

Tryptic phosphopeptides from whole casein. II. Physicochemical properties related to the solubilization of calcium

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SUMMARY. Casein phosphopeptides (CPP) were produced by tryptic hydrolysis of sodium caseinate and further purified by precipitation and chromatography on QAE-Sephadex A-25. Their physico-chemical properties were compared with the properties of an enzymically dephosphorylated equivalent preparation (DPP). Binding of Ca^{2+} to the peptides was measured using a Ca selective electrode and was found to increase with pH and to show 1/1 stoichiometry $\text{Ca}/\text{P}_{\text{org}}$ in CPP at pH 6.5 and 7.6. Klotz plots indicated equivalent binding sites at these two pH values, but some heterogeneity was seen at pH 3.5. In contrast, DPP did not bind significant amounts of Ca^{2+} .

CPP effectively inhibited the formation of insoluble calcium phosphates at different Ca/P ratios. The effective CPP concentration was 10 mg/l and complete stability of calcium phosphate solutions was obtained at about 100 mg/l. This stabilizing effect was dependent on the presence of organic P.

Casein phosphopeptides are formed during *in vivo* digestion of casein and their role in controlling luminal concentrations of soluble Ca was emphasized by Lee *et al.* (1980). They can stabilize calcium phosphate solutions *in vitro* (Reeves & Latour, 1957), and interfere with the formation of amorphous or crystalline calcium phosphate. This allows high concentrations of Ca^{2+} to exist in the presence of inorganic phosphate (Gerber & Jost, 1986).

We have produced tryptic phosphopeptides from whole casein (Juillerat *et al.* 1989) and studied their Ca binding properties as well as their power of inhibiting the formation of insoluble calcium phosphates, the specific role of peptide-linked phosphoester groups being examined by comparison with an enzymically dephosphorylated peptide preparation.

MATERIAL AND METHODS

A pool of casein phosphopeptides (CPP) and a phosphopeptide fraction (PPF) mainly composed of fragments $\beta\text{-CN}(1-28):4\text{P}$, $\beta\text{-CN}(2-28):4\text{P}$ and $\alpha_{\text{S1}}\text{-CN}(59-79):5\text{P}$, were prepared as described by Juillerat *et al.* (1989). Dephosphorylated peptides (DPP) were obtained by enzymic hydrolysis of CPP with potato acid phosphatase EC 3.1.3.2, grade II from Boehringer Mannheim GmbH, West Germany, according to Gerber & Jost (1986).

$^{45}\text{CaCl}_2$ was a product from Amersham Chemicals, U.K.

Calcium binding experiments

The Ca^{2+} -binding isotherms for each fraction CPP, PPF and DPP were obtained from measurements of free Ca^{2+} with a Ca selective electrode, Phillips IS 561, connected to an Orion EA940 IonAnalyzer. Solutions (50 ml) containing 100 mg of peptide, as the Na salt, and 186.4 mg of KCl to adjust the ionic strength were titrated with 0.1 M- CaCl_2 . The pH was kept constant at the desired value by addition of 0.25 M- NaOH or 0.25 M- HCl , and the temperature was maintained at 25 °C. Free Ca^{2+} was measured after each 50 μl addition of CaCl_2 solution, and the concentrations of total Ca^{2+} and peptide were corrected for dilution. The bound Ca^{2+} per mole of ligand was calculated by subtracting the free Ca^{2+} from the total Ca^{2+} , and dividing by the ligand concentration, using an average molecular weight of 2000 Daltons. The peptides were named respectively CPP2000 or DPP2000, for intact and dephosphorylated phosphopeptides. Alternatively, the bound Ca^{2+} was calculated on the basis of the organic phosphorus content of CPP and PPF.

The apparent association constants (K_{app}) and the maximum Ca bound per mole of ligand were calculated using a standard Klotz plot analysis (Regenstein & Regenstein, 1984).

Potentiometric titration during apatite precipitation

Solutions containing 0.008 M- NaH_2PO_4 and 0.008 M- CaCl_2 and either 0, 0.1 or 0.2 g/l CPP or 0.53 g/l of DPP were titrated in a thermostatted reactor with 0.1 M- NaOH , at 25 °C. The pH was maintained at 7.4 using a pH-stat unit Radiometer RTS 822 equipped with an autoburette Radiometer ABU 80, and a Radiometer REA160 recorder.

