 5.91 |
| Phosphorus | 25.7 | 0.58 | 3.16 |
| Amino acids | 1000.6 | 73.97 | 120.20 |

* Based on molecular weight of MPRP of 16,900.

**Fig. 3. Plot of molecular weight versus V_\text{elution} / V_{\text{in}}**

V_\text{elution} = elution volume of tritiated water (using a calibrated Bio-Gel A-1.5 column according to the procedure of Rao and Adams (22). The following cyanogen bromide peptides derived from α(1) chains of chick skin collagen served as standards. The molecular weights of CNBr peptides are given in parentheses: CB6 (7,900), CB3 (13,800), CB5 (24,800), CB7 (24,800).

35-41 eluting in the same gradient range as the PRPs and a major component (fractions 44-48) eluting in the same gradient range as statherin. The chromatographic and electrophoretic behavior of these macaque proteins indicated that their molecular size and charge are very similar to those of
Fig. 4

A

B
PRPs and statherin present in human parotid saliva. On this basis, the protein in fractions 35-41 (designated MPRP) was assumed to be the macaque counterpart of the human PRPs and that in fractions 44-48 (designated M-statherin) was assumed to be the macaque counterpart to statherin. It should be noted that the appearance of MPRP is different from that of PRPs by virtue of a characteristically broad, diffuse zone observed on all electrophoretograms at the various stages of purification.

**Isolation of MPRP**

The material in fractions 35-41 (crude MPRP) was further purified by gel filtration on Sephadex G-75 (Fig. 2A). The principal peak eluting shortly after the void volume contained MPRP together with two minor contaminants. In order to remove the minor contaminants from MPRP, the material contained in fractions 26-34 was subjected to anion exchange chromatography on DEAE-agarose (Bio-Gel A). The gradient was designed such that MPRP eluted within the shallow portion of the gradient. Electrophoretically pure MPRP was recovered from fractions 97-113. MPRP could be detected in disc gels with both the Amido Schwarz and periodic acid-Schiff stains, indicating the polypeptide chain contained a carbohydrate moiety.

**Amino Acid Composition of MPRP**

The amino acid composition of MPRP is shown in Table I. Based on 1 mol of phenylalanine/mol of protein, MPRP contains 125 amino acid residues and the polypeptide chain has a minimum molecular weight of 13,240. Proline, glutamic acid, and glycine are the most abundant amino acids, and cysteine, methionine, and tyrosine are not present. Comparison of the amino acid composition of MPRP with that of PRP I and III (Table I) reveals that all three proteins are enriched with respect to proline, glutamic acid, and glycine but lack cysteine, methionine, and tyrosine. This provides unmistakable evidence that MPRP and the PRPs are structurally related.

**Carbohydrate Composition of MPRP**

The carbohydrate composition of MPRP is given in Table II. The protein contains approximately 7 mol of neutral hexoses, 5 mol of galactosamine, 6 mol of sialic acid, and 3 mol of phosphorus/mol of protein. The combined weight of the carbohydrate units is 4,150. This indicates that the M, of MPRP is approximately 17,390 (i.e., minimum M, of polypeptide, 13,240; M, of carbohydrate, 4,150).

**Molecular Weight of MPRP**

Gel filtration of MPRP on a calibrated Sephadex G-75 column indicated that the apparent M, is approximately 40,000. This value is nearly three times greater than that expected from the amino acid and carbohydrate composition.
obtained from the amino acid sequence (PRP 111 does not

Interestingly enough, the apparent M, of PRP I11 in this system is

from the amino acid and carbohydrate compositions. Inter-

have a Carbohydrate moiety)

positions (22). The apparent M, of MPRP in this system,

calibrated with collagen CNBr peptides, was 16,400 (Fig. 3).

This is in good agreement with the value of 17,390 calculated from the amino acid sequence. The apparent M, of MPRP in this system, 10,800, also in good agreement with the true M, of 11,145, obtained from the amino acid sequence (PRP III does not have a carbohydrate moiety) (5).

