Biochem. J. (1977) **163**, 241–245
Printed in Great Britain

The Binding of Calcium to a Salivary Phosphoprotein, Protein C, and Comparison with Calcium Binding to Protein A, a Related Salivary Phosphoprotein

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(*Received* 3 *September* 1976)

The binding of Ca^{2+} to a salivary phosphoprotein, protein C, was studied by equilibrium dialysis. In 5mM-Tris/HCl buffer, pH7.5, protein C bound 190nmol of Ca^{2+}/mg of protein. The apparent dissociation constant, K, was determined to be 1.9×10^{-4}M and the binding of Ca^{2+} to the protein was non-co-operative. The binding of Ca^{2+} to protein C apparently depends on groups which ionize above pH5.0. Ca^{2+} binding decreased with increased concentration of the dialysis buffer and on addition of $SrCl_2$, $MgCl_2$ and $MnCl_2$ to the dialysis buffer. Digestion of protein C with trypsin or collagenase or heating of the protein to 60° or 100°C had little or no effect on the Ca^{2+} binding. Digestion of protein C with alkaline phosphatase caused a decrease in the amount of protein-bound Ca^{2+}. This was also found for another salivary phosphoprotein, protein A. In the absence of Ca^{2+} the $s^0_{20,w}$ for protein C was 1.29S and in the presence of Ca^{2+} it was 1.46S. Ca^{2+} may cause a conformational change in the protein or an aggregation of the protein molecules. No conformational changes of protein C in the presence of Ca^{2+} could be detected by circular dichroism or nuclear magnetic resonance.

The binding of Ca^{2+} to a proline-rich salivary phosphoprotein, protein A, has been described. The physiological importance of Ca^{2+} binding to this protein is not clear at present (Bennick, 1976), but its nature suggests potential physiological implications in the oral cavity (Bennick, 1976). Another salivary proline-rich phosphoprotein, protein C, which shows striking chemical, spectroscopic and immunological similarities to protein A, has also been described (Bennick, 1977).

Since it is likely that protein C also binds Ca^{2+}, it was decided to characterize Ca^{2+} binding to protein C and compare the results with those obtained for protein A (Bennick, 1976).

Experimental

Materials

Alkaline phosphatase from *Escherichia coli* was obtained from Worthington, Freehold, NJ, U.S.A. The sources of all other materials were the same as used previously (Bennick, 1976).

Collection of salivary secretions and purification of proteins

The collection of stimulated parotid saliva and purification of proteins A and C were performed as described previously (Bennick, 1975, 1977).

Measurement of Ca^{2+} binding

Ca^{2+} binding was measured with ^{45}Ca by equilibrium dialysis as described by Bennick (1976). In typical experiments 0.4mg of protein was dissolved in 200μl of 5mM-Tris/HCl buffer, pH7.5, placed in a dialysis bag and dialysed at 24°C against 100ml of 5mM-Tris/HCl buffer, pH7.5, containing 1mM-$CaCl_2$ and sufficient ^{45}Ca to give a specific radioactivity of approx. 100c.p.m./nmol of Ca^{2+}. The concentration of Ca^{2+} inside and outside the dialysis bag and the concentration of protein in the dialysis bag was determined as described previously (Bennick, 1976). In all calculations protein concentrations as deduced from amino acid analysis were used.

The amount of Ca^{2+} bound to protein C was determined in experiments in which the Ca^{2+} concentration was kept constant at 1mM while the protein concentration was varied from 0.8 to 4.7mg/ml, or the protein concentration was kept constant and the Ca^{2+} concentration varied from 0.001 to 5mM.