Calcium phosphate precipitation at variable Ca/P molar ratio

Formation and precipitation of insoluble calcium phosphate, and its inhibition by phosphopeptides was also measured with a ^{45}Ca isotope technique. CaCl_2 solutions and NaH_2PO_4 solutions in Tris-HCl buffer 0.05 M, pH 7.5 and 0.1 M in KCl were mixed to give different Ca/P molar ratios in the range 0.8–2.0, at a constant molar product $\text{Ca} \times \text{P}$ of 20 mM^2 . Phosphopeptides were added at concentrations ranging from 0 to 500 mg/l. The solutions were traced with ^{45}Ca , 30000 dpm/ml.

Portions (2 ml) of each solution were incubated in presence or absence of CPP at 40 °C for 30 min in a water bath. After rapid cooling of the tubes in an ice bath, precipitated calcium phosphate was removed by filtration through disposable ACRO LC 13 filters of 0.45 micron pore size (Gelman Sciences Inc. Ann Arbor, USA). Each filtrate (1 ml) was mixed in a scintillation vial with 1 ml of EDTA solution (0.01 M in Tris buffer) and 15 ml of scintillator (Optifluor, United Technology Packard, Packard Instruments International, Zürich), and was counted using a Searle Mark IV instrument.

Total, inorganic and derived organic phosphorus content of peptides was determined (Juillerat *et al.* to be published).

RESULTS

Calcium binding

The binding isotherms obtained for the system Ca-CPP2000 showed that bound Ca^{2+} increased towards a plateau as the free Ca^{2+} concentration was increased. DPP2000 did not bind Ca^{2+} to any significant extent (Fig. 1). The Klotz plots

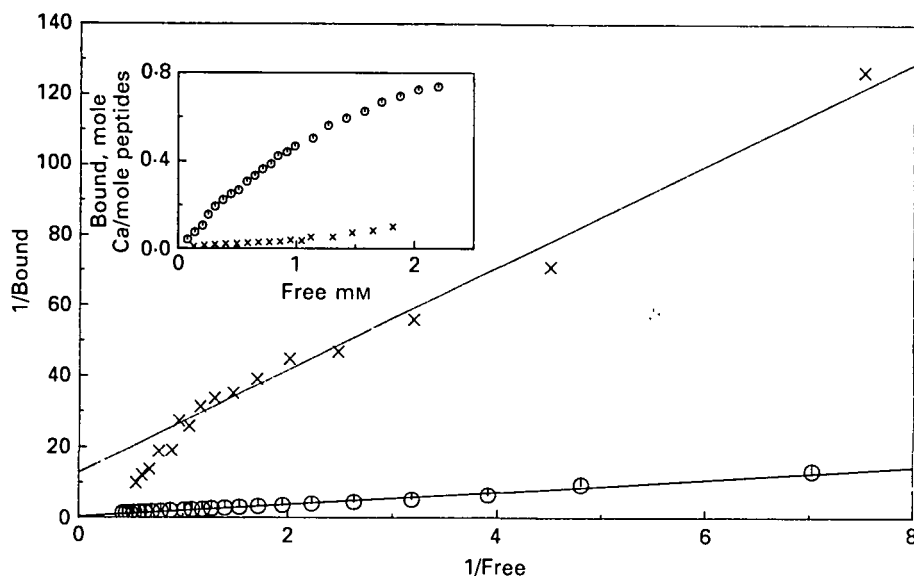


Fig. 1. Klotz plots and binding isotherms of Ca^{2+} to CPP (O) and DPP (X) at pH 6.5, 25 °C and an ionic strength of 0.1 M (KCl), using an assumed molecular weight of 2000 Daltons for the peptides.

Table 1. Apparent binding constants (K_{app}) and maximum bound Ca obtained for CPP, DPP and PPF from Klotz plot analysis

	pH	Bound Ca mole/mole	K_{app} l/mole
CPP2000*	6.5	3.0	189
DPP2000*	6.5	0.08	871
CPP†	6.5	0.81	465
PPF†	3.5	0.11	662
PPF†	6.5	0.91	640
PPF†	7.6	0.96	2060

* Per mole of peptide, molecular weight 2000.

