The presence and origin of phosphopeptides in human saliva

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The presence of phosphopeptides in whole saliva (saliva expectorated from the mouth) was demonstrated and their origin was evaluated. Whole saliva contained much larger numbers of small phosphopeptides than are found in the glandular secretions. Most of these originated from the acidic proline-rich proteins (PRPs) in the major salivary glands and were formed, after secretion into the oral cavity, as a result of rapid degradation by proteolytic enzymes from extraglandular sources contained in sediment from whole saliva. Some peptides may have been formed by cleavage of basic PRPs, but other phosphoproteins apparently contributed little to the observed phosphopeptides. Most of the enzymes that produced phosphopeptides are serine proteinases. The gel-electrophoretic band patterns of the phosphopeptides obtained from 26 individuals of various acidic-PRP phenotypes were remarkably similar, demonstrating that the enzymes responsible were generally present in the population surveyed and that similar cleavages occur regardless of the nature of the acidic PRPs. Many of these peptides were *N*-terminal proteolytic cleavage products of acidic PRPs. The *N*-terminal phosphorylated region of acidic PRPs contains various biological activities, such as inhibition of hydroxyapatite formation, calcium binding and binding to hydroxyapatite, the major mineral of teeth. The demonstration of these phosphopeptides in the saliva that is in contact with the oral surface may therefore be of biological importance.

INTRODUCTION

Proline-rich proteins (PRPs) account for approx. 70 % of the total protein in human parotid- and submandibular-gland saliva and they may be divided into acidic, basic and glycosylated PRPs (Bennick, 1982). Protein-polymorphism studies have established the presence of the following acidic PRP allelic products: Pr1, Pr2, PIF (parotid isoelectric-focusing variant), Db (double band) and Pa (acidic protein) (Azen & Oppenheim, 1973; Azen & Denniston, 1974, 1981; Friedman *et al.*, 1975). The allele Pr1 corresponds to proteins PRP1 and PRP3, Pr2 to proteins PRP2 and PRP4, PIF to proteins PIFs (slow) and PIFf (fast), Db to proteins Dbs (slow) and Dbf (fast) and Pa to protein Pa. PRP1, PRP2 and PIFs all consist of single 150-amino-acid peptide chains that are identical except for residues 4 and 50, which can be either aspartic acid or asparagine. PRP3, PRP4 and PIFf all have amino acid sequences identical with those of PRP1, PRP2 and PIFs respectively, except that they terminate at residue 106 and they may arise by post-ribosomal cleavage of the larger corresponding gene product (Wong *et al.*, 1983). Acidic PRPs will inhibit hydroxyapatite formation (Hay & Moreno, 1979), they bind Ca^{2+} (Bennick *et al.*, 1981) and adhere strongly to the hydroxyapatite forming part of the dental pellicle (Kousvelari *et al.*, 1980; Moreno *et al.*, 1982; Bennick *et al.*, 1983). These activities are all located in a 30-residue *N*-terminal sequence that can be isolated as a tryptic peptide (TX peptide). One might therefore speculate that similar *N*-terminal fragments could be the final biological product of these proteins, particularly since, at least in the case of Ca^{2+} binding, the activity in the TX peptide is higher than that of the native proteins. We have found (K. Minaguchi, G. Madapallimattam & A. Bennick, unpublished work) that

parotid saliva contains TX-like peptides, but probably in amounts too small to be of biological significance. It is possible that cleavage of acidic PRPs occurs after secretion into the oral cavity by proteinases from bacteria or sloughed-off host cells that are present in whole saliva, that is, the saliva that can be expectorated from the mouth, and the present investigation was therefore undertaken to evaluate the presence and origin of phosphopeptides in whole saliva.

