Complete Covalent Structure of Statherin, a Tyrosine-rich Acidic Peptide Which Inhibits Calcium Phosphate Precipitation from Human Parotid Saliva*

The complete amino acid sequence of human salivary statherin, a peptide which strongly inhibits precipitation from supersaturated calcium phosphate solutions, and therefore stabilizes supersaturated saliva, has been determined. The NH₂-terminal half of this Mₗ = 5380 (43 amino acids) polypeptide was determined by automated Edman degradations (liquid phase) on native statherin. The peptide was digested separately with trypsin, chymotrypsin, and Staphylococcus aureus protease, and the resulting peptides were purified by gel filtration. Manual Edman degradations on purified peptide fragments yielded peptides that completed the amino acid sequence through the penultimate COOH-terminal residue. These analyses, together with carboxypeptidase digestion of native statherin and of peptide fragments of statherin, established the complete sequence of the molecule. The 2 serine residues (positions 2 and 3) in statherin were identified as phosphoserine.

The amino acid sequence of human salivary statherin is striking in a number of ways. The NH₂-terminal one-third is highly polar and includes three polar dipeptides: H₂PO₄, H₂PO₄, Arg–Arg, and Glu–Glu. The COOH-terminal two-thirds of the molecule is hydrophobic, containing several repeating dipeptides: four of Gly–Pro, three of Tyr–Gln, two of Gly–Tyr, two of Gln–Tyr, and two of the tetrapeptide sequence Pro–Tyr–Gln–Pro. Unusual cleavage sites in the statherin sequence obtained with chymotrypsin and S. aureus protease were also noted.

Human salivary secretions are normally supersaturated with respect to the basic calcium phosphate salts that form the dental enamel, and may become saturated with acidic salts such as dicalcium phosphate dihydrate (1). This supersaturation of the saliva is important for stabilizing and protecting the surfaces enamel and for recalcifying enamel that has been demineralized by bacterial acids (2-4). Despite its supersaturated state, human saliva lacks properties characteristic of the supersaturated state. Well defined calcium phosphates do not precipitate from the saliva, and mineral accretions do not form spontaneously, without bacterial mediation, on human dental enamel surfaces. In addition, precipitation from saliva that has been seeded with a solid calcium phosphate is retarded and unpredictable compared with precipitation from equivalent synthetic solutions (5).

These anomalies, clearly favorable to the teeth, have long been recognized but poorly understood. Recently, however, we showed that the macromolecular fraction of human salivary secretions strongly inhibits precipitation from supersaturated calcium phosphate solutions, a property not possessed by some other complex protein mixtures, such as sera from humans and other mammals (6). This inhibitory activity is due to the presence of at least two macromolecules found in both the parotid and submandibular salivas. Part of the activity is associated with a complex group of proline-rich proteins (7, 8) although the exact nature of this association is undetermined. Further activity is associated with a tyrosine-rich acidic peptide to which we have given the name statherin (from the Greek statheropio meaning to stabilize). This highly purified Mₗ = 5380 polypeptide (9) was shown to be an unusually potent inhibitor of calcium phosphate precipitation. Furthermore, these inhibitors adsorb selectively from the saliva onto apatitic surfaces (10) and have therefore been regarded as possible precursors of the acquired enamel pellicle, a protein integument of the dental enamel surface. These separate properties of the inhibitors consort to enable human saliva to exist in a supersaturated but stable condition, needed for the stabilization and recalcification of the enamel, as well as for the inhibition of formation of mineral accretions on the tooth surface. In addition, the possibility cannot be overlooked that the inhibitors may function in the transport of calcium and phosphate during secretion in the salivary glands.

This paper reports the determination of the complete covalent structure of statherin as a first step in understanding the role and mechanism of action of the inhibitors.

