Biochem. J. (1976) **155**, 163–169
Printed in Great Britain

The Binding of Calcium to a Salivary Phosphoprotein, Protein A, Common to Human Parotid and Submandibular Secretions

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(*Received* 1 *September* 1975)

The binding of Ca^{2+} to a previously described phosphoprotein from human parotid saliva, protein A [Bennick (1975) *Biochem J.* **145**, 557–567] was studied by means of equilibrium dialysis. In 5mM-Tris/HCl buffer, pH7.5, protein A bound 664nmol of Ca/mg of protein. K_m was determined to be 181μM and the binding of Ca^{2+} to the protein was non-co-operative. The binding of Ca^{2+} apparently occurs to side-chain carboxyl groups in the protein, but protein phosphate is of minor if any importance in calcium binding. Hydrolysis of protein A by trypsin and collagenase or heating of the protein at 60° or 100°C did not affect Ca^{2+} binding. The Ca^{2+} binding decreases with increased concentration of the dialysis buffer and on the addition of $SrCl_2$, $MgCl_2$ or $MnCl_2$ to the dialysis buffer. Protein A does not aggregate in the presence of Ca^{2+}, since the $s_{20,w}$ was identical when determined in the presence (1.30S) and absence (1.35S) of $CaCl_2$. By use of a specific antiserum to protein A it was found that protein C [Bennick & Connell (1971) *Biochem. J.* **123**, 455–464] and perhaps minor related components cross-reacted with protein A. No other salivary proteins showed immunological similarity. Proteins A and C were also present in submandibular saliva. The possible functions of protein A are discussed.

The total concentration of calcium in human parotid saliva has been found by several investigators to be approx. 1.0mM (Windeler & Shannon, 1966; Dawes, 1969; Ahrens & Lücke, 1972; Ferguson *et al.*, 1973) and the amount of non-ultrafiltrable calcium has been estimated to be 0.33mM (Grön, 1973*a*). The ultrafiltrable calcium in saliva is ionized or forms ion pairs, and saliva is highly saturated with respect to calcium phosphate, which is considered to be important in the formation of dental calculus and remineralization of dental enamel (Grön, 1973*b*). The function of the non-ultrafiltrable calcium, which is presumably protein bound, is not clear.

Salivary amylase binds 1–2 atoms of Ca^{2+}/mol of enzyme (Vallee *et al.*, 1959), and it can be estimated, since salivary amylase has mol.wt. 55000 (Mutzbauer & Schultz, 1965) and the concentration of amylase is about 1mg/ml of parotid saliva (Schneyer, 1956), that the concentration of Ca^{2+} bound to this enzyme is 0.036mM. This leaves a substantial amount of protein-bound Ca^{2+} that is not accounted for. Little is known about other Ca^{2+}-binding proteins in saliva. Rölla & Jonsen (1967) isolated a glycoprotein from submandibular saliva that could bind Ca^{2+}, and Boat *et al.* (1974) have isolated a Ca^{2+}-precipitable protein from submandibular saliva, which may also be present in parotid saliva. A Ca^{2+}-precipitable protein has also been isolated from mixed salivary secretions by Belcourt (1975).

The purification of a phosphoprotein named protein A from human parotid saliva has been described (Bennick, 1975). No biological activities have been found for this protein, which is acidic as judged from its amino acid composition and electrophoretic mobility (Bennick & Connell, 1971). Because of the chemical properties of protein A it was decided to investigate the Ca^{2+}-binding properties of this protein, and by means of immunological techniques to demonstrate if this protein is also present in other salivary secretions.

Experimental

Materials

Distilled water for calcium-binding studies was passed through a deionizing column containing a Barnstead Ultrapure cartridge before use. Ca^{2+}-containing solutions were stored in polyethylene bottles. $^{45}CaCl_2$ and scintillation fluid (Aquasol) were obtained from New England Nuclear Corp., Boston, MA, U.S.A. Dialysis tubing [0.635cm (8/32in) flat width] was purchased from Union Carbide, Lindsay, Ont., Canada. The dialysis tubing was treated with boiling 1mM-EDTA and thoroughly rinsed in water before use. Diphenylcarbamoyl chloride-inhibited trypsin was purchased from Sigma Chemical Co., St. Louis, MO, U.S.A., and chromatographically purified collagenase was obtained from

Worthington, Freehold, NJ, U.S.A. Polyamide layer plates were from the Cheng Chin Trading Co., Taipei, Taiwan, and complete Freund's adjuvant and Agar Noble were from Difco Laboratories, Detroit, MI, U.S.A. Standard solutions of bovine serum albumin were obtained from Armour Pharmaceutical Co., Chicago, IL, U.S.A. All other chemicals were of analytical grade.