EXPERIMENTAL

Materials

N-Ethylmaleimide and phenylmethanesulphonyl fluoride were purchased from Sigma Chemical Co. Acrylamide and bisacrylamide were obtained from Bio-Rad Laboratories and Stains-All from Eastman-Kodak Co. or Sigma. AH-Sepharose was from Pharmacia, and *Escherichia coli* alkaline phosphatase was from Worthington. Dialysis tubing (M_r cut-off 1000) was supplied by Spectrum Medical Industries. Statherin was a gift from Dr. D. I. Hay (Forsyth Dental Centre, Boston, MA, U.S.A.), cysteine-containing phosphoproteins (cystatin) were donated by Dr. I. D. Mandel (Columbia University New York, NY, U.S.A.), and the basic PRP, IB-1, was kindly supplied by Dr. P. J. Keller (University of Washington, Seattle, WA, U.S.A.). All other chemicals were of reagent grade.

Methods

(1) **Collection of parotid, submandibular and whole saliva: preparation of salivary sediment.** Saliva samples were obtained from 26 individuals at least 1 h after their last meal. Before collection of saliva, the mouth was

Abbreviations used: PRPs, proline-rich proteins; AH-Sepharose, aminohexyl-Sepharose.

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rinsed for 15 s with water and 5 ml of whole saliva was then collected, while Parafilm was chewed, by expectoration into a centrifuge tube. This was followed by collection of 5 ml of parotid secretion, stimulated by sucking sour lemon candy, using an appliance described by Curby (1953). Immediately after collection, the samples of whole saliva were centrifuged at 9000 *g* for 10 min, and the supernatant, which is hereafter referred to as 'whole saliva', was removed, heated at 100 °C for 10 min to inactivate proteinases and stored frozen at -20 °C. The pellet was resuspended in 10 mM-phosphate buffer, pH 7.4, containing 50 mM-NaCl, centrifuged at 9000 *g* for 10 min, and the resulting pellet resuspended in the phosphate/NaCl buffer. This procedure was repeated once, and the pellet was resuspended in 0.1 vol. of the saliva from which it was obtained. The sediment suspension was stored at -20 °C. In addition, samples of submandibular saliva were collected from some individuals by means of the appliance described by Truelove *et al.* (1967) while secretion was stimulated by the individuals sucking sour lemon candy. On separate occasions, larger volumes of parotid and whole saliva were also collected for further protein analysis.

(2) Digestion of proteins in glandular secretion with salivary sediment suspension. To evaluate the origin of phosphopeptides in whole saliva, samples (1 ml) of parotid or submandibular secretions were incubated with 0.1 ml of salivary sediment suspension from the same individual at 37 °C for 30 s to 15 h, inactivating the enzymes in the sediment at the end of incubation by heating at 100 °C for 10 min. To evaluate the nature of the proteinases in the sediment suspension, separate experiments were done incubating parotid saliva with sediment suspension in the presence of 50 mM-EGTA, 50 mM-EDTA, 50 mM-*N*-ethylmaleimide and 8 mM-phenylmethanesulphonyl fluoride, at 37 °C for 6 h.

(3) Isoelectric focusing. For phenotyping of acidic PRPs, samples (30 μ l) of parotid saliva were subjected to isoelectric focusing as described by Azen & Denniston (1981), and the PRP phenotypes were classified as proposed by Maeda (1985). To purify various acidic PRPs, samples (10 ml) of parotid saliva were dialysed against water, freeze-dried, redissolved in 1 ml of water and subjected to isoelectric focusing on a 5.4%-(w/v)-polyacrylamide gel. After isoelectric focusing, the gel was immersed in 20% (w/v) trichloroacetic acid until the protein bands were revealed. The gel was then rinsed in water, and the bands containing acidic PRPs cut out, suspended in 50 mM-Tris/HCl buffer, pH 7.5, and the suspension placed in a dialysis sac. The sacs were placed in the Tris/HCl buffer for 20 h, and dialysis was then continued against water for 4 h. Proteins eluted from the gel slices into the fluid in the dialysis sac were freeze-dried.

To evaluate the degradation of pure acidic PRPs by sediment proteinases, samples (approx. 400 μ g) of acidic PRPs were redissolved in 1 ml of 10 mM-phosphate/NaCl buffer, pH 7.4, and incubated with 100 μ l of sediment suspension for 3 h at 37 °C.