EXPERIMENTAL PROCEDURES

Materials

Trypsin, α-chymotrypsin, carboxypeptidase A, and carboxypeptidase B were purchased from Worthington Biochemicals, Freehold,

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N. J. Stephepanthrone was purchased from Miles Laboratories, Elkhart, Ind. Sequencer reagents, of Sequencer grade and amino acid analyzer reagents were purchased from Beckaman Instruments, Palo Alto, Calif. Phosphoserine was purchased from Pierce Chemical Co., Rockford, 111.

Methods

Isolation of Statherin—The preparation of highly purified stath- erin and its partial characterization have been reported (9), but a simpler and improved method of purification was used to prepare material for this study. Larger quantities of human-stimulated parotid saliva were fractionated on columns of DEAE-Sephadex as described previously (11) to isolate statherin. Purification was achieved by salt precipitation and gel permeation chromatography on Bio-Gel P-6, and anion exchange chromatography using Bio-Rad DEAE-agarose with a chloride gradient. The product was shown to be pure by immunochemical analysis, disc electrophoresis, and NH₂-terminal analysis.

Amino Acid Analysis—Polypeptides were hydrolyzed in 6 N HCl containing 0.005% (v/v) p-mercaptoethanol at 110° in vacuo for 24 h. Amino acids were identified on a Beckman model 121M amino acid analyzer by the method of Spackman et al. (12).

Automated Sequence Analysis—Analyses were performed by the method of Edman and Berg (13) in which a double cleavage Quadrupole program was used. Statherin, 200 μmol, dissolved in 0.05 M NH₄HCO₃ (pH 9.6), was applied to and dried in the sequenator cup. Coupling with phenylisothiocyanate was performed twice before initiating automated sequencing.

Manual Sequence Analysis—Manual phenylisothiocyanate degra- dations were performed by the three-stage method of Edman (14) as modified by Sauer et al. (15). Coupling of peptide was carried out under nitrogen for 30 min at 54° in 100 μl of 0.4 M dimethylthylalami- nium in propanol/water (80/20, v/v) previously adjusted to pH 9.5 with trifluoroacetic acid. After coupling, a single extraction with 0.2 ml of benzene was performed, and the organic phase discarded. Cleavage was accomplished with 75 μl of anhydrous trifluoroacetic acid under nitrogen at 54° for 3 to 5 min. This was followed by extraction of thiazolinol products with 0.2 ml of benzene. The thiazolinolones were converted to their more stable phenylthiohydan- toin isomers in 0.2 ml of 1 M HCl at 80° for 10 min.

Identification of PTH Derivatives—This was carried out by gas (16) and thin layer chromatography (17). PTH-arginine was identified by thin layer chromatography on silica gel using the solvent system chloroform/methanol/heptafluorobutyric acid (70/30/0.5) (18). After development of the thin layer plate, intensity of the PTH derivatives was enhanced by vapors of iodine (19). The plate was transillumi- nated by ultraviolet light at 254 nm, and photographed for permanent record.

Enzymic Digestion and Purification of Peptides: Trypsin—Stath- erin, 10 mg, was dissolved in 0.2 M NH₄HCO₃ (pH 8.1) and digested with trypsin at an enzyme to substrate ratio of 1/100 (w/w) for 3 h at 37°. The digest was then acidified to pH 3 with 6 N acetic acid and lyophilized. The trypptic peptides were separated by gel permeation chromatography on Sephadex G-50 equilibrated with 0.2 M NH₄HCO₃ (pH 8.1) in a column (140 × 1.2 cm).

Staphylococcus aureus Protease Peptides—Statherin, 4 mg, was incubated with S. aureus protease in 0.2 M NH₄HCO₃ (pH 8.1) at an enzyme to substrate ratio of 1/20 for 24 h at 37°. Peptide fragments were purified by gel permeation chromography on Bio-Gel P-6 equilibrated with 0.1 M NH₄HCO₃ (pH 8.1) in a column (130 × 1.2 cm). The yield of S. aureus protease peptides ranged from 75 to 85%.

