The Nature of the Hydroxyapatite-Binding Site in Salivary Acidic Proline-Rich Proteins

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Proteins A and C, which are major components of the acidic proline-rich proteins in human saliva, were digested, before or after adsorption to hydroxyapatite, with alkaline phosphatase, trypsin, thermolysin and a proteinase preparation from salivary sediment. The results demonstrate that the binding site is located in the proline-poor *N*-terminal part of the protein, possibly between residues 3 and 25. Phosphoserine is necessary for maximal adsorption of the proteins to hydroxyapatite. When proteins A and C are adsorbed to hydroxyapatite before proteolytic digestion there is a protection of some of the susceptible bonds in the *N*-terminal part of the proteins and a gradual removal of the proline-rich *C*-terminal part. Thermolysin can cleave susceptible bonds in the part of the protein that remains bound to hydroxyapatite, but at least some of the resulting peptides are retained on the mineral. Since the ability of the proteins to inhibit hydroxyapatite formation and to bind calcium is located in the *N*-terminal proline-poor part, it is possible that these activities are retained after proteolytic digestion of the adsorbed proteins.

If salivary secretions are exposed to hydroxyapatite a selective adsorption of protein occurs. Although some proteins are almost completely adsorbed, others are only adsorbed to a small extent or not at all (Hay, 1967, 1973; Bennick & Cannon, 1978). Some of the proteins that are most readily adsorbed are the so-called 'acidic proline-rich proteins', and it has been demonstrated that they account for 42% of all of the proteins adsorbed to hydroxyapatite *in vitro* from human parotid and submaxillary saliva (Bennick & Cannon, 1978). Two of the major components of the acidic proline-rich proteins, named proteins A and C, have in particular been characterized (Bennick, 1975, 1977). The primary structure of protein A has been determined (Wong *et al.*, 1979), and it is closely related to that of protein C (Bennick *et al.*, 1977). The amino acid sequences of these proteins have a number of unique features that are not found in proteins whose primary structures are known. Both proteins A and C bind calcium, and the strength of interaction suggests that they may take part in calcium exchanges in the oral cavity (Bennick & Cannon, 1978). The proteins also have the ability to prevent transformation of calcium phosphate to more basic calcium phosphate salts such as hydroxyapatite (Hay & Grön, 1977).

The inorganic part of dental enamel, which constitute 95% of the total dry weight, consists almost exclusively of hydroxyapatite. It has also been demonstrated that there is a selective adsorption *in*

vivo of proteins from saliva to the surface of dental enamel (Mayhall, 1970). This layer of adsorbed protein has been referred to as the 'acquired pellicle'. At present it is not clear what the physiological function of the acquired pellicle is, but it is known that it will retard dissolution of the underlying enamel (Zahradnik *et al.*, 1976), and it seems likely that it will modify reactions that take place at the saliva/enamel interface. Proteins A and C can be demonstrated in the acquired pellicle (D. Tustian, unpublished work), and it would be of interest to determine whether the proteins retain their known biological activities after they have been adsorbed to the enamel surface, since these activities might be important in preventing addition of mineral to the tooth surface or dissolution of the enamel.

To evaluate these possibilities, an understanding of the nature of the binding of proteins A and C to hydroxyapatite is important. The present study describes the location of the binding site in the proteins.

Experimental

Materials

Thermolysin, sodium deoxycholate, di-isopropyl phosphorofluoridate and *N*-ethylmorpholine were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Azocoll was purchased from Calbiochem, Los Angeles, CA, U.S.A., and hydroxyapatite (Bio-

Gel HTP) from Bio-Rad, Richmond, CA, U.S.A. Dialysis tubing (mol.wt. cut-off 2000) was supplied by Spectrum Medical Industries, Los Angeles, CA, U.S.A. The sources of other materials were the same as those used in previous studies (Bennick, 1975, 1976, 1977).

Methods

Purification of proteins. Proteins A and C from human salivary secretions were purified as described previously (Bennick, 1975, 1977). Antiserum to protein C was prepared as described by Bennick (1977).

Digestion of proteins A and C by phosphatase or trypsin followed by adsorption to hydroxyapatite. Samples of 1.33 mg of protein A or C were digested with 0.053 mg of alkaline phosphatase in 0.5 ml of 1 M-Tris/HCl, pH 8.0, at 45°C for 48 h. The digests were heated at 100°C for 10 min, dialysed against water, freeze-dried and redissolved in 5 mM-Tris/HCl, pH 7.5. The amount of phosphate in the proteins was assayed as described by Bartlett (1959).

Digestion of 1 mg of protein A or C with trypsin was performed with an enzyme/substrate ratio of 1:100 (w/w) in 0.5 ml of 5 mM-Tris/HCl, pH 7.5, at room temperature for 18 h. The enzyme was inactivated by heating the digests at 100°C for 10 min. The extent of hydrolysis was evaluated by polyacrylamide-gel electrophoresis as described by Bennick (1975).

Portions (0.5 ml) of 5 mM-Tris/HCl, pH 7.5, containing 125 µg of digested protein A or 130 µg of protein C were added to samples of 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 mg of hydroxyapatite. Samples of undigested protein A or C that had been incubated at 45°C for 48 h or at 22°C for 18 h were added to hydroxyapatite in a similar manner. The hydroxyapatite was kept in suspension for 0.5 h at room temperature. This was followed by centrifugation of the suspension at 1600g for 10 min at 22°C.