† Per mole of organic phosphate.

calculated from these two isotherms are shown in Fig. 1. The system Ca-CPP2000 gave a straight line indicating a defined number of equivalent binding sites showing a low affinity for Ca^{2+} , and a maximum bound Ca^{2+} of 3 mole/mole of CPP2000. The result was different for the pair Ca-DPP2000, where the Klotz plot was not linear suggesting heterogeneous binding sites. The maximum bound Ca^{2+} and the apparent association constant (K_{app}), calculated from the first part of the binding isotherm, up to a 50% saturation are shown in Table 1. The data for Ca^{2+} binding to CPP were also analysed according to Klotz on the basis of their organic phosphorus content (P_{org}). Again, a straight line was found indicating the equivalence of all the phosphate groups present in CPP (data not shown). The maximum amounts of bound Ca^{2+} and the corresponding K_{app} are shown in Table 1.

The same determination was undertaken with the more homogeneous phosphopeptide fraction PPF. All three phosphopeptides contained in this fraction share the same highly negative charged subsequence: Glu-SerP-Leu-SerP-SerP-SerP-Glu-Glu. Binding isotherms expressed on the basis of the P_{org} of PPF and determined at

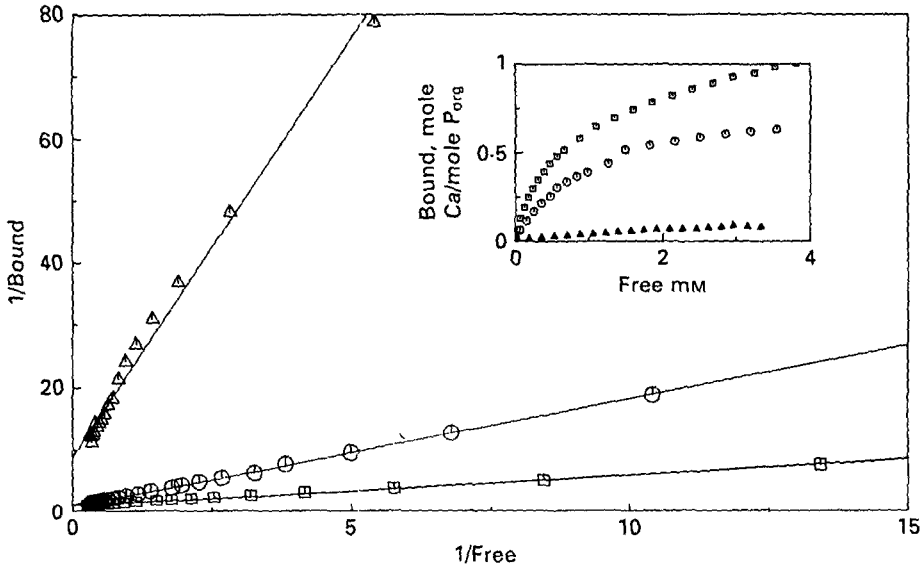


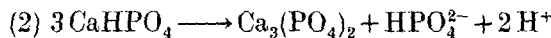
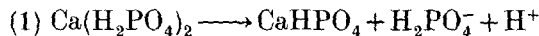
Fig. 2. Klotz plots and binding isotherms of Ca to PPF, expressed on the basis of their P_{org} , at the pH values 3.5 (Δ), 6.5 (\circ) and 7.6 (\square). Ionic strength set at 0.1 M with KCl and temperature at 25 °C.

three different pH values 3.5, 6.5 and 7.6, are shown in Fig. 2. As expected, the bound Ca^{2+} increased when the pH was raised.

The Klotz plots derived from the isotherms again gave straight lines at the pH values of 6.5 and 7.6, indicating the equivalence of all phosphate groups at these two pH values (Fig. 2) and with a stoichiometry Ca/P_{org} of practically 1/1. A slight deviation from the straight line was obtained at pH 3.5, possibly due to the low degree of dissociation of phosphate groups compared with that of carboxyl groups. Nevertheless, the linear regression coefficient obtained for the Klotz plot was still 0.989 and therefore all the data points were used for the calculations of the maximum bound Ca and the K_{app} (Table 1). The K_{app} obtained at pH values 3.5 and 6.5 were practically identical, whereas at pH 7.6 an unexplained three-fold increase of K_{app} was measured (Table 1).

