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Interaction of calcium ions and salivary acidic proline-rich proteins with hydroxyapatite

A possible aspect of inhibition of hydroxyapatite formation

Anders BENNICK, David KELLS and George MADAPALLIMATTAM
Department of Biochemistry, University of Toronto, Toronto, Ont. M5S 1A8, Canada

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The relationship between Ca^{2+}- and hydroxyapatite-binding sites in salivary acidic proline-rich phosphoproteins A and C was investigated. Coating of hydroxyapatite with protein before adsorption had no effect on Ca^{2+} binding to the mineral, but simultaneous adsorption of Ca^{2+} and protein to hydroxyapatite caused additional Ca^{2+} binding to the solid. The additional amount of Ca^{2+} adsorbed, measured in mol of Ca^{2+}/mol of protein adsorbed to hydroxyapatite, was approx. 2 for protein C, 4 for protein A, 9 for the *N*-terminal tryptic peptide and 2 for dephosphorylated protein A. It is suggested that the ability of the proteins to inhibit hydroxyapatite formation is related to the binding of the proteins to crystal growth sites on the mineral, which prevents access of Ca^{2+} from the surrounding liquid.

Human parotid-gland and submandibular-gland saliva contains a group of related acidic proline-rich proteins that constitute 28% of the total salivary protein (Bennick & Cannon, 1978). Two of the major components have been named salivary proteins A and C (Bennick & Connell, 1971) and their primary structures have been determined (Wong *et al.*, 1979; Wong & Bennick, 1980). Salivary protein A consists of a single peptide chain of 106 residues and salivary protein C contains the entire structure of protein A in its *N*-terminal part, but continues beyond residue 106 to the *C*-terminal residue, no. 150. The proteins have a highly negatively charged *N*-terminal end which can be isolated as a tryptic peptide (TX peptide). This peptide contains a total of eleven dicarboxylic amino acid residues and two phosphoserine residues. The only negatively charged amino acids found in the remainder of both proteins are two aspartic acid residues. These acidic proline-rich proteins bind Ca^{2+} (Bennick *et al.*, 1981) and they adhere readily to hydroxyapatite (Bennick *et al.*, 1979). Moreover, they can inhibit the formation of this mineral (Hay & Moreno, 1979*a*). Human saliva is usually supersaturated with Ca^{2+} and phosphate (Grön, 1973*a*), and it would therefore be expected that, under normal physiological conditions, mineral might be added to the surface of the dental enamel of the teeth, which mainly consists of hydroxyapatite. There is no indication that this occurs to any measurable extent, and it seems likely that the inhibitory ability of the acidic proline-rich proteins is at least partly responsible. The sites for Ca^{2+} and hydroxyapatite binding as well as the inhibitory action on mineral formation are all located in the TX peptide (Bennick *et al.*, 1979, 1981; Hay & Moreno, 1979*b*). We therefore decided to determine whether binding of Ca^{2+} and hydroxyapatite to the proteins occurs at the same or at different sites, and to evaluate what the relationship of these sites is to the inhibitory effect of the proteins on mineral formation. Such studies may also lead to a better understanding of the mechanism of inhibition of mineral formation by the proteins.

Abbreviation used: TX peptide, *N*-terminal tryptic peptide.

Experimental

Materials

Insoluble bovine intestinal alkaline phosphatase attached to beaded agarose, and *p*-nitrophenyl phosphate, were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Alkaline phosphatase from *Escherichia coli* was purchased from Worthington Biochemical Corp., Freehold, NJ, U.S.A. Dialysis membrane (M_r cut-off 2000) was supplied by Spectrum Medical Industries, Los Angeles, CA, U.S.A., and filters to remove hydroxyapatite par-

ticles (pore size 0.45 μm) were purchased from Millipore, Mississauga, Ont., Canada. Hydroxyapatite (Bio-Gel HTP) was supplied by Bio-Rad, Richmond, CA, U.S.A. Another sample of hydroxyapatite with a specific surface area of 68 m^2/g and a Ca/P ratio of 1.63 was a gift from Dr. D. I. Hay, Forsyth Dental Centre, Boston, MA, U.S.A. All other chemicals were of reagent grade.

Methods

Purification of proteins. Proteins A and C from human salivary secretions were purified as described previously (Bennick, 1975, 1977).

Digestion of protein A with alkaline phosphatase. To 0.6 ml of a suspension of alkaline phosphatase coupled to beaded agarose was added 8 ml of 0.2 M-$(NH_4)_2CO_3$, pH 8.9. The suspension was centrifuged at 2300 *g* for 5 min and the supernatant removed. A sample of 30 mg of protein A dissolved in 3 ml of 0.2 M-$(NH_4)_2CO_3$ was added to the insoluble enzyme. The opening of the tube was closed tightly with a dialysis membrane, and the tube was then held inverted in a beaker containing 800 ml of 0.2 M-$(NH_4)_2CO_3$. The digestion mixture was dialysed in this manner with stirring at 37°C for 24 h, centrifuged at 230 *g* for 5 min, and the supernatant removed and passed through a Millipore filter. The amount of protein recovered was measured by reading the A_{220}. The digested protein was assayed for organic phosphate by the method of Bartlett (1959), and residual alkaline phosphatase activity was determined as described by Garen & Levinthal (1960).

Isolation of TX peptide. Protein A was digested with trypsin for 5 min and the *N*-terminal 30-residue peptide was isolated as described by Bennick *et al.* (1981).

Determination of protein concentration. $A^{0.1\%}_{205,1mm}$ and $A^{0.1\%}_{220,1mm}$ were determined for protein A, protein C and peptide TX from solutions of known concentration (as determined by amino acid analysis). Dephosphorylated protein A was assumed to have the same absorption coefficient as protein A. The molecular weight of protein A has been found to be 11145 (Wong *et al.*, 1979). The molecular weights of protein C (15627) and peptide TX (3540) were calculated from the amino acid-sequence data of Wong & Bennick (1980).