The amount of protein adsorbed was calculated by subtracting the A_{220} reading of the supernatant from the corresponding value for the protein incubated without hydroxyapatite.

In order to analyse the composition of the tryptic peptides remaining on the mineral, samples of the hydroxyapatite that had been incubated with tryptic digests of protein A or C were resuspended in 5 mM-Tris/HCl, pH 7.5, and then centrifuged as described above. The supernatant was discarded and the procedure was repeated. The washed hydroxyapatite was dissolved in 1 ml of 0.2 M-EDTA, pH 7.0, dialysed exhaustively against water, freeze-dried and redissolved in 0.2 ml of water. Samples (approx. 60 µg) of peptides were hydrolysed in constant-boiling HCl in evacuated, sealed tubes at 110°C for 24 h and the amino acid composition analysed on a Beckman model 120 C amino acid analyser. To compensate for

possible interference in the amino acid analysis, a sample containing only hydroxyapatite was treated in the same manner. A comparable volume of the blank hydroxyapatite solution was dried, hydrolysed and subjected to amino acid analysis.

Tryptic digestion of protein A or C adsorbed to hydroxyapatite. Samples (6 mg) of hydroxyapatite were suspended in 1 ml portions of 5 mM-Tris/HCl, pH 7.5, containing approx. 300 µg of protein A or C. The samples were incubated on an orbital shaker for 0.5 h at 22°C. After incubation, the hydroxyapatite was sedimented by centrifugation as described above and the A_{220} of the supernatant recorded. A portion (1 ml) of 5 mM-Tris/HCl, pH 7.5, containing 6 µg of trypsin was added to the protein-coated hydroxyapatite and the samples were incubated on an orbital shaker for 0.5, 6 and 16 h. Other samples, lacking protein or protein and trypsin, were incubated for 16 h. After incubation the samples were heated at 100°C for 10 min to inactivate the trypsin. The hydroxyapatite was sedimented by centrifugation and the supernatant removed. The hydroxyapatite was resuspended, sedimented as described above and then dissolved in 1 ml of 1% HCl. The A_{220} of the supernatants and the dissolved hydroxyapatite was recorded. The dissolved mineral was dialysed in succession against 1% HCl, 0.1% HCl and water. After freeze-drying, the sample was redissolved in 0.5 ml of water.

The amount of protein released from hydroxyapatite during incubation and the amount retained on the mineral could be calculated from the A_{220} readings by using the specific absorption coefficients ($A_{1\text{mm},220}^{0.1\%}$) of 1.06 and 0.98 for proteins A and C respectively. The absorption coefficients were determined from solutions of proteins A and C of known concentration as determined from amino acid analysis. The amino acid compositions of samples of protein recovered from the hydroxyapatite were determined as described above. Other samples of the peptides retained on hydroxyapatite were analysed for immunoreactivity by double immunodiffusion with an antiserum specific to protein C (Bennick, 1977). Preliminary experiments with native protein A and C had demonstrated that the immunoreactivity of these proteins was not affected by either heating at 100°C for 10 min at neutral pH or exposure to 1% HCl at 22°C.

To investigate the effect of more exhaustive tryptic digestion, the experiment was repeated with protein C by using 0.5 and 40 h periods of incubation. The nature of the peptides retained on the hydroxyapatite was evaluated by subjecting 50 µg samples of the digests to polyacrylamide-gel electrophoresis (Bennick, 1975). Samples of the supernatants from the 0.5 and 40 h digests were removed before heating, and the tryptic activity assayed by incubation with 2 mg of Azocoll in a final volume of 1.0 ml of 0.03 M-NH₄HCO₃.

for 24 h at 37°C. The A_{520} of the digests were used as a measure of enzymic activity.

To evaluate the extent to which intact protein may become desorbed from hydroxyapatite during the incubation period, 1 ml of 5 mM-Tris/HCl, pH 7.5, containing 330 μ g of protein A or C was added to 6 mg of hydroxyapatite and maintained in suspension for 0.5 h. After centrifugation as described above, the supernatant was removed. The hydroxyapatite was resuspended in 1 ml of 5 mM-Tris/HCl, pH 7.5. This procedure was repeated 2, 6, 34 and 48 h after adsorption of the protein solution to hydroxyapatite. At the end of the experiment the hydroxyapatite was dissolved in 1 ml of 1% HCl. The concentration of protein in the supernatants and the dissolved hydroxyapatite solutions was determined from the A_{220} .

Digestion of protein C with thermolysin. Thermolysin was chosen because it is known to cleave the *N*-terminal tryptic fragment of proteins A and C at well-defined positions (Wong *et al.*, 1979).

The susceptibility of hydroxyapatite-adsorbed protein C to thermolytic digestion was tested in the same manner as that used in the tryptic digestions, with the exception that the enzyme-to-protein ratio was 1:20 (w/w), the buffer contained 2 mM-CaCl₂ and the periods of incubation were 4, 24 and 48 h. In addition, another sample was incubated for 24 h, after which the supernatant containing the enzyme was removed, replaced with the same amount of enzyme and incubated for a further 24 h. Samples in which protein C, thermolysin or hydroxyapatite had been left out, and a sample containing only protein C, were also incubated for the same periods of time. The supernatants, washes and hydroxyapatite with adsorbed peptides were treated and analysed as described in the experiment with trypsin. Thermolysin that had been adsorbed to hydroxyapatite was found not to be active after the mineral was dissolved and dialysed. Any changes in the adsorbed protein could therefore not be due to thermolytic activity remaining in the adsorbed and redissolved protein.