Chymotryptic Peptides—Statherin, 5.5 mg, was dissolved in 0.2 M NH₄HCO₃ (pH 8.1) and incubated with chymotrypsin for 4 h at 37° at an enzyme to substrate ratio of 1/100. After acidification and lyophi- lization of the digest, peptide fragments were purified on Bio-Gel P-6 as described for S. aureus protease peptides.

Detection of Peptide Peaks—Column effluents were monitored for ultraviolet absorbance at 206 nm using an LKB Ulvacmmonf for ultraviolet monitor (LKB Produktor, AB, Sweden).

COOH-terminal Analysis with Carboxypeptidase A—Carboxypeptidase A (8 μg) or carboxypeptidase B (4 μg) or both were added to NaHCO₃ (0.2 M, pH 8.1) solution containing native statherin or peptide fragments of statherin and digestion continued for 1 to 180 min. After digestion, samples were acidified to pH 3.0, using 6 N acetic acid, and lyophilized. The residue was dissolved in 0.2 M sodium citrate (pH 2.2) and applied to the amino acid analyzer columns using a physiological fluid program with a lithium citrate buffer (20) for resolution of glutamine from other amino acids. Detecc- tion of release of phosphoserine in statherin by chymotrypsin diges- tion was made by amino acid analysis using the physiological fluid program.

Determination of Phosphate Content of Statherin—Total phos- phate was determined in triplicate samples after destruction of organic matter in an oxygen generator (Plasmog, Tegel Corporation, Richmond, Calif.). Inorganic phosphate was determined by a minor modification of the method described by Sumner (21). Complete oxidation of organic matter and recovery of phosphate were con- firmed using standard samples of phosphoserine and inorganic phos- phate.

RESULTS

NH₂-terminal Analysis

Automated Analysis of Native Statherin—Native statherin (1.2 mg) was dissolved in 0.05 M NH₄HCO₃ (pH 8.6) and applied to the spinning cup of the automated sequenator and sequenced using the double cleavage protein program. The repetitive yield (average yield per cycle) through the first 22 residues of statherin is depicted in Fig. 1 with the exception of the serine phosphate residues (positions 2 and 3) which were identified independently. Positive identifications were made at each step of the degradation. The repetitive yield falls sharply as the polar residues in the first third of the molecule are removed from the remaining peptide during the degradation. All basic residues in statherin were placed within the sequence by this degradation; the single lysine residue occu- pies position 6; the 3 arginine residues occupy positions 9, 10, and 13. From this degradation all four expected trypptic peptides could be aligned in the statherin sequence, and therefore the large COOH-terminal tryptic peptide of statherin was subjected to isolation and sequence determination.

Manual Analysis of Peptide Fragments of Statherin

Tryptic Peptides—Native statherin (10 mg) was digested with trypsin, and the peptide fragments of statherin were chromatographed on Sephadex G-50. One well resolved and two partially resolved peaks were observed in the gel permeation elution profile (Fig. 2). The material in the earliest eluting peak was the large COOH-terminal tryptic peptide of statherin designated T-4. The material in the first of the two partially resolved peaks was principally the NH₂-terminal tryptic peptide (T-1); the second of these two peaks contained a
mixture of peptides T-2 and T-3, located in positions 7 through 9 and 11 through 13 in statherin, respectively. Aliquots of each of these components were sequenced using the manual Edman degradation (Table I), and aliquots of peptide T-4 and native statherin were subjected to amino acid analysis after acid hydrolysis (Table II).

Inasmuch as peptides T-2 and T-3 in the last eluting peak (Fig. 2) occurred in unequal amounts, their sequences could be unequivocally determined. An unhydrolyzed aliquot of material in the last eluting peak revealed free arginine on the basic column of the amino acid analyzer. This residue was assigned to position 10 in the intact sequence of statherin. Peptides T-1, T-2, and T-3 were sequenced through the penultimate residues whereas peptide T-4 was sequenced for 18 cycles. This degradation produced a nine amino acid overlap with that on native statherin, thus extending the sequence through residue 31 (Fig. 5).