Adsorption of Ca^{2+} to hydroxyapatite to which protein is already bound. A suspension was made containing 20 mg of hydroxyapatite/ml of 10 mM-Tris/HCl buffer, pH 7.5. The suspension was equilibrated overnight at 3°C, heated to 22°C, and maintained in uniform suspension by magnetic stirring. Equal volumes (2.7 ml) of hydroxyapatite suspension and aqueous solution of protein A, protein C or dephosphorylated protein A were mixed and incubated at 22°C for 30 min. For each experiment the concentration of protein was maintained constant, but it varied from 0.25 mg/ml to 2.18 mg/ml in different experiments. As a control, the protein solution was replaced with water. At the end of incubation the suspension was centrifuged at 2300 *g* for 5 min, the supernatant protein solution was removed, and 2.7 ml of 5 mM-Tris/HCl, pH 7.5, was added to the hydroxyapatite pellet. The mineral was resuspended, centrifuged twice and the washings and supernatant were combined and filtered through a Millipore filter. The amount of protein adsorbed to hydroxyapatite was determined as the difference between the amount of protein added to the hydroxyapatite suspension and the amount of protein in the filtrate. A 2.7 ml portion of 5 mM-Tris/HCl, pH 7.5, was added to the protein-coated hydroxyapatite, which was resuspended by magnetic stirring. Portions (250 μl) of suspension were withdrawn and transferred to 1.5 ml conical plastic tubes. To each of these tubes was added 250 μl of 5 mM-Tris/HCl, pH 7.5, containing between 0 and 10 mM-$CaCl_2$. The suspensions were incubated at 22°C for 30 min, after which they were centrifuged at 15600 *g* for 8 min. The supernatant was removed and filtered through a Millipore filter. The protein concentration of the supernatant was determined and the Ca^{2+} concentration was measured by atomic-absorption spectroscopy.

Simultaneous adsorption of protein and Ca^{2+} to hydroxyapatite. Portions (125 μl) of water containing $CaCl_2$ in concentrations from 0 to 10 mM were added to 1.5 ml conical plastic tubes together with 125 μl of an aqueous solution of protein A, protein C or dephosphorylated protein A. The concentration of protein was maintained constant in each experiment, but it varied from 0.37 mg/ml to 3.43 mg/ml in different experiments. To this mixture of $CaCl_2$ and protein was added 250 μl of hydroxyapatite suspension (20 mg/ml) in 10 mM-Tris/HCl, pH 7.5. This suspension was prepared as described above and kept in uniform suspension on a magnetic stirrer while the samples were withdrawn. The mixtures of $CaCl_2$, protein and hydroxyapatite were incubated at 22°C for 30 min, after which they were centrifuged and the supernatants filtered as described above. The protein concentration of the supernatants was determined and the Ca^{2+} concentration was evaluated by atomic-absorption spectroscopy. The amount of adsorbed protein was determined as the difference between the amount of protein added to the solution and that which was left in the supernatant at the end of the experiment. In comparable experiments the S.D. was approx. 3% of the mean amount of adsorbed protein.

Evaluation of experimental factors that affect adsorption of Ca^{2+} and protein to hydroxyapatite. The effect of length of time of incubation on the

adsorption of Ca^{2+} to hydroxyapatite was evaluated in the following manner. Portions (125 μl) of 12 mM $CaCl_2$ were mixed with equal volumes of water or of an aqueous solution of 12.7 mg of protein C/ml. To the samples was added 250 μl of a suspension of 20 mg of hydroxyapatite/ml of 10 mM-Tris/HCl, pH 7.5. The mixtures were incubated at 22°C for 10, 20, 30 or 45 min. The supernatant was removed and the amount of protein and Ca^{2+} determined as described.

The same method was used to evaluate the effect of the amount of hydroxyapatite on the adsorption of Ca^{2+} and protein. The only differences were that the protein solution added to the incubation mixture contained 13.4 mg of protein C/ml, the amounts of hydroxyapatite in the incubation mixture were 2, 4, 6, 8, 10 or 15 mg/ml and the length of incubation was 30 min.

To evaluate the effect of repeated washing of hydroxyapatite on its ability to adsorb Ca^{2+}, a 3 ml sample of the hydroxyapatite suspension was centrifuged at 12300 *g* for 5 min. The supernatant was replaced with 3 ml of 10 mM-Tris/HCl, pH 7.5. This washing was repeated twice with another suspension of the mineral.

Experiments were now performed with each of these hydroxyapatite suspensions as well as with an unwashed suspension of hydroxyapatite. In each set of experiments, samples (250 μl) of hydroxyapatite suspension were mixed with 250 μl of $CaCl_2$ solutions of various concentrations (0–10.5 mM) and incubated for 30 min. This was followed by removal of the supernatant and determination of its Ca^{2+} concentration as described above.

Since it was found that repeated washing of the hydroxyapatite *did* affect the subsequent adsorption of Ca^{2+} to the mineral, the experiment was repeated, replacing the 10 mM-Tris/HCl buffer in which the hydroxyapatite was suspended with 9.2 mM-cacodylate, pH 7.0. These two buffers have the same ionic strength.

A further evaluation of the effect of the buffer on the adsorption of Ca^{2+} and protein to hydroxyapatite was made. This was done by repeating the experiments on the adsorption of calcium to hydroxyapatite to which protein A was already bound and the simultaneous adsorption of calcium and protein A to hydroxyapatite with 9.2 mM-cacodylate, pH 7.0, instead of 10 mM-Tris/HCl, pH 7.5.

To investigate the nature of interaction of the acidic proline-rich proteins with Ca^{2+} and hydroxyapatite, the last two experiments were repeated with peptide TX rather than protein A.

To determine the amount of hydroxyapatite in the incubation mixtures, the hydroxyapatite that had been sedimented by centrifugation at the end of the incubation period and subsequently washed was dissolved in 2 M-HCl, and the amount of phosphate determined by the method of Bartlett (1959). These values were then compared with the amount of phosphate in accurately weighed samples of hydroxyapatite.

In all the adsorption experiments performed in 5 mM-Tris/HCl, pH 7.5, hydroxyapatite purchased from Bio-Rad was used. In the adsorption experiments with 4.6 mM-cacodylate, pH 7.0, the hydroxyapatite obtained from Dr. D. I. Hay was used.