In order to analyse the peptides retained on hydroxyapatite, another digestion of a 6-fold larger amount of protein C was undertaken. The digestion period was 48 h, and the enzyme was replenished after 24 h. A sample in which protein C had been deleted was also incubated. The peptide mixture recovered from the hydroxyapatite was analysed for *N*-terminal amino acids by dansylation of 6 μ g of peptide followed by hydrolysis and chromatography as previously described (Bennick, 1977). The rest of the samples were subjected to high-voltage paper electrophoresis at pH 7.4 (32 V/cm for 60 min). Guide strips were cut from the paper and stained with ninhydrin (Heilman *et al.*, 1957) and chlorine (Schwartz & Pallansch, 1958) to locate the peptides. The peptides were eluted from the paper with 1%

pyridine, freeze-dried, hydrolysed and analysed for amino acids as described above.

The ability of a solution of thermolytic digest of protein C to adhere to hydroxyapatite was evaluated by digesting 3.6 mg of protein C in the absence of hydroxyapatite at 37°C for 4 h. This time period has been found to be sufficient for extensive hydrolysis of the acidic proline-rich proteins (Wong *et al.*, 1979). After incubation, the enzyme was inactivated by heating at 100°C for 10 min and 72 mg of hydroxyapatite was added to the digest. The methods used in the incubation of the digest, and recovery of the supernatant and peptides adsorbed to hydroxyapatite were the same as used when the tryptic digest was adsorbed to hydroxyapatite. The recovered peptides were analysed by double immunodiffusion against antiserum to protein C, the *N*-terminal amino acids were determined, and the remainder were subjected to high-voltage electrophoresis as described above.

Susceptibility of hydroxyapatite adsorbed proteins to digestion by a proteolytically active fraction from salivary sediment. Samples (approx. 70 ml) of whole saliva were collected by expectoration from three persons. The flow of saliva was stimulated by chewing Parafilm. The fluids were centrifuged at 27000g at 3°C for 10 min. The supernatants were removed and the pellets, which consisted mostly of bacteria, leucocytes and desquamated epithelial cells (Kleinberg, 1970) were combined and suspended in phosphate-buffered saline (10 mM-sodium phosphate, pH 7.4, containing 0.154 M-NaCl). This procedure was repeated twice. The washed pellet was suspended in 10 ml of 0.03 M-NH₄HCO₃ containing 0.4% deoxycholate, sonicated by five pulses of 60 s each on an MSE 150 W ultrasonic disintegrator set at medium power and incubated with gentle stirring for 3 h at 22°C. This was followed by centrifugation of the suspension at 35000g at 3°C for 20 min. The supernatant was removed, and the pellet resuspended and centrifuged twice. The supernatant and washes were combined, dialysed against 0.03 M-NH₄HCO₃ and freeze-dried. The dry material was redissolved in 2 ml of 0.03 M-NH₄HCO₃ and applied to a column (2.6 cm $\times$ 18.9 cm) of Sephadex G-100, which was developed with the same solvent.

The eluate fractions were assayed for proteolytic activity with Azocoll as substrate as described above. Fractions showing high specific activity were pooled, freeze-dried and stored at -20°C.

Digestion of the acidic proline-rich proteins with the enzyme preparation was tested by incubating 50 μ g of protein C with 5, 10 or 20 μ g of enzyme preparation in 50 μ l of 0.03 M-NH₄HCO₃ at 37°C for 16 h. The digests were analysed by polyacrylamide-gel electrophoresis in 7.5% (w/v) gels at pH 9.2 (Davis, 1964). The pH-dependence of the enzyme activity was evaluated by incubating 50 μ g of protein C with 2 μ g of enzyme preparation in 80 μ l of 10 mM-Tris/HCl,

pH 8.5, 8.0, 7.4 and 7.1, and in 10 mM-imidazole/HCl, pH 6.8 and 6.0. The digests were analysed by polyacrylamide-gel electrophoresis (Davis, 1964).

Inhibition of the enzyme preparation was evaluated by performing the Azocoll digestion in the presence of 25 mM-EDTA, 10 mM-*N*-ethylmorpholine or 1 mM-di-isopropyl phosphorofluoridate. The stability of the enzyme preparation was tested by preincubation of 30 μ g of enzyme preparation in 100 μ l of 0.03 M- NH_4HCO_3 at 37°C for 1, 2, 4 and 8 days. The activity of the samples was evaluated by Azocoll assay.

Digestion by the enzyme preparation of protein C adsorbed to hydroxyapatite was performed as described for trypsin digestion of adsorbed protein with the following exceptions. Samples of 600 μ g of protein C were adsorbed to 12 mg of hydroxyapatite. After removal of non-adsorbed protein, 60 μ g of the salivary-sediment enzyme in 1 ml of 5 mM-Tris/HCl, pH 7.5, was added, and the samples were incubated at 37°C for 4, 8, 16 and 32 days. This was followed by inactivation of the enzyme, removal of supernatant and dissolution of hydroxyapatite as described. The A_{220} of the supernatant of the dissolved hydroxyapatite was recorded. The dissolved mineral was dialysed, freeze-dried and redissolved in 0.2 ml of 0.03 M- NH_4HCO_3 . Concurrent with these digestions, other samples of adsorbed protein C were incubated for 16 and 32 days, but in this case the supernatant containing the enzyme was removed and replaced with a volume of buffer containing another 60 μ g of salivary-sediment enzyme after 8, 16 and 24 days. Other samples were incubated in which either protein C, salivary-sediment enzyme or hydroxyapatite had been omitted, and samples containing only protein C were also incubated for the specified periods of time.

