The location and Nature of Calcium-binding Sites in Salivary Acids Proline-rich Phosphoproteins

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The location of the calcium-binding sites in the human acidic proline-rich proteins, salivary proteins A and C, were determined by equilibrium dialysis of the tryptic peptides with buffers containing 45Ca. All the calcium-binding sites are located in the NH2-terminal proline peptide (TX peptide). The nature of the calcium binding sites in the TX peptide and native salivary proteins A and C, as well as dephosphorylated proteins were compared. Two types of sites can be distinguished in peptide TX. Type I sites have an apparent dissociation constant (K) of 38 μM and are responsible for the binding of 2.6 mol of Ca/mol of peptide. The corresponding figures for Type II sites are 780 μM and 5.3 mol of Ca/mol of peptide. In the native proteins, the amount of calcium bound at the type II sites decreases to 3.9 mol of Ca/mol of proteins A and C and K increases to 1100 μM. The amount of calcium bound at type I sites decreases to 1.5 mol/mol of protein A and 0.6 mol/mol of protein C, but there is no change in K. Dephosphorylation affects the calcium binding at both types of sites. The experiments indicate that the COOH-terminal parts of the native proteins affect the number and the nature of the protein calcium-binding sites. Proton and phosphorous NMR data demonstrate that β-COOH in aspartic acid, as well as phosphoserine, are part of the calcium-binding sites. The difference in calcium binding to salivary proteins A and C may be due at least partially to differences in the environment of one or more aspartic acids.

Human parotid and submandibular saliva contain a group of related acidic proline-rich proteins (Bennick and Connell, 1971, Oppenheim et al., 1971). Two of the major components have been named salivary proteins A and C (Bennick and Connell, 1971) and their primary structures have been determined (Wong et al., 1979, Wong and Bennick, 1980). Salivary protein A consists of a single peptide chain of 166 residues and salivary protein C contains the entire structure of protein A in its NH2-terminal part, but continues beyond residue 166 to the COOH-terminal residue, no. 150. The proteins have a highly negatively charged NH2-terminal end which can be isolated as a tryptic peptide (TX peptide). This peptide contains a total of 11 dicarboxylic amino acids and 2 phosphoserines. The only negatively charged residues found in the remainder of both proteins are two aspartic acids. The proteins bind calcium under conditions which suggest that they take part in calcium exchange in the oral cavity (Bennick and Wong, 1979). In addition, they inhibit the formation of, and bind strongly to, hydroxyapatite (Hay and Moreno, 1979a, Hay, 1973, Bennick and Cannon, 1978). These biological activities suggest that the proteins are important for maintaining the integrity of the teeth in their physiological liquid environment. The hydroxyapatite binding site is located in the TX peptide (Bennick et al., 1979). This study was undertaken to determine the location and nature of the calcium binding sites. The results obtained prompted a reinvestigation of calcium binding to salivary proteins A and C in order to make a better comparison of the calcium binding sites in the native proteins and the peptide containing the calcium binding sites.

**EXPERIMENTAL PROCEDURES**

**Materials**

Alkaline phosphatase from *E. coli* was obtained from Worthington and dialysis tubing (molecular weight cut off: 2000) from Spectrum Medical Industries. Radioactive 45Ca was purchased from New England Nuclear and 99.8% 3H2O, 3HCl, and NaO2H from Stohler Isotope Chemicals. Aldrich was the supplier of 100% 1H2O.

**Methods**

Isolation of Salivary Proteins A and C—Salivary Proteins A and C were prepared as described by Bennick (1975, 1977a).

Tryptic Digestion of Salivary Proteins A and C: Isolation of Tryptic Peptides—Salivary proteins A or C were digested in 30-mg quantities for 5 min and the resulting peptides were purified by high voltage paper electrophoresis as described previously (Wong et al., 1979). From salivary protein A two peptides were obtained, one named TX, which contained the NH2-terminal 30 residues and a COOH-terminal peptide named TY which constituted residues 31 to 106. Digestion of salivary protein C gave a total of 3 peptides (Wong and Bennick, 1980). Two of these, CTX and CTV, were identified with TX and TY, respectively. The third peptide which was labeled CTZ constituted the COOH-terminal part of protein C (Residues 107 to 150).