Treatment of data. When hydroxyapatite is suspended and equilibrated in a buffer that does not contain Ca^{2+} and phosphate, some of the mineral dissolves so that there is equilibrium between the Ca^{2+} and phosphate in solution and the mineral. Consequently it was found that hydroxyapatite suspensions to which no soluble $CaCl_2$ had been added contained a small concentration of calcium (C_0) (approx. 40 μM). In each experiment C_0 was determined at the end of the incubation as the Ca^{2+} concentration in the supernatant of the hydroxyapatite suspension to which no $CaCl_2$ had been added. In order to evaluate the total concentration of dissolved Ca^{2+} (C_t) present at the start of the incubation period it was therefore necessary to add C_0 to the amount of $CaCl_2$ which had been added to the hydroxyapatite suspension. In any incubation mixture the amount of Ca^{2+} bound to hydroxyapatite ($\bar{v}$) may be evaluated from the difference between C_t and the amount of Ca^{2+} in the supernatant measured at the end of the incubation period (C_s). These evaluations of bound Ca^{2+} have the implicit assumption that at a free-Ca^{2+} concentration of C_0 there is no Ca^{2+} bound to the mineral. To evaluate the data further it was assumed that the binding of Ca^{2+} to hydroxyapatite when no protein is added to the suspension occurs at one type of independent binding site. Corresponding data for $\bar{v}$ and concentration of soluble Ca^{2+} at equilibrium were fitted to a hyperbolic binding curve by non-linear regression analysis on a computer by using an iterative procedure. In this analysis no assumptions were made about the location of the intercept of the binding curve with the axes. The intercept of the curve with the $[Ca^{2+}]$ axis represents C_0.

If both soluble protein and Ca^{2+} are present in the hydroxyapatite suspension, it is necessary to calculate the amount of Ca^{2+} bound to the dissolved protein in order to find C_f, the concentration of free Ca^{2+} in the solution. The number of Ca^{2+}-binding sites and the dissociation constant were determined in 5 mM-Tris/HCl, pH 7.5, for each of the two types of binding site in native proteins A, C and dephosphorylated protein A (Bennick *et al.*, 1981). The hydroxyapatite binding experiments were therefore performed in the same buffer. Ca^{2+} binding to protein A and TX peptide in 4.6 mM-cacodylate,

pH 7.0, was determined by equilibrium dialysis as described by Bennick *et al.* (1981).

From these values and the total concentration of Ca^{2+} (C_s) and protein (P_s) in solution, the concentration of free Ca^{2+}, C_f, can be calculated from the relationship:

$$\frac{C_s - C_f}{P_s} = \frac{n_1 k_1 C_f}{1 + k_1 C_f} + \frac{n_2 k_2 C_f}{1 + k_2 C_f} \quad \text{(Tanford, 1961)}$$

where n represents the number of binding sites of a given type (1 or 2) and k represents the corresponding association constant. The concentration of Ca^{2+} bound to hydroxyapatite can be calculated by subtracting C_s from C_t. The amount of bound Ca^{2+}/mg of hydroxyapatite can now be plotted as a function of C_f.

To compare binding curves obtained in the presence and absence of protein by non-linear regression analysis, it was assumed that they all had intercepts with the origin of the axes. To evaluate possible differences between these binding curves, the values obtained for maximal amount of calcium bound to hydroxyapatite ($\bar{v}_{max.}$) and the apparent dissociation constant (K) were compared by Student's t test (Snedecor & Cochran, 1967).

If the results demonstrated that there was additional Ca^{2+} binding in the presence of protein, it cannot be assumed that under these circumstances there is only one type of Ca^{2+}-binding site on the mineral surface. The amount of additionally bound Ca^{2+} can be evaluated as the difference between the experimentially determined amount of Ca^{2+} bound in the presence of protein, and that which is bound to hydroxyapatite when no protein is present, as determined from the binding curve which was fitted to these data.

Since the amount of protein bound to hydroxyapatite is known, it is possible to calculate the difference as bound Ca^{2+}/(mol of protein bound to hydroxyapatite) and to plot these values as a function of free-Ca^{2+} concentration, C_f.

The additional amount of Ca^{2+} bound to the mineral in the presence of protein was evaluated by non-linear regression analysis. For the purpose of evaluating the apparent dissociation constant, K, it was assumed that the intercept of the binding curve and the $[Ca^{2+}]$ axis represented the origin of the $[Ca^{2+}]$ axis.

Results

The following $A^{0.1\%}_{205,1mm}$ values were determined: protein A, 3.82; protein C, 3.55. $A^{0.1\%}_{220,1mm}$ values were: protein A, 1.04; protein C, 1.05; TX peptide, 0.93. As a result of phosphatase digestion of protein A it was found that all the covalently bound phosphate had been removed from the protein. To evaluate the amount of alkaline phosphatase activity remaining in the dephosphorylated protein A, the enzymic activity of this preparation was compared with that of a soluble bacterial alkaline phosphatase. These results suggested that the enzyme constituted less than 0.7% of total protein, assuming that the soluble bacterial and the insoluble intestinal alkaline phosphatase had the same specific activities. The influence of this amount of enzyme on adsorption of the dephosphorylated protein is probably negligible.

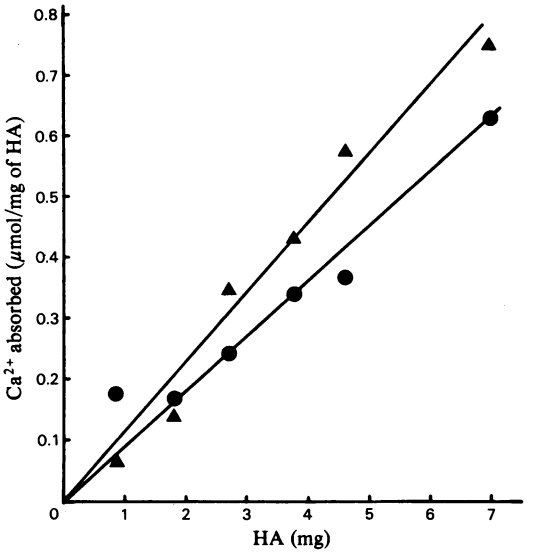

Fig. 1. *Effect of the amount of hydroxyapatite on the adsorption of Ca^{2+}*

Portions (0.5 ml) of 5 mM-Tris/HCl, pH 7.5, containing various amounts of hydroxyapatite (HA) and either 10 mM-$CaCl_2$ (●), or 3.3 mg of protein C/ml as well as 10 mM-$CaCl_2$ (▲), were incubated for 30 min. The Ca^{2+} adsorbed/mg of hydroxyapatite was plotted as a function of the amount of hydroxyapatite in the incubation mixture.