### TABLE IV

**Automated Edman degradation of M-statherin**

| Step | Experiment 1 | Experiment 2 |
|------|--------------|--------------|
|      | Deduced amino acid | Method of identification | Yield | Deduced amino acid | Method of identification | Yield |
| 1    | Asp | H, T | 131.7 | Asp | H | 80.9 |
| 2    | X   | H   | 127.2 | X   | H | 84.1 |
| 3    | X   | H   | 127.2 | X   | H | 84.1 |
| 4    | Glu | H, A | 40.5 | Glu | H | 169.0 |
| 5    | Glu | H, A | 60.6 | Glu | H | 234.0 |
| 6    | Lys | H   | 78.1 | Lys | H | 83.6 |
| 7    | Phe | H   | 73.4 | Phe | H, G | 83.2 |
| 8    | Leu | H   | 61.7 | Leu | H, G | 118.0 |
| 9    | Arg | H   | 66.8 | Arg | H | 80.9 |
| 10   | Arg | H   | 66.8 | Arg | H | 80.9 |
| 11   | Leu | H   | 58.0 | Leu | H, G | 136.0 |
| 12   | Arg | H   | 66.8 | Arg | H | 80.9 |
| 13   | Arg | H   | 66.8 | Arg | H | 80.9 |
| 14   | Phe | H   | 50.2 | Phe | H, G | 66.9 |
| 15   | Asp | H, T | 41.7 | Asp | H | 28.5 |
| 16   | Glu | H   | 36.4 | Glu | H | 62.3 |
| 17   | Gly | H   | 42.2 | Gly | H, G | 24.7 |
| 18   | Arg | H   | 66.8 | Arg | H | 80.9 |
| 19   | Tyr | H   | 42.9 | Tyr | H | 45.2 |
| 20   | Gly | H   | 41.6 | Gly | H, G | 22.1 |
| 21   | Pro | H   | 28.3 | Pro | H, G | 38.0 |
| 22   | Tyr | H   | 42.8 | Tyr | H | 40.2 |
| 23   | Gln | H   | 38.0 | Gln | H | 29.4 |
| 24   | Pro | H   | 30.6 | Pro | H, G | 28.4 |
| 25   | Phe | H   | 36.9 | Phe | H, G | 27.0 |
| 26   | Ala | H   | 23.4 | Ala | H, G | 23.2 |
| 27   | Pro | H   | 29.9 | Pro | H, G | 19.1 |
| 28   | Gln | H   | 22.7 | Gln | H | 15.2 |
| 29   | Pro | H   | 28.8 | Pro | H, G | 18.6 |
| 30   | Leu | H   | 27.7 | Leu | H, G | 26.1 |
| 31   | Tyr | H   | 23.0 | Tyr | H | 15.7 |
| 32   | Pro | H   | 21.4 | Pro | H, G | 12.6 |
| 33   | Gln | H, A | 20.4 | Gln | H | 11.8 |
| 34   | (Pro) | H, A | Pro | H | 14.7 |
| 35   | Tyr | H, A | 21.8 | Tyr | H | 14.6 |
| 36   | Gln | H   | 17.5 | Gln | H | 12.5 |
| 37   | X   | H   | 27.7 | X   | H | 13.9 |
| 38   | Tyr | H, A | 21.1 | Tyr | H | 12.6 |
| 39   | Gln | H, A | 28.2 | Gln | H | 4.9 |
| 40   | Pro | H, A | 11.6 | Pro | H | 8.4 |
| 41   | X   | H   | 11.6 | Gln | H | 10.3 |
| 42   | (Tyr) | H, A | 11.9 | Tyr | H | 6.5 |

* X indicates an amino acid residue that could not be positively identified (22).

The PTH-derivatives were determined by high pressure liquid chromatography (H), gas-liquid chromatography (G), thin layer chromatography (T), or by amino acid analysis (A) after hydrolysis in hydrochloric acid. The yield indicated refers to the first method of identification listed.

The calculated repetitive yield between steps 7 and 25 in Experiments 1 and 2 was 96.4 and 92.8%, respectively.