Digestion with Alkaline Phosphatase—Samples of protein (4 to 42 mg) were incubated with alkaline phosphatase at 70 °C for 2 h at an enzyme/substrate ratio of 1:50 (w/w) in 0.25 mM ammonium formate, pH 8.0. The final concentration of substrate varied from 0.2 to 7 mg/ml. Following incubation, the digest was transferred to a dialysis bag and dialyzed against 0.25 mM ammonium formate, pH 8.0, at room temperature for 2 h. The digest was transferred to a tube to which was added a second equivalent of alkaline phosphatase. The incubation was continued for 2 h at 70 °C. In subsequent digestions this second addition of enzyme was omitted. The completeness of protein phosphate removal was assayed by measuring the amount of organic phosphate in the digestion mixture according to the method of Bartlett (1959) and by acrylamide gel electrophoresis of the digest.

Measurement of Calcium Binding—Calcium binding to protein and peptide was studied as described previously (Bennick, 1976) except for the use of dialysis bags with a nominal retention of
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molecules of $M_c = 2000$ or larger. Furthermore, the concentration of protein in the dialysis bags was determined by reading the absorbance of the solution at 220 nm. The extinction coefficients of salivary proteins A and C and peptide TX at a wavelength of 220 nm were determined from solutions for which the protein concentrations had been determined by amino acid analysis. The extinction coefficients of dephosphorylated salivary proteins A and C were assumed to be equal to that of the corresponding native protein. The nanomoles of bound calcium/mg of protein was plotted as a function of the free calcium concentration. To evaluate the possible presence of more than one type of binding site in the protein or peptide, Scatchard plots were done of bound calcium versus the amount of bound calcium/concentration of free calcium.

To obtain the apparent dissociation constant ($K$) and number of calcium binding sites ($n$) in the proteins, the corresponding values of bound calcium/mg of protein and free calcium concentration were subjected to a computer analysis of nonlinear regression using an iterative procedure. For nonlinear Scatchard plots it was assumed that there were two types of binding sites. The sites with the smallest dissociation constant are referred to as type I sites and those with the highest dissociation constant as type II sites. Additional evaluation of $K$ and $n$ were made from the Scatchard plots by the method of Rosenthal (1967). The significance of difference between corresponding $K$ or $n$ values was evaluated by Student's $t$ test.

Proton NMR Spectroscopy—Samples of 5 mg of salivary protein A or C were freeze-dried and redissolved in 100% $^2$H$_2$O. This procedure was repeated 2 to 5 times. Immediately before use, the freeze-dried samples were redissolved in 0.16 M NaCl in 100% $^2$H$_2$O or in 100% $^3$H$_2$O containing 0.01 M NaCl and 0.05 M CaCl$_2$. The ionic strength of these two solutions was 0.15. The sample was transferred to a 5-mm-diameter NMR tube and about 1 µl of acetone was added as an internal standard. The resonance from acetone is located 2.225 ppm$^1$ downfield to 2,2-dimethyl-2-silapentane-5-sulfonate.

The pH of the solution was measured with a microelectrode. The readings of the pH meter were recorded directly without correcting for the $^2$H isotope effect. The pH of the solution was adjusted by adding small volumes of 0.5 M HCl or 0.5 M NaOH.

Proton NMR spectra were obtained at the Toronto Biomedical NMR Centre on a Varian HR-220 MHz spectrometer modified for Fourier transformation. The total number of free induction decays which were accumulated to obtain a given spectrum varied from 128 to 512. Fourier transformation was performed by a PDP-11/03 computer from 8000 data points with spectral widths of 1000 Hz. Spectra were accumulated at a probe temperature of 22 °C.

Fig. 1 illustrates a typical proton NMR spectrum. The position of the resonance of $^4$H$_2$O in the aspartic acid residues was identified as the resonance signal in the expected position in a protein with an unordered conformation (McDonald and Phillips, 1969) and the observation that the position of this signal in salivary proteins A and C was dependent on the pH of the solution (Bennick, 1977).