Collection of salivary secretions and purification of protein A

Stimulated parotid saliva was collected as described previously (Bennick & Connell, 1971), and stimulated submandibular saliva was collected with an appliance described by Truelove *et al.* (1967). Salivary flow was stimulated with sour lemon drops. Samples of parotid and submandibular saliva for immunological studies were collected from three different persons and used immediately after collection. The method of saliva collection was approved by the committee on human experimentation at the University of Toronto and was performed with the consent of the donors. Protein A was purified as described previously (Bennick, 1975).

Measurement of calcium binding

Ca^{2+} binding by protein A was studied with ^{45}Ca by means of equilibrium dialysis. In standard experiments 0.4mg of protein A was dissolved in 200μl of 5mM-Tris/HCl buffer, pH7.5, placed in a dialysis bag and dialysed against 100ml of 5mM-Tris/HCl buffer, pH7.5, containing 1mM-$CaCl_2$ and sufficient ^{45}Ca to give a specific radioactivity of about 80c.p.m./nmol of Ca. The samples were dialysed while shaking on a mechanical shaker at room temperature (24°C) for 16h. This time-period was shown in initial experiments to be sufficient for equilibrium to be reached between the concentrations of Ca^{2+} inside and outside the dialysis bag.

At the end of dialysis 150μl samples were taken from the contents of the dialysis bags and from the buffer in which the bag had been suspended. The samples were transferred to vials, to which 15ml of scintillation fluid was added, and counted for radioactivity on a Nuclear–Chicago liquid-scintillation counter. No quench correction was necessary.

The concentration of protein inside the bag was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard. Since the concentration of protein A had been determined previously by this method, as well as by amino acid analysis, it was possible to calculate the concentration of the dialysed protein by amino acid analysis. Protein concentrations determined in this manner were used in subsequent calculations of calcium binding to protein A.

The extent of Ca^{2+} binding to protein A was investigated in experiments in which the Ca^{2+} concentration was kept constant while the concentration of protein was varied from 0.08 to 5mg/ml, or the protein concentration was kept constant and the Ca^{2+} concentration varied from 0.001 to 10mM.

The effect of other metals on the binding of Ca^{2+} to protein A was investigated by adding $SrCl_2$, $MgCl_2$ or $MnCl_2$ at a concentration of 1mM to dialysis buffers containing 1mM-$CaCl_2$, and the influence of the ionic strength of the buffer was investigated by dialysing protein A in imidazole/HCl buffers, pH7.4, with concentrations varying from 5 to 200mM. Equilibrium dialysis was also performed with 5mM-Tris/HCl buffers with pH values varying from 7.0 to 9.0, with 5mM-imidazole/HCl buffer, pH6.0 or 6.5, and 5mM-sodium acetate buffers with pH varying from 3.5 to 5.8. In these experiments the concentration of calcium was kept constant at 1mM.

The effect of proteolytic digestion of protein A on its Ca^{2+}-binding ability was tested by adding trypsin or collagenase to protein A at an enzyme/protein ratio of 1:100 (w/w) before equilibrium dialysis under standard conditions.

Beside the usual determination of Ca^{2+} binding at the end of the dialysis, samples of the digests were electrophoresed on 6cm-long 4.5% (w/v) acrylamide gels in 0.1M-Tris/glycine buffer, pH9.2, separating components migrating towards the anode as described previously (Bennick, 1975).

The presence of non-collagenolytic proteinase activity in the collagenase preparation was tested by incubating collagenase with 1% casein at an enzyme/protein ratio of 1:100. The digestion proceeded in the same buffer as used in the equilibrium-dialysis experiment for 16h. The extent of hydrolysis of the casein was measured as described by Kunitz (1947) and compared with the extent of digestion by various dilutions of trypsin under the same conditions.

A sample of the digest was dansylated, hydrolysed for 4h and subjected to t.l.c. on polyamide plates by the method of Percy & Buchwald (1972). Standards of dansyl–amino acids were run with the hydrolysate.