To investigate the influence of pH on Ca^{2+} binding to protein C, equilibrium dialysis was performed in 5mM-sodium acetate buffers, pH3.5–5.8, in 5mM-imidazole/HCl buffers, pH6.0 or 6.5, and in 5mM-Tris/HCl buffers, pH7.0–9.0. The sodium acetate buffers were made by adjusting the pH of a 10mM solution of acetic acid with 100mM-NaOH and diluting to 5mM. Similarly, 10mM-solutions of imidazole or Tris were

adjusted to the appropriate pH with 100mM-HCl and diluted to 5mM. Since the ionic strength of the buffers, which varied from 0.00046 to 0.0047, might influence the Ca^{2+} binding, another set of experiments was performed in which the ionic strength of the buffers used in the previous experiment was kept constant by adding sufficient NaCl to give an ionic strength of 0.0047 for all buffers. The effect of other metals on the binding of Ca^{2+} to protein C was investigated by adding $SrCl_2$, $MgCl_2$ or $MnCl_2$ at a concentration of 1mM to dialysis buffers containing 1mM-$CaCl_2$.

In other experiments protein C was heated to 60 or 100°C for 10min before equilibrium dialysis, or the equilibrium-dialysis experiment was performed at 60°C. The effect of ionic strength was studied by equilibrium dialysis of protein C in 5mM-Tris/HCl buffer, pH7.5, containing NaCl in concentrations as high as 100mM.

Equilibrium dialysis was also performed in either 2.6mM-sodium phosphate buffer, pH7.5, containing 61mM-NaCl and 1mM-$CaCl_2$ or in 6.6mM-sodium phosphate buffer, pH5.9, containing 22mM-NaCl and 1mM-$CaCl_2$. These buffers have the same phosphate concentration and ionic strength as unstimulated parotid saliva (I 0.029) and stimulated parotid saliva (I 0.069) respectively (Grön, 1973).

The effect of proteolytic digestion of protein C was tested by adding trypsin or collagenase to protein C at an enzyme/protein ratio of 1:100 (w/v) before equilibrium dialysis, as previously described (Bennick, 1976).

In other experiments proteins A and C were digested with alkaline phosphatase by the method of Garen & Levinthal (1960) at 45°C for 48h in enzyme/protein ratios of 1:100 and 1:25 (w/w) for proteins A and C respectively. The amount of organic phosphorus released by the enzyme was assayed as described by Bartlett (1959). The presence of proteinase activity in the phosphatase preparation was tested as described previously (Bennick, 1977). No proteinase activity was detected. Incubation of proteins A and C in the absence of phosphatase at 45°C for 48h did not affect Ca^{2+} binding to the proteins. Phosphatase-digested proteins were used in equilibrium-dialysis experiments in which the Ca^{2+} concentration was varied as described for the undigested protein.

Determination of sedimentation coefficient

The sedimentation coefficient of protein C was determined in solutions containing from 1.3 to 7.7mg of protein/ml.

The protein was dissolved in 5mM-Tris/HCl buffer, pH7.5, or in 5mM-Tris/HCl buffer, pH7.5, containing 1mM-$CaCl_2$. The sedimentation coefficients were determined 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.

A total of five determinations of the sedimentation coefficient at different protein concentrations were made in the absence of Ca^{2+} as well as in the presence of Ca^{2+}. The data were analysed by the least-squares method to obtain the $s^0_{20,w}$.

Results

Calcium binding to protein C

The results obtained in equilibrium-dialysis experiments in which the Ca^{2+} concentration was kept constant at 1mM while the protein concentration was varied are illustrated in Fig. 1. A linear relationship of bound Ca^{2+} to protein concentration 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 Ca^{2+} are plotted in Fig. 2.

The parameters of Ca^{2+} binding were evaluated from Hanes (1932) plots. From this analysis the maximum amount of Ca^{2+} bound to protein C was 190nmol/mg of protein, which corresponds to 3.04g-atoms of Ca^{2+}/mol of protein, assuming mol.wt. 16300 (Bennick, 1977). The apparent dissociation constant, K, was 190μM. The Hill coefficient had an estimated value of 1.03, not significantly different from 1. The effect of pH on Ca^{2+} binding to protein C is illustrated in Fig. 3. No Ca^{2+} binding was observed in the pH range 3.5–5.0. Between pH5.0 and 9.0 there was a gradual increase in Ca^{2+} binding. No difference was observed in the results obtained with buffers in

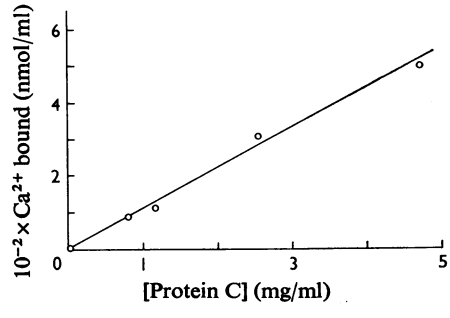

Fig. 1. *Amount of Ca^{2+} bound to protein C as a function of the concentration of protein C*

The binding of Ca^{2+} was evaluated by 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 C in the dialysis bags.