The amount of Ca^{2+} adsorbed to hydroxyapatite remains the same when the incubation time varies from 10 to 45 min. In the absence of protein, 92 ± 4 nmol of Ca^{2+}/mg of hydroxyapatite was adsorbed, and when protein was present, the value was approx. 16% higher. In the subsequent experiments an incubation time of 30 min was used.

The effect of the amount of hydroxyapatite on the adsorption of Ca^{2+} is shown in Fig. 1. Within the experimental range there is a linear relationship between the two parameters, regardless of the presence or absence of protein during adsorption. When the solution initially contained 211 μM-protein C the amount of adsorbed Ca^{2+} increased approx. 29%. The amount of adsorbed protein is plotted as a

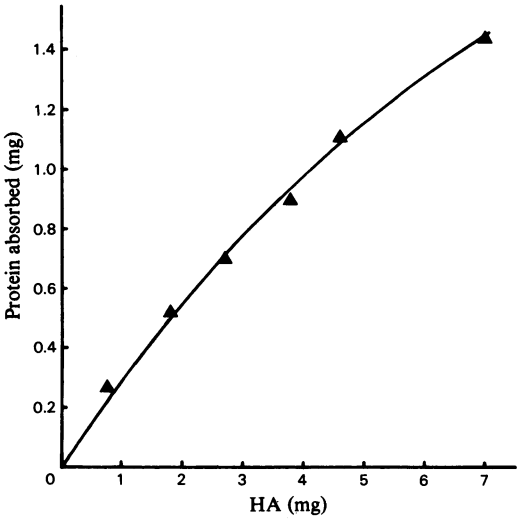

Fig. 2. *Effect of the amount of hydroxyapatite on the adsorption of protein C*
In the same mixtures that were described in the legend to Fig. 1 the total amount of protein adsorbed to hydroxyapatite was determined at the end of the incubation period (▲). The total amount of adsorbed protein was plotted as a function of the amount of hydroxyapatite present.

function of amount of hydroxyapatite in Fig. 2. Within the experimental range the relationship between the variables was almost linear.

The effect of washing hydroxyapatite suspended in 5 mM-Tris/HCl, pH 7.5, with the buffer before adsorption of Ca^{2+} is illustrated in Fig. 3. It was apparent that the amount of adsorbed Ca^{2+} decreased with the number of times that the mineral was washed in the Tris buffer.

When the same study was done replacing the Tris buffer with 4.6 mM-cacodylate, pH 7.0, it was found that repeated washing of the mineral with the cacodylate buffer had no effect on the subsequent adsorption of Ca^{2+} to hydroxyapatite (Fig. 4).

It was necessary to take this effect of washing of the mineral into account in order to study how salivary acidic proline-rich proteins affected the binding of Ca^{2+} to hydroxyapatite.

In one experiment hydroxyapatite was incubated with salivary protein A in concentrations of 24 μM, 48 μM or 96 μM. This resulted in a corresponding adsorption of 2.4, 4.4 or 6.2 nmol of protein/mg of hydroxyapatite. This hydroxyapatite coated with salivary protein A was then washed to remove any loosely adhering protein and incubated in Ca^{2+} containing buffers. During this period no desorption of protein was noticed. There were therefore no

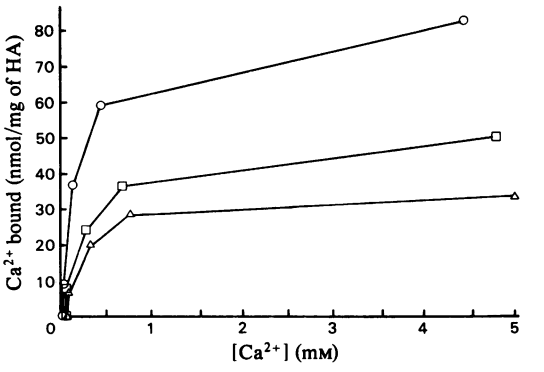

Fig. 3. *Influence of washing the hydroxyapatite in 5 mM-Tris/HCl, pH 7.5, on the adsorption of Ca^{2+}*
Portions (0.5 ml) of 5 mM-Tris/HCl, pH 7.5, containing 10 mg of hydroxyapatite (HA)/ml and various concentrations of $CaCl_2$ were incubated, and the amount of Ca^{2+} bound/mg of hydroxyapatite was plotted as a function of the concentration of Ca^{2+} in solution at equilibrium. ○, No washing of hydroxyapatite; □, hydroxyapatite washed once with 5 mM-Tris/HCl, pH 7.5; △, hydroxyapatite washed three times with the buffer.

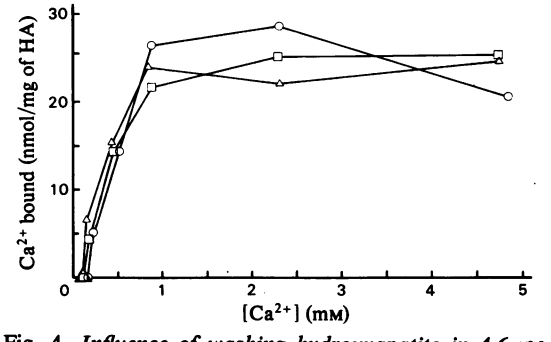

Fig. 4. *Influence of washing hydroxyapatite in 4.6 mM-cacodylate, pH 7.0, on the adsorption of Ca^{2+}*
Mixtures were prepared in the same manner as described in the legend to Fig. 3, with the exceptions that 4.6 mM-cacodylate, pH 7.0, was used instead of 5.0 mM-Tris/HCl, pH 7.5, and the adsorbant was a different preparation of hydroxyapatite (HA) (see the Experimental section). The amount of Ca^{2+} bound was plotted as a function of the concentration of Ca^{2+} in solution at equilibrium. ○, No washing of hydroxyapatite; □, hydroxyapatite washed once with 4.6 mM-cacodylate, pH 7.0; △, hydroxyapatite washed three times with the buffer.

soluble protein–calcium complexes present at the end of the incubation period.