Experiments were also performed in which protein A was heated to 60° or 100°C in a water bath for 10min before equilibrium dialysis under standard conditions.

Determination of sedimentation and diffusion coefficients

This was done in a Beckman model E analytical ultracentrifuge equipped with schlieren optics. A synthetic boundary cell was used, the speed of the rotor was 60000rev./min and the temperature was maintained at 20°C. The protein sample was dissolved in 5mM-Tris/HCl buffer, pH7.5, at a concentration of 5mg/ml. To a part of the sample was added sufficient

100mM-$CaCl_2$ to give a concentration of 1mM-$CaCl_2$. Both samples were subjected to ultracentrifugation. The viscosity and density of both buffers were identical with the corresponding values for water at 20°C. The data were analysed by the least-squares method to obtain the $s_{20,w}$.

The photographs of the schlieren peaks used for determination of the sedimentation coefficients were also used to determine the diffusion coefficients. This was done from measurements of the areas under the schlieren peaks and the maximum heights of the schlieren peaks (Chervenka, 1973).

Preparation of antiserum to protein A, immunodiffusion and immunoelectrophoresis

An adult goat was injected intramuscularly with an aqueous solution containing 4mg of protein A mixed with 2vol. of complete Freund's adjuvant. The injection was repeated 3 and 5 weeks after the initial injection. The animal was bled 1 week after the last injection, and the serum was quick-frozen and stored at −10°C.

Double radial immunodiffusion was performed on micro-Ouchterlony plates in 1% agar containing diethylbarbituric acid/sodium diethylbarbiturate buffer, pH8.6, $I = 0.02$mol/l. The distance between the wells was 4mm.

Polyacrylamide-gel electrophoresis was performed on 9cm-long 7% (w/v) polyacrylamide gels by using a 0.1M-Tris/glycine buffer, pH9.2, in the gel as well as in the electrode vessels (Bennick, 1975). A sample (100μl) of saliva containing 40% (w/v) sucrose and Bromophenol Blue was layered on top of the gel and a 2mm-high acrylamide gel was polymerized on top of the sample.

Electrophoresis of duplicate gels was conducted with the anode at the lower gel end at a potential of 150V until the tracking dye reached the lower end of the gel. This experiment was repeated, by applying the electrical potential for the same length of time as before, but with the cathode at the lower end of the gel.

After electrophoresis the sample was withdrawn from beneath the small gel and subjected to double immunodiffusion against antiserum to protein A. One acrylamide gel was stained with Coomassie Brilliant Blue as previously described (Bennick, 1975) and the other gel was imbedded in an agar gel. A trough was cut in the agar parallel to the acrylamide gel and double immunodiffusion performed with antiserum to protein A.

Results

Calcium binding to protein A

The results obtained in equilibrium-dialysis experiments in which the concentration of calcium was maintained at 1mM while the protein concentration was varied are illustrated in Fig. 1.

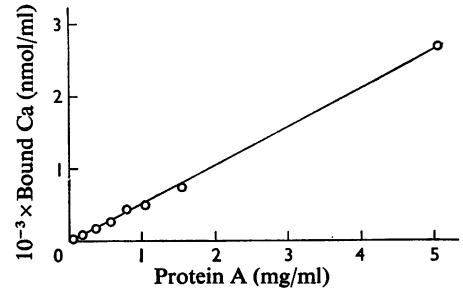


Fig. 1. *Amount of protein-bound Ca^{2+} as a function of the concentration of protein A*

The binding of Ca^{2+} was evaluated by means of equilibrium dialysis as described in the text. The concentration of free Ca^{2+} was 1mM and the buffer was 5mM-Tris/HCl, pH7.5. The concentration of bound Ca^{2+} is plotted against the concentration of protein A in the dialysis bags.

Corresponding values of concentrations of protein A and bound Ca^{2+} were plotted and a linear relationship was found within the range of protein concentrations tested.

The results from several experiments in which the amount of protein-bound Ca^{2+} was measured as a function of the concentration of free calcium are plotted in Fig. 2.