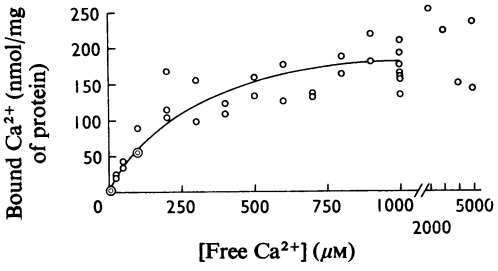

Fig. 2. *Amount of Ca^{2+} bound to protein C as a function of the concentration of free Ca^{2+}*

The binding of Ca^{2+} was evaluated by equilibrium dialysis as described in the text. The amount of bound Ca^{2+} (nmol/mg of protein) is plotted against the concentration of free Ca^{2+} (μM). All the experimental values used in evaluation of the hyperbolic binding curve have been plotted.

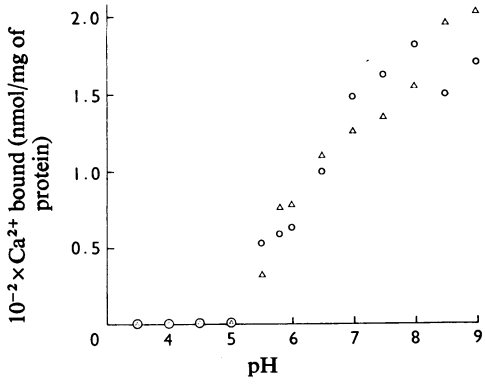

Fig. 3. *Amount of Ca^{2+} bound to protein C as a function of pH of the dialysis buffer*

The amount of protein-bound Ca^{2+} (nmol/mg of protein) is plotted against the pH of the dialysis buffers. △, Buffers of various ionic strengths; ○, buffers of constant ionic strength. The concentration of free Ca^{2+} in the buffers was 1 mM.

which the ionic strength varied and in buffers with constant ionic strength.

The presence of bivalent metals caused a decreased Ca^{2+} binding to protein C, the decrease being smallest in the presence of $SrCl_2$ and largest in the presence of $MnCl_2$. Heating protein C to 60°C before equilibrium dialysis did not affect Ca^{2+} binding to the protein, but there appeared to be a decrease in Ca^{2+} binding after heating protein C to 100°C. If the equilibrium dialysis was performed at 60°C, protein C could still bind Ca^{2+}, although in a diminished amount compared with the results obtained at 24°C. The effects of addition of bivalent metals and heating of the protein solutions are summarized in Table 1.

When NaCl was added to the dialysis buffer in concentrations as high as 100 mM there was a decrease in the amount of protein-bound Ca^{2+}, from 151 nmol/mg of protein in the absence of NaCl to 31 nmol/mg in a buffer containing 100 mM-NaCl. These results are illustrated in Fig. 4. As a result of equilibrium dialysis of protein C in phosphate buffers it was found that in 2.6 mM-phosphate, pH 7.5, containing 61 mM-NaCl, protein C bound 36 nmol of Ca^{2+}/mg. In a 5 mM-Tris/HCl/NaCl buffer, pH 7.5, of comparable ionic strength (68 mM-NaCl), protein C bound 40 nmol of Ca^{2+}/mg of protein.

In 6.6 mM-phosphate, pH 5.9, containing 22 mM-NaCl, protein C bound 40 nmol of Ca^{2+}/mg. In comparison, protein C bound 69 nmol of Ca^{2+}/mg of

Table 1. *Effect of addition of bivalent metals or heating on Ca^{2+} binding to protein C*

Ca^{2+} binding was measured by equilibrium dialysis in 5 mM-Tris/HCl buffer, pH 7.5, containing 1 mM-$CaCl_2$. For details see the Experimental section.