To evaluate the extent and nature of digestion, the amount of protein retained on the hydroxyapatite was calculated. In addition, double immunodiffusion was performed with antiserum to C against the protein recovered from hydroxyapatite and the samples incubated without hydroxyapatite.

Samples (50 μ g) of the peptides that had been recovered from hydroxyapatite were analysed for amino acids as described above.

Results

Digestion of proteins A and C with phosphatase resulted in removal of 59% and 63% respectively of the covalently bound phosphate. This result and the fact that the proteins contained a total of two phosphoserine residues (Wong *et al.* 1979) agreed with the gel-electrophoretic patterns of phosphatase-digested protein A and C, which showed the presence of two bands with slightly lower anodic mobility than the native proteins. The phosphatase preparation was not contaminated with proteolytic enzymes (Bennick,

Fig. 1. Adsorption of trypsin or phosphatase-digested protein A or C to hydroxyapatite

The percentage of total protein in the digest adsorbed to hydroxyapatite is plotted as a function of the amount of hydroxyapatite. The adsorption was performed in 5 mM-Tris/HCl, pH 7.5. (a) and (b) illustrate the results obtained for protein A and C respectively. ○, Undigested protein; Δ, protein digested with phosphatase; □, protein digested with trypsin.

1977) and the appearance of the bands is therefore not due to proteolysis.

Gel electrophoresis of tryptic digests of protein A and C in solution indicated that no native protein was left. Other results have demonstrated that 18 h digestion resulted in extensive tryptic cleavage of protein A and C (Wong *et al.*, 1979).

The amount of protein adsorbed to hydroxyapatite was plotted as a function of the total amount of hydroxyapatite (Fig. 1). Heating proteins A and C at 45°C had little if any effect on adsorption to hydroxyapatite, and all the data for the native proteins have therefore been plotted together. From Fig. 1(a) it can

be calculated that the amount of phosphatase-digested protein A adsorbed to hydroxyapatite corresponded to $49 \pm 9\%$ (s.d.) of the undigested protein adsorbed to the same amount of hydroxyapatite. The corresponding value for protein C was $32 \pm 5\%$ (Fig. 1b).

These results indicate that phosphoserine residues are necessary for optimal binding of the proteins to hydroxyapatite and point to the importance of the *N*-terminal tryptic peptide in proteins A and C, which contains both phosphoserine residues (Bennick *et al.*, 1977).

Compared with the native proteins, $23 \pm 5\%$ of the tryptic digest of protein A (Fig. 1a) and $13 \pm 5\%$ of the protein C digest (Fig. 1b) were adsorbed to hydroxyapatite. Amino acid analysis indicated small amounts of contaminants in the hydroxyapatite. These were subtracted from the composition of the tryptic peptides retained on hydroxyapatite and the values have been listed in Table 1 together with the composition of the *N*-terminal tryptic peptide of proteins A and C (Wong *et al.*, 1979). There is very good agreement between the composition of the adsorbed peptide and that of the *N*-terminal tryptic peptide of proteins A and C. It is apparent that the hydroxyapatite-binding site is located in the *N*-terminal tryptic peptide of the proteins. In subsequent experiments the small amount of contaminants in hydroxyapatite was removed before use by washing the mineral in the buffer.

Table 1. Composition of tryptic peptides adsorbed to hydroxyapatite

Amino acid	Molar ratio*		
	Peptides recovered from hydroxyapatite		<i>N</i> -Terminal peptide of protein A or C†
	Protein A	Protein C	
Lysine	0.1	0	0
Histidine	0.2	0	0
Arginine	0.9	1.0	1
Aspartic acid	6.7	6.4	7
Threonine	0.2	0.3	0
Serine	3.3	2.9	3
Glutamic acid	7.5	8.9	8
Proline	1.0	1.6	1
Glycine	3.2	3.6	2
Alanine	0.3	0	0
Valine	2.7	2.0	3
Isoleucine	1.6	1.7	2
Leucine	2.0	2.3	2
Tyrosine	0.1	0	0
Phenylalanine	1.0	1.0	1

* Assuming phenylalanine = 1.00.
† Data from Wong *et al.* (1979).

When the native protein A or C was adsorbed to hydroxyapatite before digestion with trypsin, there was a rapid loss of protein from hydroxyapatite in the first 0.5 h of incubation (Fig. 2). The loss of protein continued during the entire period of digestion, but even after 16 h incubation, 49% of protein A and 47% of protein C remained on the hydroxyapatite, and after 40 h 34% of protein C was still bound to the mineral.

The decreased rate of hydrolysis with time was not due to inactivation of the enzyme, since the same tryptic activity was found in the supernatant of the digest after 0.5 and 40 h incubation.

Incubation of $330 \mu\text{g}$ of protein A or C with hydroxyapatite in the absence of trypsin resulted in the adsorption of $316 \mu\text{g}$ of protein in 0.5 h. During the following 2 and 6 h incubation periods, a total of $14 \mu\text{g}$ of protein A and $7 \mu\text{g}$ of protein C became desorbed. No further desorption occurred during the 48 h period, and $312 \mu\text{g}$ of protein A and $288 \mu\text{g}$ of protein C were recovered from the hydroxyapatite at the end of the incubation. This demonstrates that, of the protein that was adsorbed during the initial 0.5 h period, 4% of protein A and 2% of protein C became desorbed during the following 47.5 h. The small differences between the results for protein A and C do not appear to be significant.