The parameters of calcium binding were evaluated by weighted non-linear regression analysis of the hyperbolic binding curve. From this analysis the maximum amount of Ca bound to the protein was 664±35nmol of Ca/mg of protein, which corresponds to 6.6g-atoms of Ca/mol of protein, assuming mol.wt. of 9900 (Bennick, 1975). The apparent dissociation constant, K_m, was 181±25μM. The Hill coefficient had an estimated value of 0.94±0.06, not significantly different from 1.00.

The effect of pH on calcium binding to protein A is illustrated in Fig. 3. No calcium binding was observed at pH3.5. Above this pH value the calcium binding increased in a sigmoid fashion, with a midpoint at pH4.9 until 6.0 was reached. Between pH6.0 and 7.0 about 460nmol of Ca was bound per mg of protein under the experimental conditions. From pH7.0 to 9.0 there was an increase in calcium binding. At pH9.0 approx. 600nmol of Ca was bound per mg of protein. The presence of bivalent metals caused decreased calcium binding to protein A. In the presence of 1mM-$SrCl_2$, protein A bound 338nmol of Ca/mg of protein and the corresponding values for 1mM-$MgCl_2$ and 1mM-$MnCl_2$ were 312 and 230 respectively. In the absence of bivalent metals protein A bound 612nmol of Ca/mg of protein under the experimental conditions.

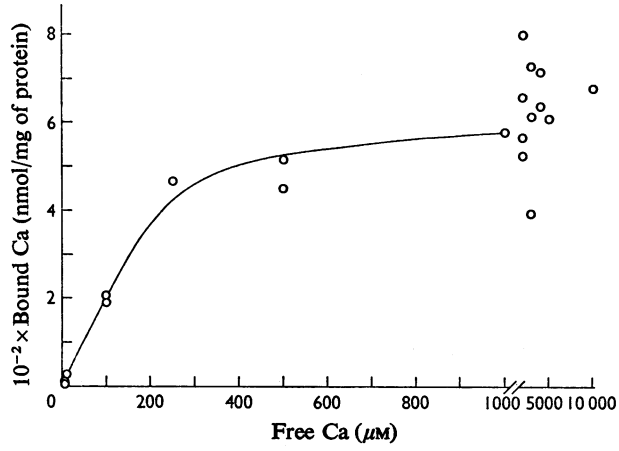


Fig. 2. *Amount of protein-bound Ca^{2+} as a function of the concentration of free calcium*

The binding of Ca^{2+} was evaluated by means of equilibrium dialysis as described in the text. The amount of bound Ca^{2+}, measured as nmol of Ca/mg of protein, is plotted against the concentration of free Ca^{2+} measured in μM. All of the experimental values have been plotted except for the data obtained with 1000 μM free Ca. In this instance the mean value of nine determinations is given [579±86 (S.D.) nmol of Ca^{2+}/mg of protein].

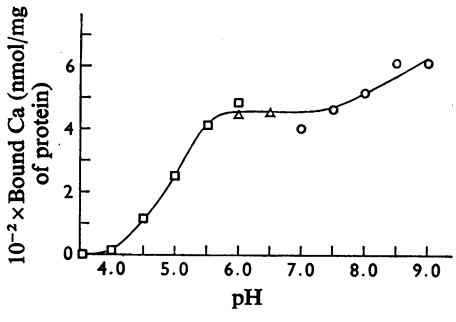


Fig. 3. *Amount of protein-bound Ca^{2+} as a function of pH of the dialysis buffer*

The amount of protein-bound Ca^{2+}, measured in nmol of Ca/mg of protein, is plotted against the pH of the dialysis buffers. The buffers used in the various experiments are indicated as follows: □, 5mM-sodium acetate buffer; △, 5mM-imidazole/HCl buffer; ○, 5mM-Tris/HCl buffer. The concentration of free Ca^{2+} in the buffers was 1mM.