Inhibition of apatite precipitation

The spontaneous transformations of calcium phosphate solutions according to the two following reactions,



both liberate H^+ ions. It is therefore possible to monitor indirectly the effect of CPP on these reactions using a pH-stat. Fig. 3 shows the inhibition of these transformations by CPP. At a molar product $Ca \times P$ of 64 mM^2 and at a molar ratio Ca/P of 1, the inhibition depends on the concentration of CPP present in solution. 0.1 g CPP/l (corresponding to 3 mg P_{org}) just delays the precipitation, while a CPP concentration of 0.2 g/l completely prevents the precipitation of calcium phosphate salts. For comparison, the same measurement was made with 0.53 g/l of DPP, which

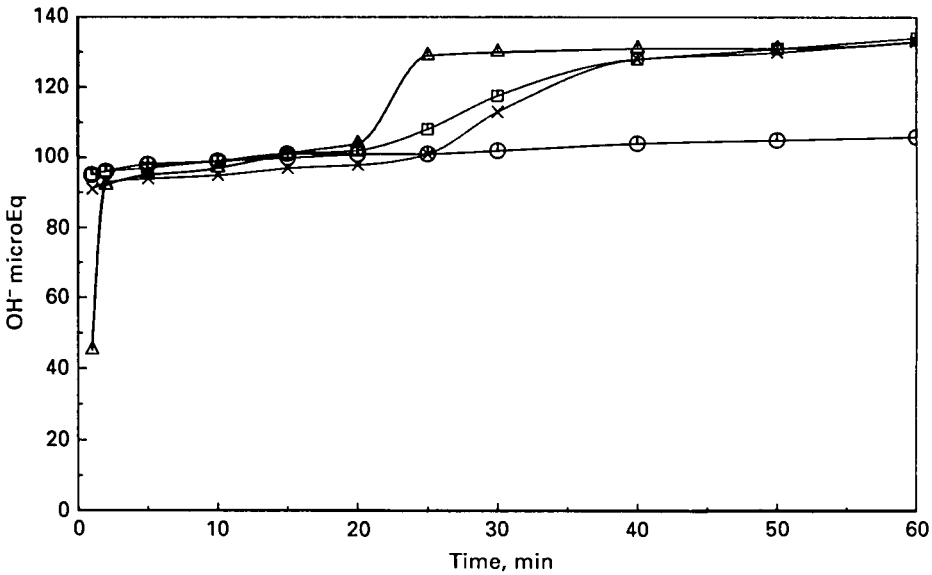


Fig. 3. Inhibition of calcium phosphate precipitation from a solution of 0.008 M-CaCl₂ and NaH₂PO₄ as measured by titration with 0.1 M-NaOH to pH at 7.4, at a temperature of 25 °C. Control (no peptides) (Δ); CPP 0.1 g/l (□); CPP 0.2 g/l (○); DPP 0.53 g/l (×).