Representative results of amino acid analysis of

Fig. 2. Tryptic digestion of protein A and C adsorbed to hydroxyapatite

Approx. $300 \mu\text{g}$ of protein A or C was adsorbed to 6mg of hydroxyapatite and then incubated in 1 ml of 5mM-Tris/HCl, pH7.5, containing $6 \mu\text{g}$ of trypsin. The percentage of protein retained or lost from hydroxyapatite is plotted as a function of period of incubation. ■, Protein A retained; ●, protein C retained; □, protein A lost; ○, protein C lost. In the case of protein C, the data from two experiments (16 and 40 h total incubation) have been combined.

these, peptide TX contains only one proline and two glycine residues (Fig. 4). The results illustrated in Fig. 3 therefore suggest a gradual removal of the C-terminal part of the adsorbed protein A and C. This is in agreement with the decrease in the amount of histidine, which is not located in peptide TX (Fig. 4), and the decrease in glutamic acid. Peptide TX contains eight residues of glutamic acid and glutamine, whereas protein A contains a total of 29 residues; the amount in protein C is 40 residues.

Polyacrylamide-gel electrophoresis demonstrated that, after 0.5 h tryptic digestion of the adsorbed protein C (Fig. 5), no native protein could be detected, but a band with the same mobility as protein A had appeared in addition to peptides with lower electrophoretic mobility. Further hydrolysis of these components after 49 h digestion was evident from the polyacrylamide-gel-electrophoretic patterns. Double immunodiffusion of the adsorbed protein digested with trypsin showed that, even after 16 h digestion of protein A, the protein retained on the hydroxyapatite gave an immunologically identical reaction with native protein. In contrast, proteins A and C cannot form precipitable immunocomplexes with antiserum to protein C after 0.5 h incubation of the protein in solution with trypsin. Tryptic digestion of adsorbed protein C for 0.5 h followed by double immunodiffusion resulted in a reaction of partial identity with native protein C, but of complete identity with

protein A (Fig. 6). After digestion of protein C for 40 h, the peptides retained on hydroxyapatite gave no visible precipitate on double immunodiffusion.

When protein C was adsorbed on hydroxyapatite and then digested with thermolysin, there was an initial rapid loss of peptides from hydroxyapatite.

Fig. 6. Tryptic digestion of protein C adsorbed to hydroxyapatite: double immunodiffusion of digest remaining on hydroxyapatite against antiserum to protein C

A 1% agarose gel containing diethylbarbituric acid/sodium diethylbarbiturate buffer, pH 8.6, 1/0.02, was used. The contents of the wells were as follows: centre well, antiserum to protein C; wells 1 and 5, native protein A; well 3, native protein C; well 2, protein remaining on hydroxyapatite after 0.5 h tryptic digestion; well 4, protein remaining on hydroxyapatite after 40 h digestion.

Fig. 5. Tryptic digestion of protein C adsorbed to hydroxyapatite: polyacrylamide-gel electrophoresis of digest remaining on hydroxyapatite

Polyacrylamide-gel electrophoresis was performed on 4.5% (w/v) gels at pH 9.2 in 0.1 M-Tris/glycine buffer, and the gels stained with Coomassie Blue (Bennick, 1975). (a) Protein recovered from hydroxyapatite after 0.5 h digestion with trypsin; (b) as (a), but the incubation period was 40 h; (c) trypsin incubated with hydroxyapatite in the absence of protein C for 40 h; (d) native protein A; (e) native protein C.

Fig. 7. Thermolytic digestion of protein C adsorbed to hydroxyapatite: amino acid composition of the digest remaining on hydroxyapatite

The amount of some of the amino acids in the digest is plotted as a function of the period of digestion with thermolysin. In the case of 48 h digestion, the enzyme solution was replaced after 24 h. The contribution from thermolysin has been subtracted as described in the text. The compositions are expressed as molar ratios (= no. of residues) assuming the presence of three valine residues. The data given at 0 h are the amounts in the intact protein (Bennick, 1977). □, Glutamic acid; ○, proline; △, glycine; ■, isoleucine; ▲, histidine.

After 4h hydrolysis only 52% of the adsorbed protein remained on the mineral. This was followed by a gradually diminished rate of loss, and 32% of the protein remained after 24h, and 24% was bound to the mineral after 48h incubation. When the enzyme was replenished after 24h, a total of 25% of the adsorbed protein remained on the hydroxyapatite after 48h. The decreased rate of hydrolysis was therefore due to exhaustive digestion of protein C rather than inactivation of the enzyme. Adsorbed protein C that had been digested with thermolysin for 4h gave a reaction of complete identity with protein C on double immunodiffusion with antiserum to protein C. After digestion of adsorbed protein C with thermolysin for 24 and 48h, the protein was unable to form an immunoprecipitate with the antiserum.