* + indicates positive identification of PTH-arginine in the aqueous phase by high pressure liquid chromatography. Yield is not given because PTH-norepinephrine internal standard is recovered in the organic phase.

### TABLE V

**Automated Edman degradation of M-statherin before and after treatment with E. coli alkaline phosphatase**

| Step | Control | Alkaline phosphatase treated |
|------|---------|------------------------------|
|      | Amino acid | Yield | Amino acid | Yield |
|      | nmol | nmol |
| 1    | Asp | 21.5 | Asp | 13.9 |
| 2    | X   | Ser | 5.6 |
| 3    | X   | Ser | 4.0 |
| 4    | Glu | 12.2 | Glu | 12.4 |

**Isolation and Characterization of M-statherin**

M-statherin was isolated by two different procedures.

**Method 1**—The protein in fractions 44-48 (Fig. 1B) was assumed to be M-statherin because it eluted from the DEAE-Sephadex A-25 column and migrated on disc gels, similarly to that of human statherin (Fig. 1A). This material was lyophilized and subjected to gel filtration on Sephadex G-75 (Fig. 4A). Electrophoretically pure M-statherin eluted in fractions 30-37.

**Method 2**—Undialyzed, lyophilized macaque parotid saliva was chromatographed on a Bio-Gel P-6 column in 3% formic acid (Fig. 4B). M-statherin (identified by amino acid analysis, see below) eluted as a distinct peak in fractions 65-68. Its high K, indicates considerable retardation. After lyophilization the recovered material was dissolved in 0.05 M Tris-HCl, pH 8.0, containing 0.025 M NaCl and 0.5% CHCl, and subjected to anion exchange chromatography on Bio-Gel DEAE-agarose for final purification (Fig. 4C).

M-statherin isolated by either of the two separate procedures was indistinguishable with respect to its amino acid composition and amino acid sequence. Preparations of M-statherin failed to yield a positive periodic acid-Schiff staining reaction even with amounts of 40 μg of pure peptide/disc gel (results not shown), and it was concluded that a carbohydrate moiety was not present.

**Amino Acid Composition of M-statherin**

The amino acid composition of M-statherin indicated that the peptide contains 39 amino acid residues based on 1 mol of lysine/mol of peptide, and that the minimum M, is 4760 (Table III). More than 50% of the residues consist of proline, glutamic acid, and tyrosine, whereas methionine, cysteine, threonine, valine, isoleucine, and histidine are absent. It is evident that the composition of M-statherin is very similar to that of human statherin (Table III).
Fig. 6. Automated Edman degradation of M-statherin-derived tryptic peptides recovered by gel filtration (see Fig. 5). The yield (nanomoles) of each PTH-derivative is given below the individual residues. PTH-derivatives were identified by high pressure liquid chromatography. X indicates an amino acid residue not positively identified. Arginine residues in parentheses were deduced by reference to sequential degradation of intact M-statherin (see Table IV).

| Residue in M-statherin | Step |
|-----------------------|------|
| 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 |
| 1 - 6  Asp X X Glu Glu Asp 5.9 7.5 8.6 3.2 |
| 12 - 18  (Arg)Phe Arg Glu Gly (Arg) 4.3 4.3 4.3 |
| 14 - 18  Phe Asp Glu Gly (Arg) 4.3 4.3 4.3 |

Values for PRPs and statherin are taken from Hay et al. (12).

Amino Acid Sequence of M-statherin

M-statherin was subjected to 44 steps of automated Edman degradation (Table IV). A PTH-derivative could not be detected at steps 2 and 3 by high pressure liquid chromatography, although alanine was observed by amino acid analysis after back hydrolysis. Since PTH-alanine was not seen by high pressure liquid chromatography, and it is known that PTH-serine is converted to alanine during hydrolysis (36), residues 2 and 3 were tentatively identified as serine. It seemed likely that residues 2 and 3 might be phosphoserine, because phosphoserine occurs at these positions in human statherin (7).