(4) Purification of PRPs. To separate PRPs from other proteins, samples (4 ml) of parotid or submandibular saliva were dialysed against water for 12 h, freeze-dried, redissolved in 0.5 ml of water and applied to a column of

AH-Sepharose to which had been coupled antibodies to acidic PRP that had been purified by affinity chromatography as described by Bennick & Madapallimattam (1983). The antibodies were coupled to the resin according to the manufacturer's instructions. The column was equilibrated, and unbound protein was eluted, with 10 mM-sodium phosphate buffer, pH 7.4, containing 0.15 M-NaCl. Bound protein was eluted by means of 0.1 M-formic acid. The column was monitored by measuring A_{220} , and the reactivity of the fractions to anti-(acidic PRPs) antibodies was tested by single radial immunodiffusion (Bennick & Cannon, 1978). To ensure that all immunoreactive proteins had bound to the column, the unbound protein was reapplied to the immunosorbent column and eluted as described above.

To evaluate the origin of phosphopeptides in whole saliva, samples (a quarter of the freeze-dried unbound and bound protein dissolved in 0.5 ml of 10 mM-phosphate/NaCl buffer, pH 7.4) were incubated with 50 μ l of sediment suspension for 15 min at 37 °C.

(5) Polyacrylamide-gel electrophoresis. Discontinuous alkaline polyacrylamide-gel electrophoresis was performed on 12.5-cm-long gel slabs as described by Smith *et al.* (1975), with the exception that the separating gel contained 35% (w/v) acrylamide and 0.7% (w/v) *NN'*-methylenebisacrylamide and the concentration of the reservoir buffer was 0.25 M and that of the gel 1 M. Polymerization of the gel was accomplished by adding 0.05 ml of *NNN'N'*-tetramethylethylenediamine and 0.83 ml of 10% (w/v) ammonium persulphate to 100 ml of gel solution. To evaluate the presence of phosphopeptides, samples (1 ml) of saliva were freeze-dried, redissolved in 30 μ l of sample buffer containing 50% (v/v) glycerol, centrifuged, and the supernatant applied to the gel. Electrophoresis was performed at 340 V for approx. 9 h until the Bromophenol Blue tracking dye had migrated 9.5 cm into the separating gel. The gel was stained with Stains-All as described by Green *et al.* (1973), resulting in blue-coloured phosphoproteins and peptides. Other proteins appear red with this stain. The gels were photographed by illuminating from beneath on an X-ray view box using Polaroid 55 film, a red filter and exposure settings of *f* 11 and 1 s.

(6) Digestion of salivary proteins with alkaline phosphatase. To evaluate whether the blue-coloured bands seen on gel electrophoresis are phosphorylated, samples (1.6 ml) of whole saliva were dialysed against water, freeze-dried, and redissolved in 3 ml of 0.25 M-ammonium formate, pH 8.4, and 16 μ l of alkaline phosphatase (as supplied by the manufacturer) was added. The mixture was incubated at 70 °C for 4 h, and then for 11 h while dialysing it against the same buffer. A further volume of alkaline phosphatase (32 μ l) was then added, and the mixture was incubated for 8 h and then dialysed against the buffer for 15 h. This procedure was repeated, except that 64 μ l of enzyme was added and the incubation period was 17 h. After digestion the samples were dialysed against water for 48 h and freeze-dried. Samples of digested as well as undigested protein were analysed for total and inorganic phosphate as described by Bartlett (1959). In addition, samples of phosphatase-digested salivary protein as well as undigested protein were subjected to polyacrylamide-gel electrophoresis and staining with Stains-All.

Fig. 1. Dephosphorylation of whole saliva

Samples (1 ml) of whole saliva were incubated for 29 h at 70 °C as described in the text in the presence (Deph WS) and absence (WS) of alkaline phosphatase. The samples were electrophoresed on a 35% (w/v) acrylamide gel and stained with Stains-All. All bands appear blue, except for the uppermost bands indicated by the bracket in the Deph WS lane.

RESULTS

In preliminary experiments it was found that degradation of proteins and peptides present in whole saliva at the time of collection could be prevented by heating the samples in a 100 °C water bath for 10 min. Moreover, it was established that whole saliva stored at -20 °C for up to 1 week did not show any signs of degradation. All experiments were therefore performed within 7 days of collection on heated samples of whole saliva that had been stored frozen.