Addition or treatment	Ca^{2+} (nmol/mg of protein)
1 mM-$SrCl_2$	90
1 mM-$MgCl_2$	73
1 mM-$MnCl_2$	27
Heating of protein C at 60°C for 10 min before dialysis	192
Heating of protein C at 100°C for 10 min before dialysis	150
Equilibrium dialysis at 60°C	114
Control	205

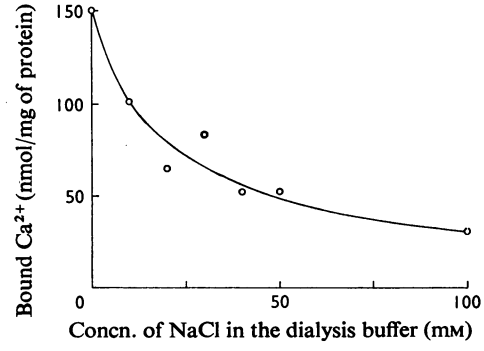

Fig. 4. *Amount of protein-bound Ca^{2+} as a function of the concentration of the dialysis buffer*

The amount of protein-bound Ca^{2+} (nmol/mg of protein) is plotted as a function of the NaCl concentration in the 5 mM-Tris/HCl, pH 7.5, dialysis buffer. The concentration of free Ca^{2+} was 1 mM.

protein in 5mM-Tris/HCl, pH7.5, containing 28mM-NaCl. The ionic strength of both these buffers is 0.029. Because of the pH-dependence of Ca^{2+} binding to protein C it can be estimated from Fig. 3 that approx. 32nmol of Ca^{2+} would be bound to 1mg of protein C in a 5mM-Tris/HCl buffer, pH5.9, containing 28mM-NaCl. The Ca^{2+} binding to protein C therefore does not appear to be notably influenced by the buffering salts, as long as the pH and ionic strength is the same.

Digestion of protein C with trypsin or collagenase had little or no effect on the Ca^{2+}-binding ability of the protein. Protein C digested with trypsin bound 197nmol of Ca^{2+}/mg of protein and after digestion with collagenase the protein bound 143nmol of Ca^{2+}/mg. Undigested protein A bound 144nmol of Ca^{2+}/mg under the experimental conditions.

As a result of incubations of samples of protein A with phosphatase, 80% of the organic phosphate was released as P_i. Similar incubations of protein C with phosphatase resulted in removal of 90% of the organic phosphate. The results of experiments with phosphatase-digested proteins in which the amount of protein-bound Ca^{2+} was measured as a function of the concentration of free Ca^{2+} are plotted in Fig. 5(*a*) for protein A and Fig. 5(*b*) for protein C. From the hyperbolic binding curve it was estimated that at a concentration of free Ca^{2+} of 1nM, protein A bound approx. 230nmol of Ca^{2+}/mg of protein. In contrast, undigested protein A binds 580nmol of Ca^{2+}/mg of protein at 1nM-free Ca^{2+}. Similarly it was found that at 1nM-free Ca^{2+}, alkaline phosphatase-digested protein C bound approx. 80nmol of Ca^{2+}/mg of protein.

In the absence of Ca^{2+}, the $s^0_{20,w}$ for protein C was 1.29±0.04S (S.E.M.) and in the presence of Ca^{2+} it was 1.46±0.04S (S.E.M.). The slope of the line fitted to plots of $s^0_{20,w}$ as a function of protein concentration in mg/ml was 0.0097±0.01 (S.E.M.) in the absence of Ca^{2+} and 0.017±0.008 (S.E.M.) in the presence of Ca^{2+}. There is thus little if any concentration-dependence for the sedimentation coefficients for protein C in the presence and absence of Ca^{2+}.

Discussion

Comparison of the physical and chemical properties of salivary proteins A and C has revealed a close relationship between these two proteins, although differences have also been noted (Bennick, 1976). With regard to the Ca^{2+}-binding characteristics, the apparent dissociation constant of 1.9×10^{-4}M for protein C is practically identical with that of protein A (1.8×10^{-4}M). But whereas protein A can bind maximally 664nmol of Ca^{2+}/mg of protein, protein C can maximally bind only 190nmol of Ca^{2+}/mg of protein. This result is surprising in view of the many chemical and physical similarities of the two proteins and the fact that protein C has mol.wt. 16300, whereas that of protein A is only 9900.