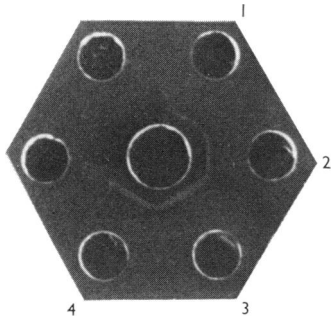
Digestion of protein A with trypsin or collagenase had little if any effect on the calcium binding of protein A. Protein A digested with trypsin bound 549nmol of Ca/mg of protein, and protein A digested with collagenase bound 619nmol of Ca/mg of protein. Trypsin and collagenase when incubated without protein A did not bind calcium and undigested protein A bound 625nmol of Ca/mg of protein. Polyacrylamide-gel electrophoresis of the enzymic digests of protein A demonstrated that all of protein A had been digested. No band with the same mobility as undigested protein A could be detected on the gels containing the proteolytic digests. On these gels bands with lower mobility than protein A could be seen. These bands faded quickly after staining. The extent of hydrolysis of casein by collagenase was the same as would have been obtained if the collagenase preparation had contained 0.3% trypsin. T.l.c. of the dansylated digest of protein A demonstrated that glycine was the *N*-terminal amino acid of the peptides in the digest, although traces of glutamic acid or aspartic acid and proline also were identified. After heating of protein A at 60° or 100°C the protein bound 652 and 682nmol of Ca/mg of protein, whereas untreated protein bound 651nmol of Ca/mg of protein. There were no apparent changes in the protein solutions after heating.

When the concentration of the dialysis buffer that was used in equilibrium dialysis was increased, the amount of Ca^{2+} bound to protein A decreased from 497nmol of Ca/mg of protein in 5mM-imidazole/HCl buffer, pH7.4, to 101nmol of Ca/mg of protein in 200mM-imidazole/HCl buffer, pH7.4 (Fig. 4).

Sedimentation and diffusion coefficients

The sedimentation coefficient $s_{20,w}$ in the absence of $CaCl_2$ was 1.35±0.06S (S.D.) and in the presence of 1mM-$CaCl_2$ it was 1.30±0.08S (S.D.). No difference in sedimentation coefficient can therefore be detected in the presence of Ca^{2+}.

The diffusion coefficient (D) in the absence of $CaCl_2$ was 9.2×10^{-7}cm^2/s and in the presence of 1mM-