Incubation of whole saliva with alkaline phosphatase resulted in the removal of all organic phosphate, as determined by chemical analysis. Fig. 1 shows the results of polyacrylamide-gel electrophoresis of untreated and alkaline-phosphatase-digested whole saliva. Several bands, all of them blue-coloured, could be seen in the sample that had not been digested with alkaline phosphatase, but only two blue-coloured peptides could be seen in the sample devoid of organic phosphate. These bands had no counterparts of the same intensity in the undigested sample, which contained two bands with slightly faster mobility and of higher intensity. The slower of these had the same mobility as the faster band in the dephosphorylated sample, but it is unlikely

Fig. 2. Digestion of submandibular and parotid saliva with salivary sediment suspension

Saliva samples were incubated with salivary sediment suspension, as described in the text, for various lengths of time and subjected to polyacrylamide-gel electrophoresis on a 35% (w/v) gel and staining with Stains-All. (a) Digestion of submandibular saliva: lanes 1 and 11, Bromophenol Blue dye; lane 2, undigested submandibular saliva; lane 3, submandibular saliva incubated for 15 h; lanes 4–9, submandibular saliva digested with sediment suspension for 30 s, 2 min, 10 min, 1 h, 2 h and 15 h respectively; lane 10, whole saliva. The samples of parotid and whole saliva and salivary sediment were all from the same person. (b) As (a), but parotid saliva was used instead of submandibular saliva. The intensely stained band in the uppermost part of the gels indicated by the bracket corresponds to acidic PRPs.

that the presence of this band in the phosphatase-digested sample was due to a residual amount of phosphate, since chemical analysis had demonstrated that all organic phosphate had been removed. It is known that some highly acidic non-phosphorylated proteins and peptides will stain blue with Stains-All, and this is probably why two components are seen in the dephosphorylated sample (Campbell *et al.*, 1983). Removal of phosphate would lead to a slightly slower electrophoretic mobility in the gel, so the two intense bands in the untreated sample may correspond to those seen in the digested sample. With the possible exception of these two components, all the bands seen in samples not exposed to phosphatase are therefore phosphorylated.

Incubation of parotid or submandibular saliva with

Table 1. Distribution of acidic PRP phenotypes in the persons surveyed and the acidic PRPs in their saliva

Acidic PRPs were identified by isoelectric focusing as described by Azen & Denniston (1981). The acidic PRP phenotypes were classified as proposed by Maeda (1985).

Genetic locus	Phenotype	Corresponding phenotypic acidic PRPs present in saliva	Observed no. of individuals
PRH1	1-4	Dbf, Dbf, PIFs, PIFf	2
	2-2	Pa	1
	2-4	Pa, PIFs, PIFf	15
	4-4	PIFs, PIFf	8
PRH2	1-1	PRP1, PRP3	9
	1-2	PRP1, PRP2, PRP3, PRP4	15
	2-2	PRP2, PRP4	2

Fig. 3. Comparison of undigested and digested parotid saliva and whole saliva from three individuals of known acidic PRP phenotype

Lanes 1-3, saliva samples from a person secreting PRP1, PRP3, PIFs, PIFf (phenotype: PRH1^{4,4}, PRH2^{1,1}); lanes 4-6, saliva samples from a person secreting PRP1, PRP2, PRP3, PRP4, PIFs, PIFf and Pa (phenotype: PRH1^{2,4}, PRH2^{1,2}); lanes 7-9, saliva samples from a person secreting PRP1, PRP2, PRP3, PRP4 and Pa (phenotype: PRH1^{2,2}, PRH2^{1,2}); lanes 1, 4 and 7, undigested parotid saliva; lanes 2, 5 and 8, parotid saliva digested with sediment suspension from the same person; lanes 3, 6 and 9, whole saliva. Electrophoresis was done on a 35%-(w/v)-polyacrylamide gel, which was stained with Stains-All. The phenotype naming is according to Maeda (1985).