Some of the results of amino acid analyses of the retained peptides are given in Fig. 7. The composition of the digest was not affected by replenishing the enzyme after 24h. Thermolysin contains threonine, which is not found in protein C (Bennick, 1977). From amino acid analysis of thermolysin adsorbed to hydroxyapatite it was therefore possible to subtract the contribution from adsorbed thermolysin to the peptides retained on hydroxyapatite. The corrected values are in molar ratios, assuming that all three residues of valine in the protein are present. The marked decrease in the amount of glutamic acid, proline, glycine and histidine, but lack of change in isoleucine content, indicates digestion of the C-terminal part of the protein, and it is the N-terminal

part that remains on the mineral. The composition of the unfractionated peptides retained on hydroxyapatite has been listed in Table 2, and Fig. 4 shows that it is close to the composition of a peptide corresponding to positions 3–34 (peptide Th6). N-Terminal analysis of the adsorbed peptides demonstrated the presence of valine and leucine. This is in agreement with the previously observed thermolytic cleavage of the aspartic acid–leucine bond in positions 2–3 and the leucine–valine bond in positions 14–15 (Wong *et al.*, 1979). High-voltage paper electrophoresis of the digest indicated the presence of three peptides that all stained with ninhydrin as well as chlorine. This indicates that none of the peptides contains the blocked N-terminal of the native protein. The results of the amino acid analyses of the peptides are presented in Table 2. The composition of the peptides is seen to agree well with previously identified peptides obtained by digestion of peptide TX and which in the intact protein occupies positions 3–14 (peptide Th2) and positions 15–25 (peptide Th3). The composition of the third peptide (peptide Th5) indicates that it occupies positions 15–34 in the protein.

When protein C was digested with thermolysin for 4h before adsorption to hydroxyapatite, only 7.8% of the total weight of the digest was adsorbed. The digest did not react with antiserum to protein C on double immunodiffusion. The result of amino acid analysis of the unfractionated adsorbed peptides is given in Table 2. The composition was similar to that of the peptides

Table 2. Composition of thermolytic peptides retained on, or adsorbed to, hydroxyapatite
Values have not been corrected for loss or incomplete cleavage during hydrolysis.

Amino acid	Peptides retained on hydroxyapatite after thermolytic digestion of adsorbed protein C	Molar ratios			Peptides adsorbed to hydroxyapatite from an unfractionated thermolytic digest of protein C
		Retained peptides separated by high-voltage electrophoresis			
		Th2	Th2+Th3	Th5	
Lysine					0.1 (0)‡
Histidine	0.1 (0)*	—	—	—	0.1 (0)
Arginine	0.6 (1)	—	—	0.7 (1)†	—
Aspartic acid	6.1 (6)	3.0 (3)†	4.9 (5)†	3.3 (3)	4.4 (5)
Threonine	—	—	—	—	—
Serine	1.1 (3)	0.8 (1)	2.1 (3)	1.3 (2)	1.7 (3)
Glutamic acid	9.0 (8)	3.0 (3)	5.2 (5)	5.0 (5)	5.2 (5)
Proline	3.8 (3)	1.0 (1)	1.6 (1)	2.8 (2)	2.0 (1)
Glycine	3.5 (3)	0.2 (0)	2.4 (2)	3.8 (3)	2.4 (2)
Alanine	—	0.1 (0)	—	—	0.1 (0)
Valine	3.0 (3)	1.9 (2)	3.7 (3)	0.7 (1)	3.0 (3)
Isoleucine	1.6 (2)	—	1.0 (1)	1.8 (2)	0.6 (1)
Leucine	1.7 (2)	1.6 (2)	2.4 (2)	0.2 (0)	1.9 (2)
Phenylalanine	0.7 (1)	—	0.7 (1)	0.8 (1)	0.2 (1)

* The values in parentheses are the amounts in the thermolytic peptide Th6 (Fig. 4).

† The values in parentheses are the amounts in the thermolytic peptide indicated (Fig. 4).

‡ The values in parentheses are the amounts in peptides Th2 and Th3 (Fig. 4).

occupying the positions between residues 3 and 25 (peptides Th2 and Th3) in the intact protein. From *N*-terminal analysis of the adsorbed peptides, valine and leucine could be identified. A third spot on the chromatogram was identified as a valine-isoleucine dipeptide; this could be due to incomplete cleavage of the *N*-terminal peptide bond in peptide Th3. High-voltage paper electrophoresis of the adsorbed peptides resulted in eight spots, four major and four minor, some of which were overlapping on the electrophoretogram. They all stained with ninhydrin as well as with chlorine. This suggests the absence of any peptide with a blocked *N*-terminus. No further purification of the peptides was undertaken.

When the extract of the pellet of salivary sediment was fractionated on Sephadex G-100, the elution

Fig. 8. Gel filtration of salivary-sediment extract on Sephadex G-100

The extract of salivary sediment from 70 ml of whole saliva was freeze-dried and redissolved in 2 ml of 0.03 M NH_4HCO_3 and applied to a column (2.6 cm $\times$ 18.9 cm) of Sephadex G-100, which was developed in the same solvent. The flow rate was 20 ml/h. The volume of the fractions was 5 ml and A_{220} (—) was read in a cell with a 1 mm optical path-length. Proteolytic activity (●) was assayed by the Azocoll method as described in the text and expressed as $A_{320}^{15\text{m}}$. $\longleftrightarrow$ indicates the fraction that was pooled to give a preparation of salivary-sediment proteinase.

pattern illustrated in Fig. 8 was obtained. Fractions with high enzymic activity were well separated from those with a high protein concentration. The fractions indicated in Fig. 8 were pooled and used for proteolytic digestion. The ability of the enzyme preparation to digest protein C is illustrated in Fig. 9. It is apparent that several peptide bonds have been cleaved in the protein. Incubation of protein C with the enzyme preparation at various (6.0–8.5) pH values demonstrated proteolytic activity over the whole pH range tested.