The amount of calcium adsorbed to the protein

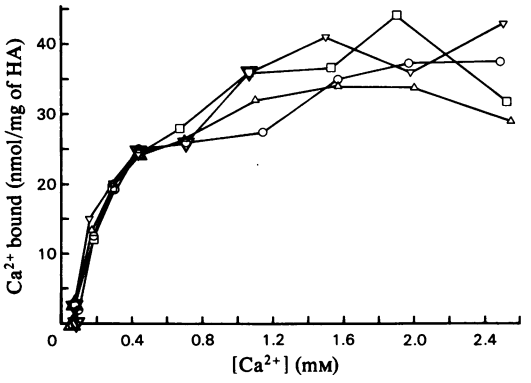

Fig. 5. *Adsorption of Ca^{2+} to hydroxyapatite coated with protein A*

Hydroxyapatite was coated with protein A before it was used in this Ca^{2+}-adsorption experiment. Portions (0.5 ml) of 5 mM-Tris/HCl, pH 7.5, containing 10 mg of hydroxyapatite/ml and various concentrations of $CaCl_2$ were then incubated and the amount of Ca^{2+} bound plotted as a function of $[Ca^{2+}]$ in solution at equilibrium. ○, Hydroxyapatite to which no protein was bound; □, hydroxyapatite to which was bound 2.4 nmol of protein A/mg of mineral; △, hydroxyapatite to which was bound 4.4 nmol of protein A/mg of mineral; ▽, hydroxyapatite to which was bound 6.2 nmol of protein A/mg of mineral.

A-coated and uncoated hydroxyapatite is plotted as a function of the concentration of soluble calcium in Fig. 5. Non-linear regression analysis was done on all the data obtained in two experiments in the presence of protein (30 values) and compared with an analysis of 10 values measured when no protein was present. Student's t test demonstrated that there were no significant differences in the values of $\bar{v}_{max.}$ ($0.2 < P < 0.1$) or K, the apparent dissociation constant ($0.4 < P < 0.2$). Fig. 6 illustrates the results obtained in an experiment in which Ca^{2+} and protein A were adsorbed simultaneously to hydroxyapatite. The experiment was performed by adding hydroxyapatite to 5 mM-Tris/HCl, pH 7.5, to which had been added only Ca^{2+} in various amounts or Ca^{2+} as well as protein A in concentrations of 88 μM, 190 μM or 255 μM. Comparison of the hyperbolic binding curves obtained in two experiments in the presence (33 values) and absence (15 values) of protein by Student's t test demonstrated significant differences in $\bar{v}_{max.}$ ($P > 0.001$) and K ($0.005 > P > 0.001$).

To evaluate the data further, the amount of protein bound to hydroxyapatite in each assay mixture was calculated (Fig. 7). From the data in Fig. 6 it was possible for each experimentally obtained value of Ca^{2+} bound to hydroxyapatite in the presence of protein to subtract the corresponding

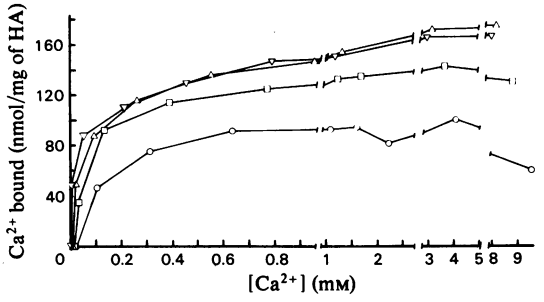

Fig. 6. *Effect of simultaneous adsorption of Ca^{2+} and protein A to hydroxyapatite on the binding of Ca^{2+} to the mineral*

Samples (5 mg) of hydroxyapatite (HA) were added to portions (0.5 ml) of 5 mM-Tris/HCl, pH 7.5, containing Protein A as well as Ca^{2+}. The amount of Ca^{2+} bound was plotted as a function of $[Ca^{2+}]$ in solution at equilibrium. ○, No protein added to the incubation mixture; □, 88 μM-protein A added to the incubation mixture; △, 190 μM-protein A added to the incubation mixture; ▽, 255 μM-protein A added to the incubation mixture. For details of the calculations, see the Experimental section.

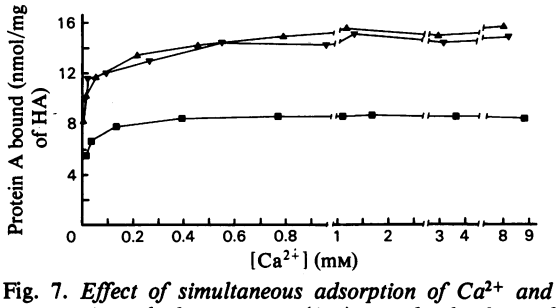

Fig. 7. *Effect of simultaneous adsorption of Ca^{2+} and protein A to hydroxyapatite (HA) on the binding of protein to the mineral*

This Figure illustrates results obtained in the experiment described in the legend to Fig. 6. The amount of protein bound has been plotted as a function of $[Ca^{2+}]$ in solution at equilibrium. ■, 88 μM-protein A added to the incubation mixture; ▲, 190 μM-protein A added to the incubation mixture; ▼, 255 μM-protein A added to the incubation mixture. For details of the calculations, see the Experimental section.

amount of Ca^{2+} bound in the absence of protein at the same concentration of soluble Ca^{2+}. This gave the difference in amount of Ca^{2+} bound to hydroxyapatite ($\Delta\bar{v}$) due to the presence of protein. From the values of $\Delta\bar{v}$ and the corresponding amount of bound protein, P_b (Fig. 7), the ratio of mol of additionally bound Ca^{2+}/mol of bound protein, $\Delta\bar{v}/P_b$, could be calculated.

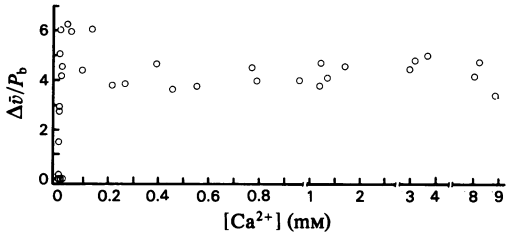

Fig. 8. *Effect of simultaneous adsorption of Ca^{2+} and protein A to hydroxyapatite: additional amount of Ca^{2+} bound due to the adsorbed protein*

From data such as presented in Figs. 6 and 7 the ratio of the difference in the amount of Ca^{2+} bound in the presence and absence of protein ($\Delta\bar{v}$) and the amount of bound protein (P_b) can be calculated. $\Delta\bar{v}/P_b$ was plotted as a function of the concentration of soluble Ca^{2+} at equilibrium. The results from two experiments have been given. For details of the calculations, see the text.