Staphylococcus aureus Protease Peptides —Native statherin (4.0 mg) was digested with S. aureus protease and the resulting peptide fragments were separated by gel permeation chromatography on Bio-Gel P-6. The elution profile is depicted in Fig. 3. Aliquots of material in peaks P-1, P-2, and P-3 were subjected to manual Edman degradation (Table III) and amino acid analysis after acid hydrolysis (Table II). Although the elution profile suggests only partial resolution, each protease peptide appeared about 90% homogeneous by the Edman degradation. Peptide P-1 was degraded for nine cycles through its COOH-terminal residue and was assigned to the statherin peptide sequence through residue 31. Peptide C-2 was degraded for seven cycles. Its NH, terminus being assigned to position 22 in statherin (Fig. 5), and confirmed the sequence through position 31. Peptide C-2 was degraded for seven cycles. Its NH, terminus was assigned to position 28 by the dipeptide overlap with peptide P-2 and the four amino acid overlap with peptide T-3. This degradation extended the sequence through residue 41 in statherin.

COOH-terminal Analysis of Statherin and Peptide Fragments —The COOH-terminal residues of native statherin and peptide fragments of statherin were identified and quantitated using carboxypeptidase A, followed by amino acid analysis employing a physiological fluid program and a lithium citrate buffer (20) for resolution of threonine and glutamine. Digestion of native statherin with carboxypeptidase A for 1 min (Table IV) suggested that the COOH-terminal residue was phenylalanine with a penultimate residue of threonine. Similar digestion of statherin for periods up to 3 h indicated removal of the COOH-terminal six amino acids. Similar amino acid ratios were obtained for peptides T-4 and P-3, indicating that they are COOH-terminal in statherin. Digestion of chymotryptic peptide C-2 with carboxypeptidase A, however, resulted in release of 2 tyrosine and 2 glutamine residues. This composition fits the COOH-terminal tetrapeptide sequence of C-2 and partial sequence of peptide P-3 (Fig. 5).

| FIG. 2. Elution profile of peptides on Sephadex G-50 obtained by digestion of statherin with trypsin. Pooled material for structural studies is enclosed in parentheses. |

<table>
<thead>
<tr>
<th>Amino acid sequence of tryptic peptides of statherin</th>
</tr>
</thead>
<tbody>
<tr>
<td>(41.1) (18.9) (10.5)</td>
</tr>
<tr>
<td>T-2: 1. Phe 2. Leu 3. Arg*</td>
</tr>
<tr>
<td>(48.0) (33.0)</td>
</tr>
<tr>
<td>T-3: 1. Ile 2. Gly 3. Arg*</td>
</tr>
<tr>
<td>(39.4) (94.0)</td>
</tr>
<tr>
<td>(82.2) (85) (84.3) (78.1) (68.3) (73.9) (43.9) (40.2) (32.9)</td>
</tr>
<tr>
<td>(28.1) (22.7) (17.0) (10.3) (12.0) (3.4) (2.5)</td>
</tr>
</tbody>
</table>

* Residues in parentheses were identified by overlap with automated Edman degradation on native statherin and by trypsin specificity and are not quantitated in this degradation.

* Yield in nanomoles of selected residues in the sequences.
Structure of Human Salivary Statherin

Table II
Amino acid composition of statherin and of peptide fragments of statherin produced by cleavage with trypsin (T-4), chymotrypsin (C-1, C-2), and Staphylococcus aureus protease (P-1, P-2, P-3)