sediment suspension resulted in the patterns illustrated in Figs. 2(a) and 2(b). Phosphopeptides had already appeared after 30 s incubation in both submandibular and parotid saliva, and the number and intensity of these bands increased with time and there was a gradual shift towards the presence of lower- M_r peptides. Whereas only a few phosphopeptides were present in glandular saliva, at least 20 phosphopeptides could be discerned in whole saliva, and, after incubation of glandular saliva with sediment suspension for more than 1 h, the band

Fig. 4. Digestion of pure acidic PRPs with salivary sediment

Electrophoresis of the digests was performed on a 35%-(w/v)-polyacrylamide gel, which was stained with Stains-All. (a) The contents of the lanes are as follows: PS, parotid saliva; WS, whole saliva; Pr4, digest of PRP4; PIFf, digest of PIFf; Pr3, digest of PRP3; Pa, digest of Pa; Dbf, digest of Dbf; Pr2, digest of PRP2; PIFs, digest of PIFs; Pr1, digest of PRP1; BPB, Bromophenol Blue. (b) Lanes 1, 2 and 3 contain parotid saliva from representative phenotypes digested with salivary-sediment suspension. Lane 1, parotid saliva containing Dbf, Dbf, PRP1, PRP3, PIFs and PIFf; lane 2, parotid saliva containing PRP1, PRP2, PRP3, PRP4, PIFs, PIFf and Pa; lane 3, parotid saliva containing PRP1, PRP3, PIFs and PIFf. Pr2, PIFs, Pr1 and Pa refer to the samples in (a), and the content of lane Dbf is a digest of Dbf.

pattern was almost identical with that of whole saliva from the same person. No bands were seen when sediment suspension was electrophoresed separately.

An incubation time of 30 min was initially chosen in the subsequent comparison of parotid saliva, parotid saliva incubated with sediment suspension and whole saliva from 26 persons. If necessary this was followed by incubation of glandular saliva with sediment for different time periods until a band pattern was obtained that was similar to that given by whole saliva. Table 1 shows the distribution of acidic PRP phenotypes in the persons

surveyed and demonstrates that all acidic PRPs are present in at least two persons. Fig. 3 shows the gel-electrophoretic results obtained from three individuals of different acidic PRP phenotypes. In each person most of the bands that were present in whole saliva were also found in digested parotid saliva, regardless of the phenotype. This result was obtained for all individuals in the sample. Although the intensity of staining varied, there was little difference in the overall patterns obtained from whole saliva of the individuals, except for minor phenotypically related variations.

It is apparent that many, if not all, phosphopeptides in whole saliva originate from salivary-gland secretions, and it is therefore of interest to evaluate from which proteins they arise.

Fig. 4(a) shows the result of gel electrophoresis of pure acidic PRPs digested with sediment suspension. Since it is known that the acidic PRPs, PRP1, PRP2, PRP3, PRP4, PIFs and PIFf and possibly also Dbs, Dbf and Pa, contain phosphoserine at positions 8 and 22, the peptides that are revealed on the gel should contain at least one of these residues. It is interesting to note that the peptides obtained from PRP4 and PRP2 were the same, and identical patterns were also obtained with PRP3 and PRP1 and with PIFf and PIFs. The amino acid sequences of PRP4 and PRP2 are identical, except that PRP4 terminates at residue 106 but PRP2 has a 44-amino-acid C-terminal extension, and the same primary structural relationship exists for PRP3+PRP1 and also for PIFf+PIFs. None of the peptides revealed on the gel, therefore, contain parts of the C-terminal extension found in PRP1, PRP2 and PIFs.