The effects of enzyme inhibitors and preincubation of the enzyme preparation on the proteinase activity are listed in Table 3. From the results it is apparent that only di-isopropyl phosphorofluoridate can inhibit the proteinase preparation, suggesting that only serine proteinases are present. EDTA apparently caused a slight activation of the proteinases. The activity of the enzyme preparation had not diminished after preincubation for 8 days at 37°C. In fact there was an increase in the activity, which was highest after 4 days. It is possible that limited proteolysis caused activation of proteinases during the incubation.

The amount of protein (adsorbed protein C and enzyme) retained on hydroxyapatite after digestion for various periods of time is plotted in Fig. 10. The rate of removal decreased with time. The removal of the supernatant and addition of new enzyme solution

Fig. 9. Digestion of protein C with salivary-sediment proteinase

A portion (50 μg) of protein C was digested with the salivary-sediment proteinase in 50 μl of 0.03 M NH_4HCO_3 at 37°C for 16 h. The digests were analysed by electrophoresis in 7 cm-long 7% (w/c) polyacrylamide gels at pH 9.2 as described by Davis (1964). Components migrating toward the anode were separated. (a) Protein C digested with 20 μg of enzyme preparation; (b) as (a), but 10 μg of enzyme was used; (c) as (a), but 5 μg of enzyme was used; (d) protein C incubated without enzyme.

Fig. 10. Digestion of protein C adsorbed to hydroxyapatite by salivary-sediment proteinase

Approx. 600 μ g of protein C was adsorbed to hydroxyapatite and digested with 60 μ g of salivary-sediment proteinase in 5 mM-Tris/HCl, pH 7.5. The percentage of protein retained on hydroxyapatite is plotted as a function of the period of incubation. ●, No replenishment of enzyme during incubation; ■, enzyme replenished after 8 or 8, 16 and 24 days of incubation.

after 8 or 8, 16 and 24 days caused a further decrease in the amount of protein retained on hydroxyapatite.

Double immunodiffusion demonstrated that, compared with native protein C, there was no change in the immunoreactivity of protein C incubated without enzyme in solution or adsorbed to hydroxyapatite for 32 days. The preparation of salivary-sediment enzyme did not react with antiserum to protein C. When protein C in solution was incubated with the salivary-sediment enzyme for 16 days, the digest gave only a partially identical reaction with protein A and C on double immunodiffusion (Fig. 11a). In contrast, protein C adsorbed to hydroxyapatite before digestion with the enzyme preparation for 16 days gave a reaction of complete identity with protein A and C on double immunodiffusion, and after digestion of the adsorbed protein for 32 days the digest gave a reaction of complete identity with protein A, but partial identity with protein C (Fig. 11b). This pattern of reaction was also obtained with a sample of adsorbed protein C digested for 16 days, the enzyme being renewed after 8 days (Fig. 11c). If protein C was incubated for 32 days, renewing the enzyme after 8,

Fig. 11. Digestion of protein C adsorbed to hydroxyapatite by salivary-sediment proteinase: double immunodiffusion of digest remaining on hydroxyapatite against antiserum to protein C

The conditions for double immunodiffusion were the same as those described in Fig. 6. (a) Protein C in solution digested with the enzyme. Centre well, antiserum to protein C; wells 1 and 5, native protein C; well 2, 16 days digest; well 3, native protein A; well 4, 32 days digest. (b) Protein C adsorbed to hydroxyapatite and then digested with the enzyme. Centre well, antiserum to protein C; wells 1 and 5, native protein A; well 2, 16 days digest; well 3, native protein C; well 4, 32 days digest. (c) Protein C adsorbed to hydroxyapatite and then digested with the enzyme. The enzyme was replenished after 8 or 8, 16 and 24 days of incubation. Centre well, antiserum to protein C; wells 1 and 5, native protein A; well 2, 16 days digest; well 3, native protein C; well 4, 32 days digest. Well 6 was empty in all cases.

Table 3. Hydrolysis of Azocoll by a preparation of proteinases from salivary sediment

The enzymic activity (ΔA_{520}) was measured after incubating the enzyme preparation with Azocoll in 0.5 ml of 0.03 M- NH_4HCO_3 at 37°C for 24 h. The A_{520} of the supernatant was measured in a cell with a 1 cm optical path length. The value obtained with an appropriate blank was subtracted to give ΔA_{520} .

Enzyme preparation	Enzymic activity (ΔA_{520})
Proteinase (30 μ g)	0.725
in 1 mM-di-isopropyl phosphorofluoridate	0.010
in 10 mM-N-ethylmorpholine	0.817
in 25 mM-EDTA	1.072
preincubated 24 h at 37°C	1.171
48 h at 37°C	2.596
96 h at 37°C	2.986
192 h at 37°C	1.891

Fig. 12. Digestion of protein C adsorbed to hydroxyapatite by salivary-sediment proteinase: amino acid composition of digest remaining on hydroxyapatite

The results obtained when there was no replenishment of enzyme during the incubation period are illustrated in (a) and those obtained when the enzyme was replenished after 8, or 8, 16 and 24 days in (b). The compositions are expressed as molar ratios (= no. of residues), assuming the presence of one phenylalanine residue. □, Glutamic acid; ○, proline; △, glycine; ●, valine; ▲, histidine.

16 and 24 days, the digest was unable to form an immunoprecipitate (Fig. 11c).