The effects of digestion of protein C with trypsin and collagenase, and the addition of other bivalent metals, or heating, on Ca^{2+} binding to protein C are similar to those previously found for protein A (Bennick, 1976). In addition it was found that protein C still retained some Ca^{2+}-binding ability at 60°C. The Ca^{2+}-binding sites in protein C therefore appear to be located within short stretches of the peptide chain and the tertiary structure may not be important for Ca^{2+} binding.

The results obtained for Ca^{2+} binding to phosphatase-digested proteins A and C clearly show a diminished ability of the proteins to bind Ca^{2+} after the phosphate has been removed, and phosphorylation of the proteins is therefore necessary to ensure maximal Ca^{2+} binding. After removal of approx. 80% of the phosphate, protein A only binds about 40% of the amount of Ca^{2+} bound to undigested protein. Protein C from which 91% of the phosphate has been

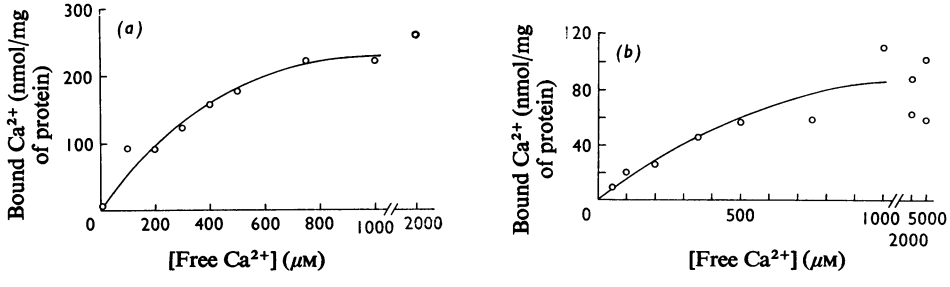

Fig. 5. *Amount of Ca^{2+} bound to phosphatase-digested protein A (a) and phosphatase digested protein C (b) as a function of the concentration of free Ca^{2+}*

The amount of bound Ca^{2+} (nmol/mg of protein) is plotted against the concentration of free Ca^{2+} (μM).

removed also binds approx. 40% of the amount of Ca^{2+} bound to undigested protein.

Although the results obtained for protein A (Bennick, 1976) suggest the necessity of ionization of groups with approximate pK4.9, an essential feature of Ca^{2+} binding to protein C appears to be groups which ionize above pH5.0. The difference in ionic strength of buffers with the same pH used in the experiments is so small that it would be difficult to observe any difference in the amount of Ca^{2+} bound to protein C by the method used. The variation in the amount of protein-bound Ca^{2+} with changes in pH is therefore not due to variation in ionic strength.

The differences in the magnitude of $s^0_{20,w}$ suggest that in the presence of Ca^{2+} there is either a conformational change of protein C or an aggregation. It is possible that there is a conformational change in protein C on Ca^{2+} binding, although c.d. (circular dichroism) and n.m.r. both failed to demonstrate such a change in protein C in the presence of Ca^{2+} (Bennick, 1977). For protein A, $s^0_{20,w}$ in the absence of Ca^{2+} was 1.28S and in the presence of Ca^{2+} it was 1.30S. No difference could therefore be found in the magnitude of the sedimentation coefficient for this protein. This finding, together with the observations from c.d. and n.m.r. studies, suggests neither aggregation nor conformational changes of protein A in the presence of Ca^{2+}.

These studies have thus demonstrated that although proteins A and C share a number of characteristics in their Ca^{2+}-binding ability, differences exist in their Ca^{2+}-binding sites.

I thank Mrs. M. Cannon for invaluable technical assistance and Dr. D. Kells and Mr. M. Paull for performing the ultracentrifugations. This work was supported by grant no. MA 4920 from the Canadian Medical Research Council.

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