The overall peptide patterns of PRP1+PRP3, PRP2+PRP4 and PIFs+PIFf were very similar, and the minor variation in electrophoretic mobility could be related to differences in the primary structures of these proteins (A. Bennick, K. Minaguchi, D. I. Hay, D. Schlesinger, S. Schlakebier & G. Madapallimattam, unpublished work):

	N			C
	4	50	106	150
PRP1	— Asp —	Asn —	—————	
PRP3	— Asp —	Asn —	—————	
PRP2	— Asp —	Asp —	—————	
PRP4	— Asp —	Asp —	—————	
PIFs	— Asn —	Asp —	—————	
PIFf	— Asn —	Asp —	—————	

In the region of rapidly migrating components (Fig. 4a), peptides from PIFs+PIFf migrated slightly slower than the corresponding fragments from PRP1+PRP3 and PRP2+PRP4, suggesting that these peptides contained residue 4, which is asparagine in PIFs+PIFf, but aspartic acid in the other acid PRPs. The peptides did not extend to residue 50, since in that case the fragments of PRP1+PRP3 and PIFs+PIFf should have the same mobility. Db and Pa digests showed the same bands as PIFf+PIFs, suggesting that they were most closely related to these acidic PRPs, but additional peptides were also present in the Pa digest. In the region of slowly migrating peptides (Fig. 4a), the rate of mobility of corresponding peptides from PRP2, PRP1 and PIFs did not appear different, presumably because they were larger, and small differences in charge may not give rise to clearly discernible rates of mobility in this gel. The

Fig. 5. Chromatography of saliva on an immunosorbent column

Samples (4 ml) of parotid or submandibular saliva were applied to an AH-Sepharose column to which had been coupled anti-(acidic PRPs) antibodies. The elution profile of submandibular saliva is shown. The column (8 ml bed volume) was equilibrated and eluted with 10 mM-sodium phosphate buffer, pH 7.4, containing 0.15 M-NaCl. At the arrow, elution with 0.1 M-formic acid was started. The column was monitored by measuring A_{220} . Fractions 7–16 (UBI) and 17–32 (UBII) were pooled as shown; tubes 57–62 were pooled as bound proteins ('B').

Dbf and Dbs digests showed a different pattern of slowly migrating peptides, perhaps owing to the larger size of Dbf+Dbs (Figs. 4a and 4b).

The results reflect the high degree of similarity of the amino acid sequences of acidic PRPs and indicates that cleavages by salivary sediment enzyme occur of the same bonds in all acidic PRPs. The band patterns of purified PRP1, PRP2, PIFs, Pa and Dbf digested with sediment suspension are shown in Fig. 4(b). For comparison, samples of parotid salivas of various representative acidic PRP phenotypes digested with the same sediment suspension are also illustrated. All the bands obtained by digesting pure acidic PRP with sediment suspension could also be seen in at least one of the digested parotid salivas. The latter contained only a few rapidly migrating bands that were not present in the acidic PRP digests. Most of the bands seen in digested parotid saliva could therefore arise by digestion of acidic PRPs after secretion into the oral cavity.

To evaluate the origin of peptides not originating from acidic PRPs, parotid saliva was fractionated on the immunosorbent column and the resulting elution profile is illustrated in Fig. 5. Single radial immunodiffusion demonstrated the absence of PRPs from 10-fold-concentrated unbound protein, and a total of 3600 μ g of PRP was eluted with 0.1 M-formic acid in the case of parotid saliva. A similar result was obtained with submandibular saliva, except that 1600 μ g of PRP bound to the column.

Fig. 6. Digestion of proteins obtained by fractionation on the immunosorbent column (Fig. 5)

The content of the lanes (right to left) is as follows: UBI and UBII, unbound protein from the immunosorbent column; WS, whole saliva; B, protein bound to the immunosorbent column; PRP1 + PIFs, a mixture of PIFs and PRP1 digested with sediment suspension; IB-1, the basic PRP, IB-1, digested with sediment suspension. The whole and parotid saliva as well as the sediment suspension were all from the same person. Digestion with sediment suspension was at 37 °C for 15 min.