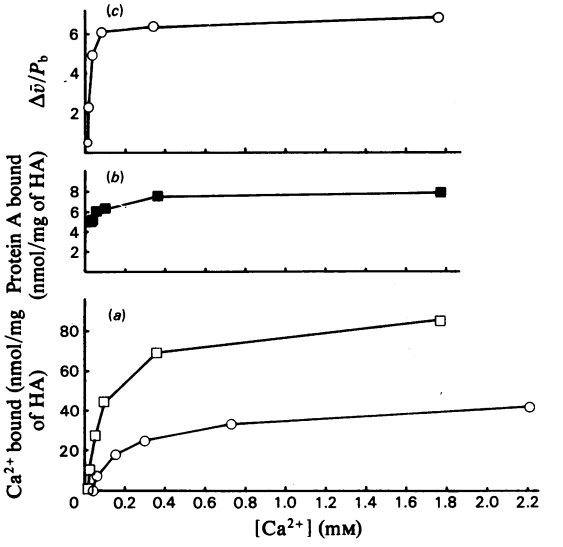

Fig. 9. *Effect of simultaneous adsorption of Ca^{2+} and protein A to hydroxyapatite (HA) that had been washed previously in 5 mM-Tris/HCl, pH 7.5*

(*a*) The amount of Ca^{2+} bound to hydroxyapatite. ○, No protein added to the incubation mixture; □, protein A added (83 μM). The amount of bound Ca^{2+} has been plotted as a function of the concentration of Ca^{2+} in solution at equilibrium. (*b*) ■, Amount of protein bound to hydroxyapatite (P_b). (*c*) ○, Additional amount of bound Ca^{2+} ($\Delta\bar{v}/P_b$) caused by the simultaneous adsorption of Ca^{2+} and protein A. For details of the calculations, consult the text.

In Fig. 8 the values for $\Delta\bar{v}/P_b$ have been plotted as a function of C_f. Assuming one type of independent binding site, a computer analysis of the data by an iterative procedure gave the following values: $K = 2.6 \pm 1.4$ (S.D.) μM and $n = 4.4 \pm 0.3$ (S.D.) mol/mol.

From these two types of experiments it could be seen that it was only when protein and Ca^{2+} were adsorbed simultaneously to the mineral that there was an increased binding of Ca^{2+}. The lack of increase in Ca^{2+} binding when the protein was bound to mineral before adsorbing Ca^{2+} was not due to the additional washing of hydroxyapatite with buffer. Fig. 9(*a*) illustrates the results obtained when hydroxyapatite was washed with the Tris/HCl buffer before simultaneous adsorption of Protein A (83 μM) and Ca^{2+} to hydroxyapatite. Comparison of binding curves by Student's *t* test obtained in the presence (six values) and absence (six values) of protein demonstrated that there was a significant difference in the values of both $\bar{v}_{max.}$ ($P > 0.001$) and K ($P > 0.001$). The total amount of adsorbed Ca^{2+} decreased in both the presence and absence of protein, but the difference was similar to that which is illustrated in Fig. 6. Values for $\Delta\bar{v}$ obtained from Fig. 9(*a*) and for P_b (Fig. 9*b*) were used to calculate $\Delta\bar{v}/P_b$. This parameter has been plotted as a function of [soluble Ca^{2+}] (Fig. 9*c*). Assuming one type of independent binding site, values of $n = 6.9 \pm 0.2$ (S.D.) mol/mol and $K = 12.5 \pm 2.1$ (S.D.) μM for this additionally bound Ca^{2+} were obtained.

To further substantiate the effect of protein A on Ca^{2+}-binding to hydroxyapatite, the Tris buffer was replaced with 4.6 mM-cacodylate, pH 7.0, and another preparation of hydroxyapatite was used. To estimate the amount of free Ca^{2+}, parameters for the binding of Ca^{2+} to protein A in 4.6 mM-cacodylate, pH 7.0, were determined experimentally. The following values were obtained: $n = 252 \pm 104$ (S.D.) nmol/mg, $K = 120 \pm 69$ (S.D.) μM for site I, and $n = 394 \pm 63$ (S.D.) nmol/mg, $K = 2070 \pm 1658$ (S.D.) μM for site II. The effect of protein on Ca^{2+} adsorption to hydroxyapatite suspended in cacodylate is illustrated in Fig. 10. Comparison of computer-fitted binding curves based on six values each by Student's *t*-test demonstrated that binding of protein A to hydroxyapatite before Ca^{2+} adsorption had no statistically significant effect on the values for $\bar{v}_{max.}$ ($0.1 > P > 0.05$) and K ($P < 0.5$) (Fig. 10*a*). In contrast, the value for $\bar{v}_{max.}$ increased significantly ($P > 0.001$) when the adsorption of protein and Ca^{2+} occurred simultaneously (seven values) (Fig. 10*a*). Values for $\Delta\bar{v}$ calculated from Fig. 10(*a*), together with the corresponding data for bound protein (P_b) (Fig. 10*b*), were used to calculate $\Delta\bar{v}/P_b$, which is plotted in Fig. 10(*c*). From these data, values of $n = 4.0 \pm 0.3$ (S.D.) mol/mol and $K = 30.6 \pm 1.0$ (S.D.) μM were obtained. It is therefore apparent that additional Ca^{2+} adsorption is not dependent on a particular preparation of hydroxyapatite or on the nature of the buffering ions.