<table>
<thead>
<tr>
<th></th>
<th>Statherin</th>
<th>T-4</th>
<th>P-1</th>
<th>P-2</th>
<th>P-3</th>
<th>C-1</th>
<th>C-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp</td>
<td>1.00 (1)</td>
<td>0.20 (0)</td>
<td>0.10 (0)</td>
<td>0.10 (0)</td>
<td>0.07 (0)</td>
<td>0.20 (0)</td>
<td>0.01 (0)</td>
</tr>
<tr>
<td>Thr</td>
<td>1.00 (1)</td>
<td>0.80 (1)</td>
<td>0.07 (0)</td>
<td>0.07 (0)</td>
<td>0.06 (0)</td>
<td>0.02 (0)</td>
<td>0.02 (0)</td>
</tr>
<tr>
<td>Ser</td>
<td>1.55 (2)</td>
<td>0.01 (0)</td>
<td>0.01 (0)</td>
<td>0.01 (0)</td>
<td>0.01 (0)</td>
<td>0.01 (0)</td>
<td>0.01 (0)</td>
</tr>
<tr>
<td>Glu</td>
<td>10.16 (10)</td>
<td>8.00 (8)</td>
<td>2.02 (2)</td>
<td>4.30 (4)</td>
<td>2.30 (2)</td>
<td>4.10 (4)</td>
<td>4.00 (4)</td>
</tr>
<tr>
<td>Pro</td>
<td>6.64 (7)</td>
<td>6.20 (7)</td>
<td>2.72 (3)</td>
<td>3.81 (4)</td>
<td>0.40 (0)</td>
<td>4.70 (3)</td>
<td>0.81 (1)</td>
</tr>
<tr>
<td>Gly</td>
<td>4.08 (4)</td>
<td>3.00 (3)</td>
<td>1.00 (1)</td>
<td>0.11 (0)</td>
<td>0.13 (0)</td>
<td>0.1 (0)</td>
<td>0.40 (0)</td>
</tr>
<tr>
<td>Val</td>
<td>1.04 (1)</td>
<td>1.10 (1)</td>
<td>1.01 (1)</td>
<td>0.03 (0)</td>
<td>0.02 (0)</td>
<td>0.70 (1)</td>
<td>0.01 (0)</td>
</tr>
<tr>
<td>Ile</td>
<td>0.96 (1)</td>
<td>0.05 (0)</td>
<td>0.01 (0)</td>
<td>0.02 (0)</td>
<td>0.01 (0)</td>
<td>0.10 (0)</td>
<td>0.02 (0)</td>
</tr>
<tr>
<td>Lys</td>
<td>2.00 (3)</td>
<td>1.06 (2)</td>
<td>0.13 (0)</td>
<td>1.02 (2)</td>
<td>0.13 (0)</td>
<td>1.00 (1)</td>
<td>0.12 (0)</td>
</tr>
<tr>
<td>Tyr</td>
<td>6.60 (7)</td>
<td>6.89 (7)</td>
<td>2.20 (2)</td>
<td>2.60 (2)</td>
<td>1.90 (2)</td>
<td>1.72 (2)</td>
<td>2.30 (2)</td>
</tr>
<tr>
<td>Phe</td>
<td>3.00 (3)</td>
<td>1.91 (2)</td>
<td>0.01 (0)</td>
<td>0.12 (0)</td>
<td>1.01 (1)</td>
<td>0.21 (0)</td>
<td>0.11 (0)</td>
</tr>
<tr>
<td>Arg</td>
<td>2.80 (3)</td>
<td>0.12 (0)</td>
<td>0.00 (0)</td>
<td>0.01 (0)</td>
<td>0.01 (0)</td>
<td>0.00 (0)</td>
<td>0.10 (0)</td>
</tr>
<tr>
<td>Total</td>
<td>43</td>
<td>30</td>
<td>9</td>
<td>11</td>
<td>6</td>
<td>13</td>
<td>7</td>
</tr>
</tbody>
</table>

* Numbers in parentheses represent number of residues found by sequence analysis.