Therefore, M-statherin was sequenced 4 steps, before and after incubation with E. coli alkaline phosphatase (Table V). PTH-serine could be positively identified at steps 2 and 3 (high pressure liquid chromatography) in the sample treated with enzyme, but not in the untreated sample. This experiment positively identified residues 2 and 3 as phosphoserine.

The amino acid sequence of M-statherin could be unambiguously deduced to residue 36 (Experiment 1) and residue 42 (Experiment 2) by automated Edman degradation of the intact peptide (Table IV). However, amino acid analyses indicated the peptide contains 39 amino acids (Table III) and the carboxyl-terminal region (Table IV) consisted entirely of repeating tripeptides containing proline. To confirm the amino acid sequence of the carboxyl-terminal region, tryptic peptides were prepared because cleavage at arginine at residue 18 would be expected to yield a large peptide containing the entire carboxyl-terminal region.

The elution profile of tryptic peptides from a Bio-Gel P-2 column is shown in Fig. 5. The material in the major peak was recovered and sequenced (Fig. 6). The sample contained a mixture of four tryptic peptides in nearly equal amounts.

One of these was the carboxyl-terminal peptide and the other three were small peptides with 5 or 6 amino acid residues. Nevertheless, the complete amino acid sequence of the carboxyl-terminal tryptic peptide could be deduced because at steps 1-6 the PTH-derivatives in the small peptides were different from those in the large peptide, and at steps 7-24 only one PTH-derivative was observed, yielding exactly the same amino acid sequence as that deduced by automated Edman degradation of the intact peptide.

Digestion of M-statherin with carboxypeptidase A released only tyrosine, as predicted from the carboxyl-terminal sequence, Pro-Gln-Tyr-COOH (see Table IV and Fig. 6).

Inhibition of Calcium Phosphate Precipitation

Both MPRP and M-statherin are potent inhibitors of spontaneous precipitation as well as crystal growth of calcium phosphate salts (Table VI). The 50% inhibition values indicate that MPRP is considerably more active in both assays than M-statherin. Its inhibitory activity is higher than M-statherin by factors of approximately 3 and 8 for spontaneous precipitation and crystal growth, respectively. As described (12) the four PRPs, assayed under similar conditions, show no inhibition of spontaneous calcium phosphate precipitation. However, the PRPs are effective inhibitors of crystal growth, but they require a protein concentration approximately 20-80 times greater than that of MPRP to cause 50% inhibition.

DISCUSSION

Several immunological and compositional similarities between partially purified components of salivary secretions from subhuman primates and man have been observed by other investigators (37). The present investigation is the first in which identical amounts of parotid saliva protein from a subhuman primate and man are compared under identical fractionation conditions. Both the anion exchange chromatograms and the electrophoretic patterns of the proteins from the two species reveal considerable similarities in molecular size and charge for most parotid proteins (Fig. 1, A and B). The elution patterns and the electrophoretograms of parotid saliva proteins of both M. fascicularis and man are of sufficient closeness that most components of the subhuman primate can be readily assigned to a group of proteins already described for human salivary secretions. The parameters of molecular size and charge were therefore used to predict which components in macaque parotid secretion represent the counterparts of the proline-rich proteins (I-IV) and statherin in parotid saliva of humans.

Both the proline-rich phosphoglycoprotein, MPRP, and the proline-rich phosphopeptide, M-statherin, have been purified to homogeneity from parotid saliva of M. fascicularis. It is noteworthy that both of these components could be identified chromatographically and electrophoretically in extraparotid saliva as well. This suggests that MPRP and M-statherin are
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also synthesized and secreted by the submandibular gland. Using a rabbit antiserum to MPRP (38), the concentration of MPRP was found to be 9% mg which contained 16% of the total pr in macaque parotid fluid.