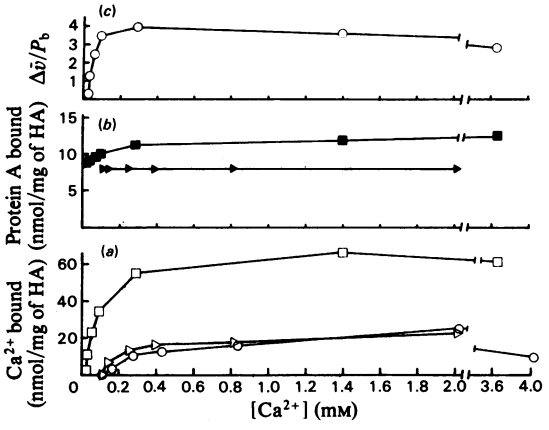

Fig. 10. *Adsorption of Ca^{2+} and protein A to hydroxyapatite (HA) suspended in 4.6 mM-cacodylate, pH 7.0*
(*a*) The amount of Ca^{2+} bound to hydroxyapatite: O, no protein present; ▷, hydroxyapatite coated with protein A before Ca^{2+} adsorption; □, simultaneous adsorption of Ca^{2+} and protein A (180 μM). The amount of Ca^{2+} bound was plotted as a function of the concentration of Ca^{2+} in solution at equilibrium. (*b*) ▶, Amount of protein A bound to hydroxyapatite (this adsorption was done before binding of Ca^{2+} to the mineral); ■, amount of protein A bound to hydroxyapatite (P_b) when protein and Ca^{2+} were adsorbed simultaneously. (*c*) O, Additional amount of Ca^{2+} ($\Delta\bar{v}/P_b$) bound to hydroxyapatite because of the simultaneous adsorption of Ca^{2+} and protein A. Details of the calculations are given in the text.

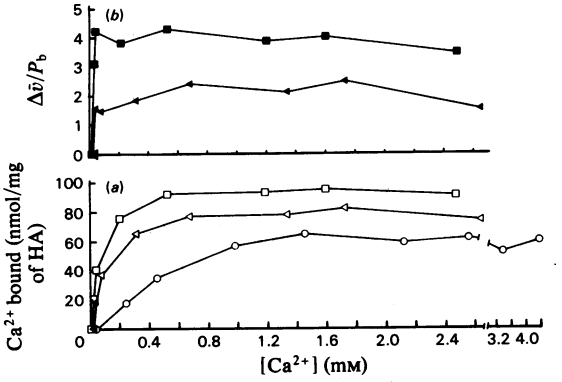

Fig. 11. *Simultaneous adsorption of native or dephosphorylated protein A and calcium to hydroxyapatite (HA)*
The experimental conditions are described in Fig. 7. (*a*) O, No protein added to the incubation mixture; □, Native protein A added in a concentration of 90 μM; ◁, Dephosphorylated protein A added in a concentration of 91 μM. The amount of Ca^{2+} bound was plotted as a function of the free $[Ca^{2+}]$ in solution at equilibrium. (*b*) ■, Additional amount of Ca^{2+} bound to hydroxyapatite in the presence of native protein A: ◀, additional amount of Ca^{2+} bound when dephosphorylated protein A was present.

Ca^{2+} binding to uncoated hydroxyapatite and to protein C-coated hydroxyapatite (nine values each) was analysed by non-linear regression and compared by Student's *t* test. No significant differences were found in the values for $\bar{v}_{max.}$ ($P<0.5$) and K ($0.4<P<0.2$), indicating that prior adsorption of protein C to the mineral had no effect on the subsequent binding of Ca^{2+} to the solid.

In another experiment hydroxyapatite was added to solutions containing only Ca^{2+} or Ca^{2+} as well as 70 μM-, 149 μM- or 219 μM-protein C. Comparison of the data obtained in three experiments in the absence (24 values) and presence (60 values) of protein C by non-linear regression analysis and Student's *t* test demonstrated significant differences in the values of $\bar{v}_{max.}$ ($P>0.001$) as well as K ($P>0.001$).

The amount of protein adsorbed to hydroxyapatite in the same experiment was also measured and $\Delta\bar{v}/P_b$ was plotted as a function of C_f. When C_f was increased to 0.1 mM, there was a gradual increase in $\Delta\bar{v}/P_b$, which changed little when C_f was further increased to 2 mM. Above that concentration there was a slow additional increase in $\Delta\bar{v}/P_b$. The data obtained at values of C_f lower than 2 mM were evaluated by non-linear regression analysis, assuming one type of independent binding site. This gave a value of $n=1.9\pm0.2$ (S.D.) mol/mol and $K=5.3\pm5.5$ (S.D.) μM.

The hydroxyapatite- and Ca^{2+}-binding sites have been located in the *N*-terminal 30-residue TX tryptic peptide (Bennick *et al.*, 1979, 1981). We therefore decided to examine the effect of this peptide on Ca^{2+} binding to hydroxyapatite. The experiments were done in 4.6 mM-cacodylate, pH 7.0. Studies on the binding of Ca^{2+} to peptide TX in this buffer gave values for site I of 531 nmol/mg for n and 27 μM for K. For site II the value for n was 2312 nmol/mg and for K, 618 μM.

Adsorption of the peptide to mineral before exposure to Ca^{2+} gave rise to a binding curve (seven values) with a value for $\bar{v}_{max.}$ [36.5 ± 2.7 (S.D.) nmol/mg] that was slightly higher than that obtained with the uncoated hydroxyapatite [30.3 ± 0.5 (S.D.) nmol/mg]. The two values were, however, significantly different ($P>0.001$). Simultaneous adsorption of peptide and Ca^{2+} (seven values) gave a significant ($P>0.001$) and much larger increase in $\bar{v}_{max.}$ to 65.4 ± 2.0 (S.D.) nmol/mg. The amounts of peptide adsorbed in the experiments were also

measured and calculated and plotted as a function of C_f. Simultaneous adsorption of Ca^{2+} and peptide TX caused a substantial increase in $\Delta\bar{v}/P_b$. Non-linear regression analysis, assuming one type of independent binding site, gave a value of $n = 9.0 \pm 1.0$ (S.D.) mol/mol and of $K = 66 \pm 25$ (S.D.) μM. When Ca^{2+} was adsorbed to peptide-TX-coated hydroxyapatite the maximal value found for $\Delta\bar{v}/P_b$ was 1.1 mol/mol, which was substantially lower than that obtained when Ca^{2+} and TX peptide were adsorbed simultaneously.