At a magnetic field strength of 220 MHz with respect to protons, it is not possible to distinguish signals from individual aspartic acid residues. All measurements were therefore made to the midpoint of the signal. The distance from the acetone reference signal to the resonance position of the $^4$H$_2$O in the aspartic acid residues was measured in hertz and plotted as a function of pH. The apparent $pK$ was measured as the midpoint of the chemical shift range.

Phosphorous NMR Spectroscopy—Samples of 30 mg of salivary proteins A or C were freeze-dried and redissolved in 99.8%$^2$H$_2$O containing 0.1 M NaCl. The pH of the solution was measured with a microelectrode and was not corrected for the $^2$H isotope effect. The pH was adjusted by adding small aliquots of 0.3 M HCl or 0.3 M NaOH.

$^3$P NMR spectra were recorded on a Bruker WH-360 spectrometer operating at 145.7 MHz for phosphorus. The spectrometer was equipped with a Bruker BST-100/700 temperature control unit. All spectra were recorded at 20 °C ± 1 °C. Low power broad band $^1$H decoupling was employed. The chemical shift of the signals was measured with respect to an external glycerophosphorylcholine sample. The position of the signals with respect to 85% phosphoric acid was calculated assuming that the glycerophosphorylcholine signal is at $-0.51$ ppm with respect to 85% phosphoric acid.

RESULTS

Assays of the dephosphorylated salivary proteins demonstrated that all organic phosphate had been removed. This

$^1$ The abbreviation used is: ppm, parts per million.

was confirmed in gel electrophoresis which demonstrated a single band with a slightly lower anodic mobility than the native protein. There was no sign of proteolysis of the salivary proteins. The extinction coefficients at 220 nm, $E_{1}^{1cm}$ was

![Fig. 1. Proton NMR spectrum of a solution of salivary protein C, nominal pH 7.12. The position of various resonances has been given as measured in Hz downfield from the internal acetone standard. H$_2$, ASP indicates the position of the H$_2$ in aspartic acid. For orientation the positions of the H$_2$ in lysine (H$_2$, LYS) and H$_2$ in arginine (H$_2$, ARG) have also been indicated.](http://www.jbc.org/)

![Fig. 2. Calcium binding to peptides derived from salivary proteins A and C. A, calcium binding to the tryptic peptides TX (C—O) and TY (C—O) from salivary protein A, and B, to the tryptic peptides CTX (C—O), CTY (C—O), and CTZ (V—V) from salivary protein C. The amount of bound calcium has been plotted as a function of free calcium.](http://www.jbc.org/)
found to be 0.86 for TX, 1.00 for salivary protein A, and 1.05 for salivary protein C.

The amount of calcium bound to the tryptic peptides has been plotted as a function of free calcium concentration in Fig. 2. There was no calcium binding to peptide TY and peptide CTY bound maximally a negligible amount of 23 nmol/mg of protein at a free calcium concentration of 2 mM. This corresponds to 0.2 mol of bound calcium/mol of peptide.

No calcium binding to peptide CTZ was observed. In contrast, there was considerable calcium binding to peptides TX and CTX. No apparent difference in calcium binding to peptides TX and CTX could be observed (Fig. 2). A Scatchard plot of all the data from the two peptides is illustrated in Fig. 3. The plot is clearly nonlinear and analysis of the binding data gave the values for $n$ and $K$ which are listed in Table I. The peptides contain 2 or 3 calcium binding sites (type I sites) with an apparent dissociation constant of about 38 µM. In addition there are evidently 5 sites with a lower affinity for calcium. For these sites, $K$ is approximately 780 µM. The Scatchard plots of the data obtained for native salivary proteins A and C (Figs. 4 and 5) are also nonlinear. Table I shows that both proteins have some binding sites (type I) with dissociation constants of about 45 µM and other sites (type II) with a dissociation constant of about 1100 µM. A statistical evaluation (Table II) demonstrated that the number of both type I and type II binding sites (Table I) is larger in peptide TX than either salivary proteins A or C. A comparison of salivary proteins A and C shows that the number of type I binding sites is significantly smaller in salivary protein C than A, but no difference can be detected in the number of type II sites.