The composition of the salivary-sediment enzyme adsorbed to hydroxyapatite was determined, and since this preparation contains threonine, which is absent in protein C, it was possible to subtract the contribution of the enzyme. Some of the amino acid analyses of adsorbed and digested protein corrected in this manner are given in Fig. 12a. The results are given in molar ratios assuming that there is one residue of phenylalanine present. There was no change in the valine content and the results also showed no change in the amount of leucine and isoleucine, indicating that the *N*-terminal part of the protein had been retained on hydroxyapatite. In contrast there was a marked decrease in the amount of proline, glycine,

glutamic acid and histidine, indicating loss of the *C*-terminal part of the protein. If further additions of enzyme were made during incubation, an additional loss occurred of the amino acids primarily located in the *C*-terminal part (Fig. 12b). The amino acid analysis of the sample of adsorbed protein C exhaustively digested for 32 days suggests that only peptides located in the *N*-terminal proline-poor part of the protein remain on the hydroxyapatite.

Discussion

Bernardi (1971) demonstrated that binding of protein to hydroxyapatite is mediated by negatively charged groups, such as phosphate and carboxylate, or by positive charges on the protein. These charged groups probably interact with positive or negative charges on the hydroxyapatite. The decreased binding of protein A and C after phosphatase digestion is in agreement with findings of Bernardi (1971), although it is not possible to determine whether the phosphoserine residues are necessary to maintain the conformation of the proteins or are directly involved in the binding to mineral. It is also possible that carboxy groups in aspartic and glutamic acids are part of the hydroxyapatite-binding site, since most of these residues are located in the *N*-terminal tryptic peptide. The positively charged residues in the proteins are not essential for the binding of the proteins to hydroxyapatite. Except for arginine in position 30, there are no basic amino acids in the *N*-terminal tryptic peptide, and none of the other tryptic peptides that contain lysine, histidine and arginine bind to the mineral.

If a solution of protein C is digested with trypsin, the peptide bonds at arginine-30 and -106 are both rapidly cleaved (Bennick *et al.*, 1977), and the peptides are unable to form immunoprecipitate with the antibodies. The results demonstrate that, when protein C is adsorbed to hydroxyapatite, there is a protection of the peptide bond at arginine-30, but the bond at arginine-106 is cleaved, giving rise to a fragment that is identical with protein A as determined by gel electrophoresis and double immunodiffusion. It could be argued that the adsorbed protein is not digested while bound on the mineral, but that it will slowly become desorbed and then be digested by the enzyme. This could then be followed by adsorption of the *N*-terminal peptide. If this were correct we would not expect to detect protein A in the digest of adsorbed protein C, because of the equally rapid cleavage of the bonds at arginine-30 and -106 in a solution of protein C. The negligible removal of adsorbed protein C during the incubation period in the absence of trypsin, compared with the rapid fall in the glycine, proline and glutamic acid content of adsorbed protein digested with trypsin, also makes it unlikely that the digestion occurred after desorption of the protein.

This argument is supported by the observation

that, after exhaustive digestion of adsorbed protein C with thermolysin, 25% of the protein remained on the mineral, but only 8% of protein C digested in solution with thermolysin bound to hydroxyapatite. If the adsorbed protein had been desorbed before the exhaustive digestion, we would expect the same amount of protein bound to hydroxyapatite in both experiments. It is apparent that the part of adsorbed protein C that was retained on hydroxyapatite is still susceptible to hydrolysis by thermolysin, since free *N*-termini and three different peptides were found in the adsorbed protein after exhaustive thermolytic digestion. This demonstrates that the part of the protein that contains the binding site does not have to be intact in order to remain on the hydroxyapatite. The similarity of peptides Th2 and Th3 to the peptides adsorbed to hydroxyapatite from a solution of thermolytic digest of protein C suggests that the hydroxyapatite-binding site is located between residues 3 and 25. It is possible that there is more than one binding site, since both *N*-terminal leucine and valine residues were detected in these adsorbed peptides, indicating adsorption of both peptides Th2 and Th3.

The digestion with the salivary-sediment enzyme was undertaken in order to evaluate to what extent the adsorbed proteins may be digested under physiological conditions. It is therefore initially not desirable to fractionate individual proteinases. Because of the low activity of the unfractionated enzymes, a partial purification by gel filtration was undertaken. The activity of this proteinase preparation was only 3% of that of trypsin as measured by the Azocoll assay. This low activity could be compensated for by prolonged incubations of the protein for 32 days, exchanging the enzyme every eighth day, because the enzyme was stable over this period of time. The results from double immunodiffusion demonstrate that, as with digestion by trypsin and thermolysin, there is protection of susceptible bonds when protein C is adsorbed to hydroxyapatite, and that it is the *C*-terminal part that is removed.

It is noteworthy that both the ability of the protein to inhibit hydroxyapatite formation (D. I. Hay, personal communication) and to bind calcium (Bennick & Madapallimattam, 1979) are located in the *N*-terminal tryptic fragment. It is possible that

some of the calcium-binding sites also are part of the hydroxyapatite-binding site, but at least part of the calcium-binding capacity may be retained in the adsorbed protein. It would be interesting to determine whether the adsorbed protein possess their known biological activities after proteolytic removal of the *C*-terminal part.

The newly formed pellicle has a high content of proline (14% of the total number of residues), but it decreases to 6% when the pellicle ages (Mayhall, 1970). The present results suggest that this change may in part be explained by proteolytic removal of the *C*-terminal proline-rich part of proteins A and C.

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