MPRP—Our data show that there is a single, major anionic proline-rich protein in the parotid secretion of M. fascicularis. Pooled human parotid saliva, however, contains four distinct anionic proline-rich proteins (I-IV) lacking carbohydrate moieties. Azen and Oppenheim (39) have shown that these four major PRPs display genetic polymorphism as indicated by the fact that individual saliva samples from a large human population exhibited three phenotypes by either containing PRP I and III, PRP II and IV, or all four PRPs. Genetic analysis of the data revealed the inheritance of two codominant autosomal genes where the allele Pr; codes for PRP I and III and the allele Pr; codes for PRP II and IV, explaining the existence of 2 homozygous and 1 heterozygous phenotypes. As noted above, MPRP always displayed a single, broad band on disc gels. Definitive evidence that MPRP is a single polypeptide chain is derived from a single structural gene was obtained in preliminary work by NH2-terminal sequence determination (34) and immunochemical analysis (38). Furthermore, it can be demonstrated by proper alignment that at least 23 of the first 40 residues at the NH2 terminus of MPRP (34) and the PRPs (4-6, 35) are identical, showing that these proteins are phylogenetically related. The broad band of MPRP noted in disc gels, even after extensive purification, may be explained by the fact that MPRP is a glycoprotein whose electrophoretic pattern may be the result of microheterogeneity in the carbohydrate units.

Differences in degrees of glycosylation of MPRP could also be affected by the nature and strength of the secretory stimulus. Levine et al. (40) reported on the presence of a cationic proline-rich protein (different from the anionic PRPs) in human parotid secretion, which under gustatory stimulation appeared to undergo incomplete glycosylation. Muenzer et al. (41) described an acidic proline-rich protein isolated from rat parotid tissue homogenates which could only be detected after chronic administration of isoproterenol. Macaque saliva in this study was obtained under pilocarpine stimulation and the effect of this drug on glycosylation is not known. The effects of stimulation on glycosylation of proteins in salivary secretions have not been clarified but the availability of a well-characterized phosphoglycoprotein (i.e. MPRP) present as a major constituent of parotid secretion make the investigation of the parameters affecting glycosylation and phosphorylation feasible. Large scale screening of monkey salivas has not been practical but purification conducted with salivary samples from 5 different monkeys all show only one major proline-rich protein, MPRP.

Difficulties with the determination of the molecular weight of various salivary proline-rich proteins have been noted by several investigators (1, 5, 41). High speed equilibrium sedimentation of the PRPs gave molecular weights which were too low (1, 5) and the elution behavior on Sephadex G-75 resulted in molecular weight values of MPRP and the PRPs which were too high (present investigation). Structural proteins, such as collagen, have been found to exhibit atypical behavior in electrophoretic (42) and gel filtration (23, 43) systems even in the presence of denaturing agents. While collagen and collagen-derived peptides display a linear, semilogarithmic relationship between the molecular weight and the migration distance in sodium dodecyl sulfate gels, or the elution volume from agarose columns, the slope and intercept of such linear plots differ from that obtained with globular proteins. As a consequence the molecular weight values calculated for collagen peptides tend to be too large when such determinations are based on the behavior of globular protein standards. This is believed to relate to the unusual composition of collagen in which the high proportion of imino acid residues is responsible for a more rigid, rodlike structure even in the denatured state (42). Reliable molecular weight values can be obtained if standards consisting of well characterized collagen peptides, or synthetic polypeptides such as (Pro-Pro-Gly), are used (22). The influence of carbohydrate moieties has not yet been elucidated in this system but glycosylated polypeptide regions are likely to be in an extended conformation lacking significant amounts of higher ordered structure (44). The close agreement between the apparent molecular weights of PRP III and MPRP (Fig. 3), and the true molecular weight of PRP III calculated from the amino acid sequence (5), indicates that this method should provide a useful tool for the molecular weight estimates of this class of salivary proteins.