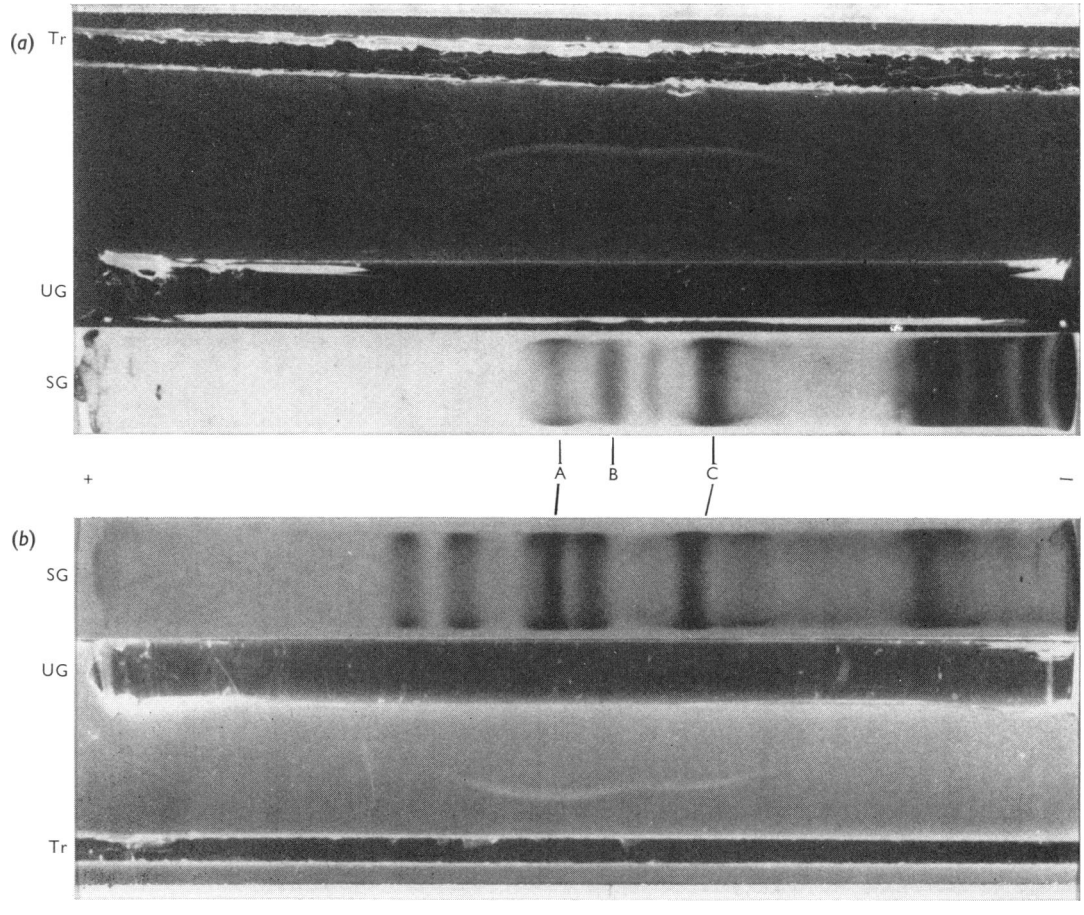


EXPLANATION OF PLATE 1

Double immunodiffusion of salivary proteins against antiserum to protein A

A 1% agar gel containing diethylbarbituric acid/sodium diethylbarbiturate buffer, pH 8.6, $I = 0.02$ mol/l, was used. The contents of the wells are as follows: centre well, antiserum to protein A; well 1, parotid saliva; well 2, submandibular saliva; well 3, protein A; well 4, protein C.

(*facing p.* 166)



EXPLANATION OF PLATE 2

Polyacrylamide-gel electrophoresis of salivary secretions followed by double immunodiffusion

Electrophoresis of parotid or submandibular saliva was performed as indicated in the text. Plate 2(*a*) illustrates the experiment with parotid saliva, and Plate 2(*b*) the experiment with submandibular saliva. In both instances electrophoresis was performed with the anode at the lower end of the gel. Electrophoresis was terminated when the Bromophenol Blue tracking dye had reached the lower end of the gel. A photograph of the gel (SG) which had been stained with Coomassie Blue has been aligned with a photograph of the polyacrylamide gel (UG) which was embedded in an agar gel before immunodiffusion against antiserum to protein A. A, B and C indicate the location of proteins A, B and C respectively in the stained gels (SG). UG is the unstained gel used in double immunodiffusion. Tr is the trough in the agar gels containing antiserum to protein A. Note the location of the double-arched immunoprecipitation line corresponding to the location of protein A and C in the stained gels.

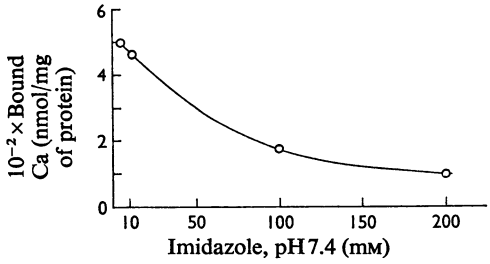


Fig. 4. *Amount of protein-bound Ca^{2+} as a function of the concentration of the dialysis buffer*

The amount of protein-bound calcium, measured in nmol of Ca/mg of protein, plotted as a function of the concentration of the imidazole/HCl dialysis buffer, pH7.4. The concentration of free Ca^{2+} was 1mM.

$CaCl_2$ it was $9.0\times10^{-7}cm^2/s$. No apparent difference can therefore be detected in the diffusion coefficient in the absence or presence of Ca^{2+}.

Reactivity with antiserum to protein A

In double-immunodiffusion experiments the antiserum to protein A gave a single precipitation line with protein A, which was continuous with precipitation lines with unfractionated parotid and submandibular saliva (Plate 1). Double immunodiffusion also demonstrated that a previously described protein C, purified by a procedure modified from that of Bennick & Connell (1971), reacted immunologically in a manner identical with protein A with antiserum to protein A.

To investigate further the reactivity of the antiserum to protein A with salivary proteins, polyacrylamide-gel electrophoresis followed by immunodiffusion was performed with unfractionated parotid and submandibular saliva. Two fused arcs of immunoprecipitate can be seen in experiments in which components migrating towards the anode were separated (Plate 2). The arc that is closest to the anode corresponds to protein A. The other arc of immunoprecipitate was due to precipitation of protein C with the antiserum to protein A. Protein B, which has been isolated from parotid saliva (Bennick & Connell, 1971), appears on the gel as a heavily stained band between proteins A and C. In separate tests protein B did not react with the antiserum to protein A.

In gel electrophoresis in which components migrating towards the cathode were separated, subsequent immunodiffusion gave no precipitation reactions with gels containing parotid or submandibular saliva. There was no reaction with antiserum to protein A in double immunodiffusion of the components in the salivary samples that stayed at the origin. The results were identical for all three persons tested.