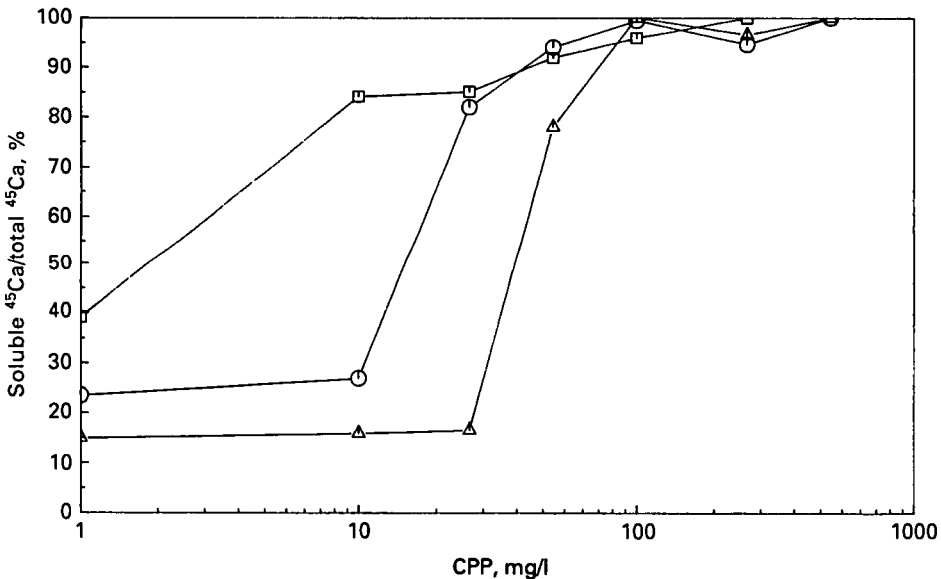


Fig. 4. Effect of CPP concentration on calcium phosphate solubility ($\text{Ca} \times \text{P} = 20 \text{ mM}^2$) at molar Ca/P ratios of 0.8 (Δ); 1.25 (○) and 1.95 (□). Further conditions were: 40 °C for 30 min, at pH 7.4 and an ionic strength of 0.1 M; tracing with 30000 dpm/ml of ⁴⁵CaCl₂.

even at the higher concentration, was unable to prevent the precipitation of calcium phosphates (Fig. 3).

CPP were shown to inhibit formation of insoluble calcium phosphate, as measured by the loss of soluble ⁴⁵Ca during incubation of calcium phosphate solutions at 40 °C. All solutions with molar products $\text{Ca} \times \text{P}$ higher or equal to 16 mM²

lost up to 80% of their Ca^{2+} by precipitation within 20 min of incubation. CPP at concentrations of 10 mg/l or higher inhibited this precipitation reaction effectively. Depending on the Ca/P molar ratio, different dose-response curves were obtained (Fig. 4). In our experimental conditions, complete stability was achieved with 100 mg/l of CPP. DPP were ineffective, even at concentrations higher than 100 mg/l.

DISCUSSION AND CONCLUSIONS

The results on Ca^{2+} -binding (Table 1), show that CPP2000 binds Ca weakly, with a K_{app} equal to 185 l/mole, lower than the first binding constant of 398 l/mole for α_{s1} -casein (Sillen & Martel, 1964) but very close to the 200 l/mole reported by the same authors for the system Ca-phosphoserine. Also, the binding constants of 640 l/mole and 465 l/mole determined at pH 6.5 for Ca-PPF and Ca-CPP respectively, on the basis of their P_{org} , can also be compared to the average binding constant of 574 and 519 l/mole reported by Parker & Dalgleish (1980) for Ca^{2+} binding to phosphoserine residues of α_{s1} - and β -casein at pH 7 and 20 °C. This observation suggests that the phosphoserine residues in caseins and the derived CPP and PPF are similarly accessible. Our results show clearly that the binding sites for Ca^{2+} are essentially the phosphoserine residues. They all appear equivalent and the stoichiometry Ca/ P_{org} is approximately 1/1 at pH 6.5 and 7.6. The Klotz plot obtained at pH 3.5 may suggest more than one class of binding sites, but the phosphoserine residues are preferentially filled. pH affects Ca^{2+} binding to phosphopeptides similarly to its effect on caseins (Dickson & Perkins, 1971), the lower stoichiometry found at pH 3.5 being related to the low extent of dissociation of phosphoserine residues at this pH. On the other hand, the carboxyl groups seem unable to bind a significant amount of Ca, since the Ca bound by DPP2000 is very low, and the calculated K_{app} has not perhaps any real physical significance.

The ability of 0.2 g/l of CPP to prevent the precipitation of 0.008 M of calcium phosphate salts, and the ineffectiveness of 0.53 g/l of DPP, confirm the essential role played by the phosphoserine groups in Ca^{2+} -phosphopeptide interactions. The mechanism by which CPP can maintain high concentrations of soluble calcium in solution, even in presence of high concentrations of inorganic phosphate is not yet fully understood. Nevertheless, the stabilizing effect depends on both the molar ratio Ca/P and the CPP concentration present in solution (Fig. 4). Our results support the work previously reported by Reeves & Latour (1958) and confirm those reported by Gerber and Jost (1986), showing the same phenomenon in an *in vitro* model. Furthermore, it may explain the observations of Naito *et al.* (1972) and Lee *et al.* (1980) who noted the ability of phosphopeptides detected *in vivo*, in rats on a casein diet, to increase luminal Ca concentration.

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