M-statherin—An unusual feature of M-statherin is its gel filtration behavior on Bio-Gel P-6 (fractionation range 5000-1000 daltons) in 3% formic acid (Fig. 4B). Based on a molecular weight of 5368, obtained from the amino acid sequence, M-statherin was expected to elute with a K, ranging between 0.05-0.20. The observed K, of 0.90 suggests that the residues in the hydrophobic region of M-statherin may be involved in H-bonding or other interactions. A similar retardation was observed during the chromatography of tryptic peptides (Fig. 5) where the large carboxyl-terminal peptide (24 residues) did not elute in the void volume of the Bio-Gel P-2 column, as expected, but was recovered later, together with peptides containing 5 or 6 amino acid residues. Such anomalous behavior could be explained by a hydrophobic affinity of M-statherin for the acrylamide matrix. This property may be significant for formation of relatively insoluble aggregates as exemplified by those in the acquired enamel pellicle.

The amino acid sequence of M-statherin is the first complete sequence of any component in the parotid secretion of a subhuman primate, and is only the second amino acid sequence determined for a statherin. M-statherin exhibits a strong polarization of the polypeptide chain into a highly charged NH2-terminal segment and a hydrophobic carboxyterminal portion. The acidic and basic amino acids of M-statherin amount to 13 residues which are all located within the NH2-terminal 18 amino acids. The remaining 24 residues, comprising the hydrophobic carboxy-terminal portion of the molecule, exhibit tripeptides of the general sequence (X-Y-Pro) with X being occupied by Tyr (4X), Phe (1X), and Gln (1X), while the position of Y is occupied by Gln (3X), Gly (1X), Ala (1X), or Tyr (1X). In addition the dipeptide Gln-Pro occurs twice and Gln-Tyr forms the carboxyl terminus.

Comparison of the amino acid sequences of human and macaque statherin shows that the first 10 residues (containing the two vicinal phosphoserines in position 2 and 3) and residues 13, 14, 17, 38, and 39 are identical (Scheme 1). Proper alignment of the two statherin molecules to give maximum homology shows that 33 of 42 amino acid residues are identical. This leaves no doubt that human and macaque statherin are derived from the same ancestral gene.

The secondary structure as predicted by the Chou-Fasman method (46, 47) is shown in Fig. 7. This analysis predicts an a helix in the NH2-terminal region (residues 4-16) and a b pleated sheet in the carboxyl-terminal region (residues 22-26, 38-42). Whether such a clear division of M-statherin into two distinct structural domains occurs under physiological conditions is not known. If correct, one could visualize that the structural domains containing a helix and b pleated sheet are responsible for different biological functions. This would correlate nicely with the fact that the NH2-terminal region of
human statherin (containing phosphoserine residues) is the portion of the molecule in which the activity related to inhibition of calcium phosphate precipitation and adsorption to hydroxyapatite resides (12, 45). It is also possible that the carboxyl-terminal regions of human and macaque statherin undergo significant conformational changes during adsorption and may therefore play a structural role in the outermost layers of dental enamel.

Function—Both MPRP and M-statherin share structural features which appear to be related to their function. Both molecules demonstrate a clustering of the anionic residues such as phosphoserine, glutamic acid, and aspartic acid in the NH₂-terminal portion of the polypeptide chain. The biological activities of the PRPs, statherin, and other inhibitors of calcium phosphate precipitation have been linked to the anionic character of these macromolecules (12). In crystal growth inhibition assays, the NH₂-terminal tryptic fragments of the PRPs exhibit more activity than the intact proteins (12). Similarly the mechanism of selective binding of these constituents to hydroxyapatite and enamel surfaces seems to be related to their highly charged NH₂-terminal regions (45). The precise role of the proline-rich, hydrophobic carboxyl-terminal moiety, however, has not been positively elucidated.

The only oral function of the PRPs and statherin so far uncovered is their inhibitory activity of calcium phosphate precipitation. The assays developed to quantitate such activity clearly show that MPRP and M-statherin described in this study are highly effective inhibitors and therefore are the functional equivalents of the human PRPs and statherin. The elucidation of the molecular mechanism of this inhibition has only recently begun. The highly active macaque components structurally characterized in this work may be useful tools in such investigations. Moreover, the structural and functional parallelism between human and M. fascicularis parotid proteins establishes this primate as an excellent animal model in which to investigate the synthesis and secretion mechanism of these salivary proteins and their role in the oral environment.

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