The effect of dephosphorylation of protein A on the interaction of protein and Ca^{2+} is illustrated in Fig. 11. Simultaneous adsorption of dephosphorylated protein A and Ca^{2+} (nine values) caused adsorption of less Ca^{2+} than that which occurred in the presence of the same concentration of native protein A. Whereas $\bar{v}_{max.}$ was 95.4 ± 1.2 nmol/mg in the presence of native protein A, it was 78.2 ± 2.6 nmol/mg when dephosphorylated protein was used. This decrease was significantly different ($P > 0.001$) (eight values). From plots of $\Delta\bar{v}/P_b$ it could be evaluated that n decreased from 4.2 ± 0.4 (S.D.) mol of Ca^{2+}/mol of native protein A to 2.3 ± 0.2 (S.D.) mol of Ca^{2+}/mol of dephosphorylated protein A. K, on the other hand, increased from 9.4 ± 5.6 (S.D.) μM in native protein A to 22 ± 12 (S.D.) μM in the dephosphorylated protein.

Discussion

The lack of a difference in the amount of Ca^{2+} adsorbed to untreated hydroxyapatite and to hydroxyapatite that had been absorbed with protein A or C before exposure to soluble Ca^{2+} indicates that protein and Ca^{2+} bind at different sites on the mineral surface.

A similar observation has been made by Rölla & Bowen (1978). They found that a salivary glycoprotein had no effect on the desorption of radioactive Ca^{2+} that had previously been adsorbed to hydroxyapatite.

Pearce (1981) observed that addition of Ca^{2+}- and phosphate-containing solutions of carboxymethylcellulose or bovine submandibular mucin to hydroxyapatite suspensions caused adsorption of macromolecules as well as Ca^{2+}. It is not known if a similar binding could occur to hydroxyapatite that had been coated with these macromolecules, and it is therefore difficult to relate the results obtained by Pearce (1981) to those obtained in the present experiments.

The additional Ca^{2+} binding observed when proteins A or C and Ca^{2+} are adsorbed simultaneously to hydroxyapatite must be due to the creation of additional Ca^{2+}-binding sites because of the presence of protein. These Ca^{2+}-binding sites are not accessible to ions in solution because of the lack of additional Ca^{2+} binding to protein-coated hydroxyapatite. Exposure of peptide-TX-coated hydroxyapatite to Ca^{2+} did cause binding of a small additional amount of the ion, but the effect was much larger when peptide and Ca^{2+} bound simultaneously. These additional Ca^{2+}-binding sites are therefore, as in proteins A and C, not accessible to Ca^{2+} in the surrounding solution. In solutions of proteins A and C it has been observed that Ca^{2+} binding decreases upon dephosphorylation (Bennick *et al.*, 1981). Similarly it was found that the amount of Ca^{2+} bound to adsorbed protein decreased significantly upon dephosphorylation, indicating that the protein phosphoserine residues are involved in the creation of additional Ca^{2+}-binding sites. The amount of Ca^{2+} bound to adsorbed protein C is less than that which is bound to adsorbed protein A, at least when the concentration of soluble Ca^{2+} is less than 2 mM. In contrast, the amount of Ca^{2+} bound to adsorbed peptide TX was greater than that bound to either of the native proteins. A parallel difference in Ca^{2+}-binding to the soluble proteins has been observed (Bennick *et al.*, 1981). A closer evaluation of the binding parameters derived from the experimental data in the present investigation is difficult because of the inevitable error arising from the subtraction of corresponding binding data obtained in the presence and absence of protein.

Other explanations may be given for the observed results. It is, for example, possible that the lack of adsorption of additional Ca^{2+} to hydroxyapatite already coated with protein is due to a decrease in the amount of Ca^{2+} bound directly to hydroxyapatite, which is cancelled by a simultaneous binding of Ca^{2+} to the adsorbed protein. Barring such possibilities, the interpretation of the results suggests an explanation for the ability of these proteins to inhibit hydroxyapatite formation.

It has been observed that various inhibitors of hydroxyapatite crystal growth are effective when only a small fraction of the total surface area of the crystal is covered by the inhibitor (Meyer & Nancollas, 1973). This adsorption may occur at dislocations or kinks on the hydroxyapatite surface where crystal growth is thought to be initiated.

Acidic proline-rich proteins have also been demonstrated to effectively inhibit crystal growth when only a fraction of the hydroxyapatite surface has been covered, presumably by blocking crystal-growth sites (Moreno *et al.*, 1979), since hydroxyapatite 'seeds' that had been pretreated with acidic proline-rich proteins were unable to initiate crystal growth in a supersaturated solution of calcium phosphate. Dephosphorylation destroys the inhibiting activity of the proteins (Hay & Moreno, 1979*b*). This finding, and the observation in the present study that dephosphorylation decreases the additionally

bound Ca^{2+} in the presence of protein, suggests that part of this Ca^{2+} is bound close to, or at, the crystal-growth site. The adsorption of acidic proline-rich proteins to hydroxyapatite would prevent access of Ca^{2+} from solution to these sites, thereby inhibiting hydroxyapatite formation.

If this mechanism is correct, it is perhaps surprising that the binding of protein to the mineral surface does not decrease the amount of Ca^{2+} that binds to the mineral in the absence of protein, since it might be expected that, under those circumstances, Ca^{2+} would also bind at the crystal-growth sites. The reason why this is not observed may be that binding of Ca^{2+} at the growth sites requires the co-operation of phosphate, which is not present in these experiments.

The acidic proline-rich proteins constitute a substantial amount of the proteins in the newly acquired dental pellicle that forms by adsorption of proteins from saliva on to clean tooth surfaces (Bennick *et al.*, 1983). This pellicle will retard movement of ions between the dental enamel and the fluid environment and decrease the rate of acid dissolution of hydroxyapatite in dental enamel (Zahradnik *et al.*, 1976). It is possible that the ability of the acidic proline-rich proteins to bind Ca^{2+} at the mineral surface is part of these processes. The Tris and cacodylate buffers we have used are at I 0.004, but in saliva I can be as high as 0.669 (Grön, 1973*b*). Under the ionic conditions that exist in saliva, acidic proline-rich proteins bind 28–99 nmol of Ca^{2+}/mg of protein (Bennick & Cannon, 1978). It can therefore be expected that adsorption of the proteins *in vivo* to the tooth surface will also lead to binding of additional Ca^{2+}.

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