No significant difference could be detected in the dissociation constant for type I sites in peptides TX and salivary proteins A and C. In contrast, the dissociation constant for type II sites was significantly smaller for peptide TX than either salivary protein A or C, but the differences in the dissociation constants between salivary proteins A and C were not significant. Dephosphorylation of salivary protein A gave rise to a nonlinear Scatchard plot of the calcium binding data (Fig. 6). Compared to native protein A (Tables I and II), there is no significant change in the number of either type I or type II binding sites, but there is a significant increase in the dissociation constant for both types of sites as a result of dephosphorylation. Dephosphorylated protein C was the only one of the proteins for which there was no clear evidence of a curved Scatchard plot of the binding data (Fig. 7). These results were therefore analyzed on the premise that only one type of binding site was present. The results showed that the total amount of bound calcium decreased in the dephosphorylated protein and the apparent dissociation constant was in between the values of those of the native protein.

In general, there was good agreement between the data obtained for $n$ and $K$ from the computer evaluation and those calculated from the Scatchard plots which have been listed in brackets in Table I. The largest deviations were seen in the magnitude of $K$ for type II sites.

The position of the NMR signal of the βH₂ in aspartic acid of salivary proteins A and C as a function of pH is illustrated in Figs. 8 and 9. The apparent pK values have been given in Table III. In the absence of calcium, pK is 4.27 in salivary proteins A and C. If the titration is performed in 50 mM CaCl₂
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The maximum amount of bound Ca(n) ± S.D. and the apparent dissociation constant (K) ± S.D. as calculated by nonlinear regression computer analysis have been listed. The values in brackets of n or K were calculated by resolving the Scatchard plots as described by Rosenthal (1967). The molecular weights used in calculation of n were determined from sequence analysis (Wong and Bennick, 1980) to be: TX 3540, salivary protein A 11145, salivary protein C 15827.

### Table I

**Calcium binding to salivary proteins A and C and their derivatives**

| Polypeptide | Type I site | Type II site | Total |
|-------------|-------------|--------------|-------|
|             | n         | K            | n    | K         | mol/mol | μM | n    | K         | mol/mol | μM |
| TX          | 2.56 ± 1.03 (2.34) | 38 ± 27 (37) | 5.31 ± 0.84 (4.64) | 784 ± 363 (546) | 7.87 (6.98) |
| Native protein A | 1.49 ± 1.17 (1.58) | 47 ± 68 (65) | 3.89 ± 0.90 (3.85) | 1118 ± 850 (863) | 5.38 (5.43) |
| Native protein C* | 0.64 ± 0.28 (0.50) | 44 ± 30 (39) | 3.81 ± 0.22 (3.09) | 1076 ± 329 (671) | 4.45 (3.59) |
| Dephosphorylated protein A* | 1.08 ± 0.99 (1.00) | 158 ± 192 (188) | 3.42 ± 0.86 (3.17) | 3038 ± 3901 (2219) | 4.51 (4.20) |
| Dephosphorylated protein C | 1.38 ± 0.19 (1.31) | 379 ± 227 (394) | 1.38 (1.31) |

*These results were calculated after eliminating the 3 values in protein C and 5 values in dephosphorylated protein A which in the initial nonlinear regression analysis showed the largest deviation from the computer calculated value.

### Table II

**Statistical analysis of calcium binding parameters in salivary proteins A and C and their derivatives**

For explanation of n and K, see Table I. The values of n or K in salivary proteins A and C and dephosphorylated salivary protein A were compared with the corresponding parameter in salivary protein A or peptide TX by the Student's t test. The percentage level of significance is given in the table. n.s., not significant.

| Protein | n | K | n | K | n | K |
|---------|---|---|---|---|---|---|
| TX      | 0.1 | n.s. | 0.1 | n.s. | 0.1 | 0.5 |
| Site 1  | 0.1 | 2.5 | 0.1 | 0.5 |
| Site 2  | 0.5 | n.s. | n.s. | 0.5 |
| Protein A | Site 1 | 0.5 | n.s. | n.s. | 0.5 |
| Site 2  | 0.5 | n.s. | n.s. | 0.5 |

### Fig. 6

Scatchard plot of the calcium binding data obtained for dephosphorylated protein A.