Discussion

Compared with a number of other animal Ca^{2+}-binding proteins, which have dissociation constants of the order of 1×10^{-5}–1×10^{-7}M (Wasserman *et al.*, 1968; MacLennan & Wang, 1971; Benzonana *et al.*, 1972; Hitchman & Harrison, 1972; Wolff & Siegel, 1972; Oldham *et al.*, 1974; Hermsdorf & Bronner, 1975), the dissociation constant for protein A (1.8×10^{-4}M) is comparatively high, although Ca^{2+}-binding proteins with dissociation constants of 1×10^{-4}–1×10^{-3}M have been described (Callissano *et al.*, 1969; Ostwald & MacLennan, 1974). The relatively weak binding of Ca^{2+} to protein A must be seen in relation to the concentration of Ca^{2+} in saliva. The concentration of calcium not bound to macromolecules in stimulated parotid saliva has been estimated to be 0.64mM (Grön, 1973*a*), and the pH of stimulated parotid saliva is 7.5 (Grön, 1973*a*). At such a calcium concentration and at such a pH, protein A would be almost saturated with Ca^{2+} under the experimental conditions used in the present study. The Ca^{2+} binding in imidazole buffer, pH7.5, decreased with increased concentration of the buffer. The ionic strength of a 0.2M-imidazole buffer, pH7.4, the most concentrated buffer that was used, is close to that of stimulated parotid saliva, as calculated from the data of Dawes (1969). It is therefore likely that less Ca^{2+} binds to protein A under the physiological conditions in the oral cavity than can be observed in 5mM-Tris/HCl buffer, pH7.5. Further experimentation is needed to establish the extent of calcium binding to protein A in parotid saliva *in vivo*.

The value of the Hill coefficient indicates absence of positive co-operativity in calcium binding to protein A. There is also no indication of negative co-operativity or that the binding sites are heterogeneous, since the value of the Hill coefficient is not significantly different from 1.00.

If the binding is non-co-operative, digestion with proteolytic enzymes would not necessarily affect the Ca^{2+} binding of the enzymic fragments, since each calcium-binding site may be contained within short stretches of the polypeptide chain.

The demonstration that non-collagenolytic enzyme is only a very minor contaminant of the collagenase preparation indicates that at least part of the hydrolysis of protein A by collagenase is due to collagenolytic activity. This is confirmed by the observation that glycine is the *N*-terminal amino acid in the peptides of the digest. Clostridial collagenase is known to break the bond indicated by an arrow in sequences of the type –X–Gly–R–, where X can be several amino acids and R most often is proline (Nordwig, 1970). Oppenheim *et al.* (1971) observed that a protein preparation (protein IV), which is very similar to or identical with protein A, was susceptible to hydrolysis by bacterial collagenase. Both of these studies indi-

cate the presence of collagen-like sequences in the salivary proteins, but hydrolysis of susceptible bonds in these sequences does not affect Ca^{2+} binding.

The lack of an effect on calcium binding of protein A owing to heating before dialysis may indicate that the tertiary structure of the protein is not important for Ca^{2+} binding. This agrees with the observation that there is no positive co-operativity in the binding of calcium.

The decreased binding of calcium to protein A in the presence of equimolar concentrations of Sr^{2+}, Mg^{2+} and Mn^{2+} suggests that these metals bind to the same sites as Ca^{2+}. Because of the large difference in ionic radii of the metal ions the Ca^{2+}-binding sites seem to be relatively non-restricted with regard to the size of the ions that can be accommodated. Since the concentrations of Sr^{2+}, Mg^{2+} or Mn^{2+} in saliva are very small compared with the Ca^{2+} concentration (Arwill *et al.*, 1967; Dawes & Ong, 1973), they would have no noticeable effect on the binding of Ca^{2+} to protein A in saliva.

The dependence of Ca^{2+} binding on pH indicates that most of the binding involves dissociable groups in the protein with approximate p*K*4.9. These would most likely be side-chain carboxyl groups on aspartic acid and glutamic acid, which have been found in a number of proteins to have intrinsic p*K* values varying from 4.06 to 4.8 (Tanford, 1962). Data obtained from *O*-phosphorylated peptides suggested intrinsic p*K* values for $-PO_3H_2$ in the pH range 0.5–1.0 and for $-PO_3H^-$ between pH 5.5 and 6.2 (Fölsch & Österberg, 1959). From the present data it is not possible to determine if phosphate is involved in calcium binding to the protein. The pH-dependence of the calcium binding and the fact that there are only two serine phosphate residues in the protein indicates that most if not all of the calcium binding occurs to side-chain carboxyl groups.