Electrophoresis of unbound and bound protein from parotid saliva digested with sediment suspension gave rise to the electrophoretogram illustrated in Fig. 6. Only a few rapidly migrating bands could be seen in the digest of unbound proteins, but these were also present in the digest of bound proteins. The pattern of peptides obtained from bound protein corresponded closely to that of whole saliva, suggesting that almost all the phosphopeptides in whole saliva arose from PRPs. A few bands present in digested bound protein did not have a counterpart in the digests of acidic PRPs. A digest of a phosphorylated basic PRP, IB-1, that has a 10-residue *N*-terminal sequence identical with that of PIF, except for the presence of asparagine rather than aspartic acid at position 2 (Kauffman *et al.*, 1986), gave rise to a peptide with the same mobility as one of the peptides from whole saliva that had no counterpart in the digests of acidic PRPs (Fig. 6). Digestion of statherin, another salivary phosphoprotein (Schlesinger & Hay, 1977), did not give rise to any of the peptides seen in whole saliva. Native cystatin, which also is phosphorylated (Shomers *et al.*, 1982), as well as cystatin digested with sediment suspension, both contained two phosphopeptides that corresponded to phosphopeptides in whole saliva. At present it is therefore not clear if these peptides are part of cystatin or a contaminant of the preparation. Fig. 7 shows the result of electrophoresis of parotid saliva digested with sediment suspension in the presence and absence of phenylmethanesulphonyl fluoride. The number and intensity of bands was greatly decreased in

Fig. 7. Prevention of degradation of parotid proteins by phenylmethanesulphonyl fluoride

Samples (1 ml) of parotid saliva were incubated with 0.1 ml of sediment suspension in the presence and absence of phenylmethanesulphonyl fluoride (8 mM) at 37 °C for 6 h. The following samples were electrophoresed on a 35%-(w/v)-polyacrylamide gel: lane 1: parotid saliva incubated without sediment; lane 2, parotid saliva incubated with sediment; lane 3, as lane 2, but phenylmethanesulphonyl fluoride was added.

the presence of phenylmethanesulphonyl fluoride, but addition of other inhibitors did not prevent the degradation of parotid protein (results not shown), demonstrating that most of the enzymes that produce the phosphopeptides were serine proteinases.

DISCUSSION

The collection of 5 ml of whole saliva takes about 5 min. It is unlikely that the peptides observed in whole saliva are formed after expectoration from the oral cavity, since samples of whole saliva collected every 30 s and immediately heated at 100 °C showed the same gel-electrophoretic pattern as that of a 5 ml sample. The phosphopeptides therefore most likely are present in the saliva that wets the oral surfaces. This proposal is supported by the observation that several of the phosphopeptides are formed by incubation of parotid saliva for only 30 s with the same relative amount of sediment as found in whole saliva, and the estimation from a mathematical model (Dawes, 1983) that, at a normal flow rate of 0.3 ml/min, the saliva present in the mouth at zero time will have been diluted by half in 2 min (C. Dawes, personal communication); thus the transient time of saliva in the mouth is sufficient for these peptides to be formed.

The revelation of the phosphopeptides was only possible because of the use of very concentrated acrylamide gels and the use of Stains-All. The acrylamide gels usually used to reveal proteins contain from 7 to 15% (w/v) acrylamide, but in such gels these peptides run in the dye front and therefore are likely to be overlooked. Moreover they do not stain readily with conventional protein stains, including the various silver stains, and it is therefore hardly surprising that they have not been observed before. Although the intensity of the bands varied from person to person, the patterns

obtained were remarkably similar, demonstrating that the enzymes responsible for the cleavage of the phosphorylated proteins occur generally in the population, and so also therefore, do the salivary phosphopeptides. Most of the peptides originate from acidic PRPs, although at least one may stem from basic PRPs; but further analysis of basic PRPs is necessary to determine their contribution to phosphopeptides in whole saliva. Other known phosphoproteins in saliva, such as statherin and cystatin, apparently contribute little to these peptides, but the presence of a few phosphopeptides that do not bind to the immunosorbent column demonstrates that some phosphopeptides apparently do not originate from PRP. It remains to be demonstrated if these phosphopeptides have the same biological activities as the *N*-terminal TX peptide from acidic PRPs and if the concentration of these peptides is sufficient to be of physiological importance. Hay & Grön (1976) demonstrated that the ability to inhibit hydroxyapatite formation associated with acidic PRPs in whole saliva occurred in fractions with increasingly lower M_r , the longer the whole saliva was incubated, and it is possible that these active fractions are indeed the phosphopeptides we have demonstrated in the present study. We have shown (Bennick *et al.*, 1983) that acidic PRPs are part of the newly formed dental pellicle, but are only present in minute concentrations in old pellicle. In addition, we have found that if acidic PRPs are adsorbed to hydroxyapatite, the *N*-terminal region which mediates binding to the mineral is protected from degradation. It is therefore possible that the old pellicle contains phosphopeptides from acidic PRPs that are no longer immunoreactive.