### Fig. 7

Scatchard plot of the data obtained for calcium binding to dephosphorylated protein C.

### Fig. 8

Variation in the proton NMR spectral position of the Hβ resonance in the aspartic acid (Hβ, ASP) in protein A as a function of pH. The distance of Hβ, ASP from the reference acetone resonance has been plotted in the absence (V — V) and presence (C — C) of 50 mM CaCl₂.

in a solution containing 10 mM NaCl, the pK decreases to 3.78 in salivary protein A and to 4.08 in salivary protein C. Analysis of the change in chemical shift as a function of the concentration of protons by means of a Hill plot demonstrated that the Hill coefficient was 0.93 in the case of protein A, but 0.83 for protein C. In the presence of calcium there is a small, but consistent shift to a higher magnetic field of the βH2 resonance at the limit of the chemical shift obtained at higher pH.

Fig. 10 illustrates a typical 31P NMR spectra of protein A. The resonances of the two phosphoserines are resolved and fall within the range where such resonances have been observed in other proteins (Ho et al., 1969). The resonance at lower field strength was designated peak I and the other peak II. At the limits of the chemical shifts each signal is a single peak (Fig. 10a), but in the middle of the range of shift the upfield resonance (peak II) appears as a doublet (Fig. 10b). The reason for this is not clear, but it could be due to coupling to a proton outside the range of the broad band proton decoupler. The chemical shift measured in ppm downfield from phosphoric acid has been plotted as a function of pH for protein A in Fig. 11 and for protein C in Fig. 12.

The Hill coefficient as calculated from these plots varied between 0.92 and 1.07. This agrees well with the expected values of 1.0 from titration of a single proton.
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**TABLE III**

Apparent pK values of β-carboxyl groups of aspartic acids in salivary proteins A and C as measured by proton NMR

| Salivary protein | Concentration | Apparent pK |
|------------------|---------------|-------------|
|                  | NaCl mM | CaCl₂ mM |         |
| A                | 160       | 0         | 4.27     |
| A                | 10        | 50        | 3.78     |
| C                | 160       | 0         | 4.27     |
| C                | 10        | 50        | 4.08     |

**DISCUSSION**

The limited number of data in previous studies (Bennick, 1976; Bennick, 1977b) suggested the presence of a single type of binding site in salivary proteins A and C with an apparent dissociation constant of 200 μM. From the present studies, it is clear that both the native proteins and peptide TX contain two different classes of binding sites. From the available data it is not possible to determine if the sites are independent of each other or if there is negative cooperativity. Proteolytic cleavage of protein A or C modifies the calcium binding. The removal of peptide CTZ from salivary protein C, resulting in the formation of salivary protein A, causes an increase in the number of type I sites. Further cleavage of salivary protein A into peptide TX and TY causes an increase in the number of type I as well as type II sites and the affinity of calcium for the type II sites is increased. These results indicate that the COOH-terminal ends of salivary proteins A and C which by themselves cannot bind calcium cause an alteration in the number and nature of the calcium binding sites of the proteins. This could be due to conformational differences in the NH₂-terminal part of the proteins or to an ionic interaction such as salt bridge formation. The smaller number of type I sites in protein C compared to protein A could be due to such interactions of positive charges in the COOH-terminal end in

**Fig. 9.** Variation in the proton NMR spectral position of the βH resonance in aspartic acid (Hβ, ASP) in protein C as a function of pH. The distance of Hβ, ASP from the reference acetone resonance has been plotted in the absence (△—△) and the presence (□—□) of 50 mM CaCl₂.

**Fig. 10.** Phosphorous NMR spectra of protein A at pH 4.45 (A) and pH 6.03 (B). The measurements are in Hz from H₃PO₄. The resonances have been labeled I and II as indicated on the figure.

From the Hill plots, the pK for peak I in proteins A and C was found to be 6.51 and for peak II it was 6.56 in both proteins. In the presence of 50 mM CaCl₂ the pK values for the phosphoserines dropped to between 5.85 and 5.92.

It is noteworthy that in the presence of calcium the chemical shift of the deprotonated phosphoserine is displaced approximately 0.4 ppm upfield relative to the position in absence of calcium.