Identification of the 2 serine residues (positions 2 and 3) as phosphoserine was obtained in a surprising way. Initially, it was hoped that digestion of statherin with aminopeptidase M (22) would release aspartic acid, phosphoserine, and other NH₂-terminal residues. This release, however, did not occur. Unexpectedly, release of both phosphoserine residues in statherin by enzymatic means was obtained by digestion of statherin with chymotrypsin. After digestion of statherin with chymotrypsin, an aliquot of unpurified products of the digestion was subjected to amino acid analysis without hydrolysis, whereas another aliquot was subjected to a single step of Edman degradation followed by amino acid analysis (see "COOH-terminal Analysis of Statherin"). Amino acid analysis directly after digestion revealed the release of phosphoserine as the only free amino acid. Amino acid analysis following digestion and a single step of Edman degradation similarly showed release of phosphoserine as well as phenylalanine and tyrosine in equivalents of 1/1/2, respectively. This surprising result indicates that chymotrypsin cleaves the bond between the 2 vicinal phosphoserine residues in statherin as well as at the COOH-terminal side of the phosphoserine in position 3.

Discussion
Statherin, an unusual polypeptide in human parotid and submandibular salivas, is a potent inhibitor of calcium phosphate precipitation. The way in which statherin and other calcium phosphate precipitation inhibitors act is not fully understood (23). The most probable mechanism, however, is that the inhibitors bind to the first formed aggregates of calcium and phosphate ions or nuclei, block their growth, and so delay precipitation (24). A similar mechanism was proposed to explain both inhibition of precipitation in seeded systems and inhibition of transformation of unstable calcium phosphate phases to stable calcium phosphate salts (25).
Structure of Human Salivary Statherin

TABLE III
Amino acid sequence of Staphylococcus aureus protease (P-1, P-2, P-3) and chymotryptic peptides (C-1, C-2)

<table>
<thead>
<tr>
<th></th>
<th>F-1: Tyr - Gly - Pro - Tyr - Gln - Pro - Val - Pro - Glu</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(21.9) (21.6) (18.6) (14.5) (11.1) (11.1) (7.0)</td>
</tr>
<tr>
<td></td>
<td>P-2: Gln - Pro - Leu - Tyr - Pro - Gln - Pro - Tyr - Gln - Pro - Prol</td>
</tr>
<tr>
<td></td>
<td>(15.6) (12.4) (1.0) (8.2) (6.0) (5.0) (1.7)</td>
</tr>
<tr>
<td></td>
<td>P-3: Tyr - Gln - Gln - Tyr - Thr -</td>
</tr>
<tr>
<td></td>
<td>(20.9) (5.4)</td>
</tr>
<tr>
<td></td>
<td>C-1: Gln - Pro - Val - Pro - Glu - Gln - Pro - Leu - Tyr - Pro -</td>
</tr>
<tr>
<td></td>
<td>(26.0) (29.0) (23.6) (9.2) (13.0) (3.5) (3.2)</td>
</tr>
<tr>
<td></td>
<td>C-2: Gln - Pro - Gln - Tyr - Cln - Cln - Tyr -</td>
</tr>
<tr>
<td></td>
<td>(20.9) (9.9)</td>
</tr>
</tbody>
</table>

* Yield in nanomoles of selected residues in the sequence.