If these phosphopeptides are biologically important, one might wonder why they are not formed before secretion from the glands. Perhaps the answer is that the need for the organism to acquire such enzymes never arose during evolution, because the micro-organisms that inhabit the oral cavity possessed enzymes that would very quickly form the peptides after secretion. These questions need to be addressed in future studies. If these peptides are not biologically active, evaluation of their rate of appearance is still important in order to estimate how quickly the acidic PRPs are inactivated after secretion into the oral cavity.

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REFERENCES

- Azen, E. A. & Denniston, C. L. (1974) *Biochem. Genet.* **12**, 109–120
- Azen, E. A. & Denniston, C. L. (1981) *Biochem. Genet.* **19**, 475–485
- Azen, E. A. & Oppenheim, F. G. (1973) *Science* **180**, 1067–1069
- Bartlett, G. R. (1959) *J. Biol. Chem.* **234**, 466–468
- Bennick, A. (1982) *Mol. Cell. Biochem.* **45**, 83–99
- Bennick, A. & Cannon, M. (1978) *Caries Res.* **12**, 159–169
- Bennick, A. & Madapallimattam, G. (1983) *Arch. Oral Biol.* **28**, 13–17
- Bennick, A., McLaughlin, A. C., Grey, A. A. & Madapallimattam, G. (1981) *J. Biol. Chem.* **256**, 4741–4746
- Bennick, A., Chau, G., Goodlin, R., Abrams, S., Tustian, D. & Madapallimattam, G. (1983) *Arch. Oral Biol.* **28**, 19–27
- Campbell, K. P., MacLennan, D. H., Jorgensen, A. O. & Mintzer, M. C. (1983) *J. Biol. Chem.* **258**, 1197–1204
- Curby, W. A. (1953) *J. Lab. Clin. Med.* **41**, 493–496
- Dawes, C. (1983) *Caries Res.* **17**, 321–334
- Friedman, R. D., Merritt, A. D. & Rivas, M. L. (1975) *Am. J. Hum. Genet.* **27**, 292–303
- Green, M. R., Pastewka, J. V. & Peacock, A. C. (1973) *Anal. Biochem.* **56**, 43–51
- Hay, D. I. & Grön, P. (1977) in *Microbial Aspects of Dental Caries* (Stiles, W. J., Loesche, W. J. & O'Brien, T. C., eds.), vol. 1, pp. 143–150, Information Retrieval, Washington
- Hay, D. I. & Moreno, E. C. (1979) *J. Dent. Res.* **58**, 930–940
- Kauffman, D., Hofmann, T., Bennick, A. & Keller, P. (1986) *Biochemistry* **25**, 2387–2392
- Kousvelari, E. E., Baratz, R. S., Burke, B. & Oppenheim, F. G. (1980) *J. Dent. Res.* **59**, 1430–1438
- Maeda, N. (1985) *Biochem. Genet.* **23**, 455–464
- Moreno, E. C., Kresak, M. & Hay, D. I. (1982) *J. Biol. Chem.* **257**, 2981–2989
- Schlesinger, D. & Hay, D. I. (1977) *J. Biol. Chem.* **252**, 1689–1695
- Shomers, J. P., Tabak, L. A., Levine, M. J., Mandel, I. D. & Ellison, S. A. (1982) *J. Dent. Res.* **61**, 764–767
- Smith, Q. T., Shapiro, B. L. & Hamilton, M. J. (1975) *Arch. Oral Biol.* **20**, 369–373
- Truelove, E. L., Bixler, D. & Merritt, A. D. (1967) *J. Dent. Res.* **46**, 1400–1403
- Wong, R. S. C., Madapallimattam, G. & Bennick, A. (1983) *Biochem. J.* **211**, 35–44

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