**Fig. 11.** The downfield chemical shift of the phosphorous resonances in protein A measured relative to H₃PO₄ plotted as a function of pH. □—□ indicates the values obtained for peak I and △—△ those obtained for peak II in the absence of calcium. ■—■ denotes the results for peak I and ▲—▲ those for peak II in the presence of 50 mM CaCl₂.

**Fig. 12.** The downfield chemical shift of the phosphorous resonances in protein C relative to H₃PO₄ plotted as a function of pH. For explanation of the legend see Fig. 11.
protein C with aspartic acid or phosphoserine which would be part of a calcium binding site in protein A. In that case, a
difference could be expected in the titration curves of the
serine phosphate or β-COOH in aspartic acid in salivary
proteins A and C.

The 31P NMR spectra clearly resolves the resonances from
the two phosphoserine residues. The environment of the
phosphoserines must therefore be different. This could, for
example, be due to a ring current shift because of interaction
of one of the phosphoserines with the phenylalanine or the
histidines. The fact that the pK values are practically identical
in the two proteins suggests that the environment of corre-
sponding phosphoserines in the two proteins is very similar or
identical. The lowering of the pK values of the phosphoserines
in the native proteins in the presence of calcium is indicative
of the residues being part of the calcium binding sites or that
they are necessary to maintain the conformation of the sites.
These changes in pK are identical in salivary proteins A and C
suggesting that the difference in calcium binding to the
proteins does not involve the phosphoserines.

No difference was observed in the apparent pK value for
the β-COOH in the aspartic acids in proteins A and C, but the
different negative values of the Hill coefficients are probably
due to variations in the pK values of individual carboxyl
groups. Because the amino acid sequences containing the
aspartic acids in proteins A and C are identical, the results
suggest that one or more aspartic acids occupying the same
position in the two proteins have different pK values. This
may at least partly explain the difference in calcium binding
to the two proteins. This suggestion is in agreement with the
observation that upon addition of calcium there is a larger
drop in the apparent pK value of the aspartic acid β-COOH
in protein A than protein C. This suggests that aspartic acid
is part of the type I site which is present in protein A, but
absent in protein C.

The necessity of the serine phosphates in calcium binding
is also demonstrated by the effect of dephosphorylation. The
serine phosphates are not uniquely associated with either type
I or type II binding sites. In that case, one of the two types of
sites should be absent in the dephosphorylated protein, but
the retained type of sites should have the same characteristics
as in the corresponding native protein.

Both the dephosphorylated proteins have binding sites with
a dissociation constant intermediate to those of sites I and II
in the native proteins. In addition, dephosphorylated salivary
protein A has binding sites with a dissociation constant ap-
proximately 3 times larger than those of type II sites in the
native proteins. Dephosphorylation of the proteins may cause
a conformational change or the serine phosphates may be part
of both types of binding sites. It is also possible that the
chemical shift changes observed in the native proteins upon
addition of calcium to the deprotonated aspartic acids and
phosphoserines are due to a conformational change.

Under ionic conditions which exist in saliva, calcium binding
to salivary proteins A and C has been demonstrated (Bennick
and Cannon, 1978) and it has been suggested that the proteins
may function to maintain the concentration of ionic calcium
in saliva (Bennick and Wong, 1979). If that is correct, the
presence of the two types of binding sites would extend the
effective range of the proteins. The concentration of ultrafiltrable
calcium in saliva varies from 0.64 mM to 0.96 mM (Gron,
1973). Under these conditions, the type II sites would be
partly occupied and an increase in the calcium concentration
in the fluid would be counteracted by binding of additional
calcium to the proteins. If the calcium concentration falls
below the exchange range of the type II sites, the calcium
exchange capability of the type I sites would become effective.

The biological advantage of having both salivary proteins
A and C present in the secretion is not clear at the moment
and it is not known if peptide TX, the most effective of these
calcium binding macromolecules, is present in the secretion
in vivo.

Since the calcium binding sites, the hydroxyapatite binding
site (Bennick et al., 1979), and the ability to inhibit hydroxy-
apatite formation (Hay and Moreno, 1979b) are located in the
TX peptide it would be important to investigate the relation-
ship of these sites to each other.

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