Fig. 4. Elution profile of peptides on Bio-Gel P-6 obtained by cleavage of statherin with chymotrypsin. Pooled material for structural studies is enclosed in parentheses. Mechanism requires that the inhibitory molecules bind to the surfaces of the various calcium phosphate phases involved in these processes, and the inhibitors may therefore be expected to exhibit unusual interactions with calcium phosphate salts, as does statherin, which can be selectively adsorbed from human saliva, in nearly pure form, using apatitic minerals (10). We undertook the primary structure determinations of statherin as a first step in determining the molecular basis for its unusual properties. The NH₂-terminal half of the molecule was determined by an automated Edman degradation of 200 nmol of intact native polypeptide. Every residue through the first 22 residues was identified by the degradation, except for the serine residues, which were identified as phosphoserines on subsequent studies (see "Phosphate Content and Identification of Phosphoserine in Statherin"). This automated degradation stands in marked contrast to that of ubiquitin (18), in which the first 61 residues were determined in a single analysis on a 74 amino acid polypeptide. Ubiquitin, possessing 2 arginine residues in its COOH-terminal tripeptide as well as other charged residues in the COOH-terminal region is not readily extracted by solvent washings in the spinning cup of the sequenator. Statherin, on the other hand, loses all its arginine residues and all but 1 of its remaining charged residues (glutamic acid, position 26) after 13 cycles of the Edman degradation and thereafter is readily extracted by solvent washings, as can be seen by the sharply falling slope of the repetitive yield curve (Fig. 1). Therefore, the entire COOH-terminal half of statherin was determined using manual Edman degradations, which greatly reduce peptide extractive losses, on peptide fragments of statherin produced by enzymic cleavage. Digestion of statherin with trypsin and subsequent purification of the tryptic peptides on Sephadex G-50 resulted in the isolation of the COOH-terminal tryptic peptide (positions 14 through 43) in a homogeneous form. Manual sequence analysis of this COOH-terminal tryptic peptide extended knowledge of the sequence through residue 31 and revealed
peptide overlap between the S. aureus protease peptides P-1 and P-2 (Fig. 5). Manual sequence analysis of peptide P-2 for 10 cycles completed the sequence of statherin through residue 36. Manual sequence analysis of chymotryptic peptide C-2 revealed overlap between protease peptides P-2 and P-3, whereas sequence analysis of peptide C-2 and P-3 provided sequence determination of statherin through position 42, its penultimate COOH-terminal residue. COOH-terminal analysis of statherin and of peptide fragments of statherin using carboxypeptidase A showed that peptides T-4, C-2, and P-3 were COOH-terminal in statherin and thus completed the sequence of statherin. Analysis of phosphate content in statherin confirmed that only the 2 serine residues, identified as phosphoserine residues by chymotrypsin digestion, possess phosphate groups. With respect to peptides P-2, C-1, and C-2 which possess an NH₂-terminal glutamine, it was fortunate that the degree of NH₂-terminal cyclization was not substantial enough to preclude sequence analysis, peptides C-1 and C-2 being obtained in yields about 60%. Peptide P-2 was obtained in substantially lower yields of about 35%; the lengthened S. aureus protease digestion period (24 h) presumably increasing the degree of cyclization. Confidence that each of these peptides possessed an NH₂-terminal glutamine and not glutamic acid is strengthened by the observations that (a) glutamine (position 22 and NH₂-terminal in peptide C-1) was identified as the amide in the degradations on native statherin and peptides T-4 and P-1; (b) glutamine (position 27 and NH₂-terminal in P-2) was identified as the amide in the degradations on peptide T-4 and C-1; (c) glutamine (position 35 and NH₂-terminal in C-2) was identified as such in the degradation on peptide P-2.

A number of structural features of statherin are striking. First, statherin possesses a high content of tyrosine, proline, and glutamic acid that together account for more than 50% of the molecule. Second, the NH₂-terminal one-third of the molecule contains all but 1 of the polar residues in statherin; the native molecule is quite acidic, having an isoelectric point of 4.2. In addition to these features, enzymatic studies suggest that statherin possesses an unusual conformation. For example, S. aureus protease, which reportedly cleaves the molecule specifically at the COOH-terminal side of glutamic acid residues under conditions performed in this study (28), also cleaves one of the two glycine-tyrosine bonds (positions 17 and 18) in statherin. Although S. aureus protease was observed to cleave at the COOH-terminal side of selected glutamine residues (18), a glycine-tyrosine cleavage site is very unusual, the closest precedent being a glycine—alanine bond in porcine parathyroid hormone (19). These unusual cleavage sites suggest an unusual conformation that produces stress at these sites in the sequence, subjecting them to facile enzymic cleavage.