At present it is not known how large a fraction of the protein-bound Ca in the salivary secretions is in the form of Ca–protein A complexes. In a previous study 10mg of protein A as determined from amino acid analysis (Bennick, 1975) was recovered from 100ml of parotid saliva. The minimum amount of Ca bound to protein A as determined in 5mM-Tris/HCl buffer, pH7.5, would therefore be 66μmol/litre. This would correspond to about 13μmol/litre in 0.2M-imidazole/HCl buffer, pH7.4, which has an ionic strength similar to that of stimulated parotid saliva (Dawes, 1969). Since the concentration of protein A in parotid saliva is not known, it is difficult to evaluate how much of the 330μM non-ultrafiltrable calcium in stimulated parotid saliva is complexed to protein A.

The magnitude of the sedimentation coefficients obtained in the presence and the absence of Ca^{2+} indicates that under the experimental conditions there is no aggregation of protein A in the presence of Ca^{2+}. Moreover the similarity of the diffusion coefficients in the absence and the presence of Ca^{2+} indicates that there is no conformational changes in the protein in the presence of Ca^{2+}.

The immunological experiments clearly demonstrate that protein A is present in submandibular as well as parotid secretions. The cross-reactivity with protein C is not surprising in view of the strikingly similar composition of proteins A and C (Bennick & Connell, 1971). Proteins A and C are probably identical with two of the proline-rich proteins from parotid saliva described by Oppenheim *et al.* (1971). These proteins are part of a genetically related group of proteins (Azen & Oppenheim, 1973), and on the basis of the presence in submandibular saliva of components that have similar unusual staining properties as these proline-rich parotid proteins, it has been suggested that the proline-rich proteins were present in submandibular saliva.

The immunological results also indicate that beside proteins A and C and perhaps some minor components of similar composition and gel-electrophoretic mobility (Hay & Oppenheim, 1974), no other proteins in parotid and submandibular secretions react with the antiserum. A number of basic glycoproteins with amino acid composition strikingly similar to that of proteins A and C, but with isoelectric points pI$\geqslant$9 have been isolated (Arneberg, 1975). These proteins apparently do not react with the antiserum, since their electrophoretic mobility would be noticeably different from that of proteins A and C.

A carbohydrate-containing protein fraction with an amino acid composition similar to that of protein A has been isolated from rat saliva (Keller *et al.*, 1975). This protein is very similar in amino acid composition to proteins associated with the secretory granule membrane isolated from rat parotid glands (Robinovitch *et al.*, 1975) and it is possible that protein A is associated with the secretory granule membrane in the human salivary glands. In this connexion the calcium-binding capacity of protein A is interesting because of the concomitant release of calcium and protein content of the rat salivary secretory granule (Wallach & Schramm, 1971). These authors have suggested that calcium in the rat salivary glands serves to aggregate the exportable proteins.

Because of the influence of the ionic strength of the buffer on the calcium binding to protein A, it is not possible from the results in this study to evaluate the physiological importance of calcium binding to protein A in saliva. Salivary proteins that are very similar to or identical with protein A have been shown to adsorb selectively to hydroxyapatite, the main mineral of dental enamel (Hay, 1973). It is also noteworthy that McGaughey & Stowell (1971) have reported the simultaneous binding of protein and calcium to hydroxyapatite. These problems are under investigation at present.

Another salivary calcium-binding protein has been described by Boat *et al.* (1974), and Henkin *et al.* (1975) have demonstrated a zinc-binding protein in saliva. The compositions of these two proteins are quite different from protein A and they are clearly not identical with protein A.

I thank Mrs. M. Cannon for invaluable technical assistance. A part of this work was performed by Mr. T. K. Leung employed as a Canadian Medical Research Council summer student. I also thank Dr. D. Kells for performing the ultracentrifugations and Dr. L. Endrenyi for the regression analysis of the calcium-binding data. This work was supported by Grant no. MA 4920 from the Canadian Medical Research Council.

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