Another striking feature of statherin is the high repetition of dipeptide sequences in the COOH-terminal two-thirds of the sequence, such as two adjacent Gly—Tyr sequences (only one of which cleaves with S. aureus protease), four Gin—Pro sequences, and three Tyr—Gln, and two Pro—Tyr—Gln—Pro—sequences. This contrasts strongly with the dipeptides located in the NH₂-terminal one-third of the molecule, which are composed of 2 identical polar residues:

\[
\begin{align*}
\text{H}_2\text{PO}_4^- & \quad \text{H}_2\text{PO}_4^- \\
\text{Ser} & \quad \text{Ser}
\end{align*}
\]

\[-\text{Glu—Glu—} \quad -\text{Arg—Arg—}\]

Other anionic macromolecules, such as polyaspartate and phosvitin, also inhibit calcium phosphate precipitation. It seems significant, therefore, that the 5 NH₂-terminal residues of statherin are all anionic. It is probable that the interaction between statherin and calcium phosphate nuclei and surfaces will involve the polar NH₂-terminal third of the molecule, as this segment contains 10 of the 12 charged sites present in statherin. Both negatively and positively charged groups have been implicated in the binding of polyampholytes to apatitic surfaces (27), although inhibitory activity appears to be associated with anionic, rather than with cationic polymers.

Studies of the binding of inhibitors to calcium phosphate surfaces indicate that the surfaces do not have to be completely covered before they lose their ability to seed precipitation from supersaturated solutions. Consequently, it is suggested that binding occurs at active growth sites, such as dislocations and kinks in the crystal surface (28). This view seems particularly significant when considering macromolecular inhibitors. Although many macromolecules form oriented monolayers at interfaces, it seems unlikely that statherin, with 25% of the molecule consisting of relatively bulky aromatic residues (these forming over 35% of the COOH-terminal two-thirds of the molecule), would become oriented at the solid/solution interface and screen the crystal surface from solution interactions. A more plausible concept involves binding of the charged NH₂-terminal end of the molecule to the active growth sites on the crystal. The vicinal serine phosphate residues may be expected to play a particularly important role in such binding, similar to that proposed for the phosphate groups in the highly inhibitory multidentate phosphonates (28). It will be interesting, therefore, to determine if the unusual serine phosphate dipeptide sequence is present in the other salivary inhibitors, and in other inhibitory macromolecules. It is of interest, in this connection, that the mode of action of the diphasphonates is considered to involve chelation of a calcium ion at the crystal growth site. We have found that statherin binds calcium and may therefore act in a similar way. This finding, and the presence of a glutamic acid dipeptide sequence, led to the analysis of statherin for γ-carboxylglutamic acid (29), but this constituent of several other calcium-binding proteins (30) was absent from statherin.

Human urine also contains inhibitors of calcium phosphate precipitation (31, 32) and two small active peptides have been isolated. Their compositions (aspartic, 1; glutamic acid, 1; serine, 1; threonine, 1; glycine, 1; alanine, 1; and asparagine, 1; glutamic acid, 3; serine, 1; cystine, 1; glycine, 2) are unlike statherin, but they are acidic and there is potential for them to

\[\text{M. S. Rosemblatt, and D. I. Hay, unpublished data.}\]
partially mimic the NH₂-terminal segment of statherin. Present studies have focused principally upon human saliva, but recently we have found that the parotid and submandibular salivas of the monkey, Macaca arctoides, contain two inhibitory macromolecules, and that the salivas of the rat, Rattus rattus, and the hamster, Mesocricetus auratus, will also inhibit precipitation. It will be interesting to determine at what point in evolutionary development that this unusual property appeared in the saliva.

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REFERENCES

21. Sumner, J. B. (1944) Science 100, 413-414
Complete covalent structure of statherin, a tyrosine-rich acidic peptide which inhibits calcium phosphate precipitation from human parotid saliva.

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