Physicochemical Characterization of Casein Phosphopeptide-Amorphous Calcium Phosphate Nanocomplexes*

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Management of tooth surface lesions in the oral cavity is of extreme importance in human health. The process of remineralization, which involves the absorption of calcium and phosphate into tooth enamel, is dependent upon the delivery of these ions in a bioavailable form. Casein micelles, which are the primary transporters of calcium and phosphate in milk, provide an easy means of delivering calcium and phosphorus to the neonate. The ability of casein micelles to maintain calcium and phosphate ions in solution is due to the presence of phosphopeptides that stabilize the calcium phosphate phase by electrostatic interactions (1). These phosphopeptides are derived from the casein molecules by endogenous tryptic digestion; the predominant tryptic phosphopeptides of caseins are: (sequence 1 below) with smaller amounts of (sequence 2 below) and (sequence 3 below) (4). The caseins resemble soap micelles in that they form a "hairy layer" that sterically stabilizes the complexes (8). The casein micelles serve as a carrier of calcium phosphate providing the neonate with a bioavailable source of calcium and phosphate ions for bone and teeth formation (3). It has been postulated that the ability of casein to form stable complexes with calcium phosphate is intrinsic to a general mechanism for avoiding pathological calcification and regulating calcium flow in tissues and biological fluids containing high concentrations of calcium (11).

Many techniques have been used to investigate the ultrastructure of the casein micelles. Although the structural details are still being elucidated, the casein micelles are believed to be roughly spherical particles with a radius of ~100 nm, dispersed in a continuous phase of water, salt, lactose, and whey proteins (4). The calcium phosphate isolated after exhaustive hydrazine deproteinization of micelles has been reported to exhibit a fine and uniform granularity under the electron microscope with the particles consisting of small subunits of 2.5-5 nm diameter (5, 6). The calcium phosphate, present as nanometer-sized ion clusters, and caseins are not covalently bound; hence the casein micelle is known as an association colloid (7). Nevertheless, the casein micelles are extremely stable and can withstand boiling, freeze-drying, and the addition of salt and ethanol. It is believed that the amphipathic, glycosylated C-terminal end of k-casein protrudes from the micelle surface forming a so-called "hairy layer" that sterically stabilizes the complexes (8).

The literature on casein interactions has been reviewed by Horne (9), and a model of the casein micelle has been formulated that accounts for many of the physicochemical properties of the micelle. The model involves electrostatic interactions between colloidal calcium phosphate particles and multiple α- and β-casein molecules and hydrophobic interactions between the α-, β-, and k-caseins forming a cross-linked network (9). Electron microscopy of casein micelles (10) has provided evidence that the caseins are organized into tubular structures within the micelle.

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The ability of casein micelles to maintain calcium and phosphate ions in a soluble and bioavailable state is retained by the tryptic multiphosphorylated peptides of the caseins known as the casein phosphopeptides (CPP) (4). The major tryptic CPP are β-CN(1–25) (sequence 1 below) and αs1-CN(59–79) (sequence 2 below) with smaller amounts of αs2-CN(46–70) (sequence 3 below) and αs2-CN(1–21) (sequence 4 below) (12, 14).

Phosphopeptides from bovine casein can be isolated using a number of methods including selective acidification, tryptic digestion, and binding to phosphopeptide affinity columns. Application of these phosphopeptide isolates to studies of calcium binding and calcium phosphate stabilization by casein phosphopeptides has revealed that although the fully phosphorylated seryl-cluster motif Ser(P) 3-Glu2, that is involved in the interaction with calcium have a number of Ser(P) residues in a specific motif, Ser(P) 3-Glu2, that is involved in the interaction with calcium phosphate (3).

Bovine milk contains ~30 mM calcium and 22 mM inorganic phosphate in solution with most of the calcium (68%) and phosphate (47%) associated with the proteins αs1-, αs2-, β-, and k-casein in casein micelles (1, 2). The αs1-, αs2-, and β-caseins have a number of Ser(P) residues in a specific motif, Ser(P) 3-Glu2, that is involved in the interaction with calcium phosphate (3).

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† The abbreviations used are: CPP, casein phosphopeptide(s); ACP, amorphous calcium phosphate; CN, casein; DCPD, dicalcium phosphate dihydrate; HA, hydroxyapatite; OCP, octacalcium phosphate; SEM, scanning electron microscopy; TEM, transmission electron microscopy; HPLC, high performance liquid chromatography.
casein tryptic phosphopeptides are shown with the motif underlined: sequence 1 (β-CN(1–25)), Arg-Glu-Leu-Glu-Leu-Leu-Asn-Val-Pro-Glu-Gly-Ile-Val-Glu-Ser(P)L-Leu-Ser(P)5-Glu2-Ser(Ile-Thr-Arg)3; sequence 2 (αS1-CN(59–79)), Glu-Met-Ala-Glu-Ser(P)2-Ile-Ser(P)2-Val-Ile-Pro-Asn-Ser(P)6-Val-Glu-Gln-Lys2; sequence 3 (αS2-CN(46–70)), Asn-Ala-Asn-Glu-Glu-Tyr-Ser-Ile-Gly-Ser(P)2-Glu2-Ser(Ile-Ala-Val-Thr-Glu-Glu-Val-Lys)2; and sequence 4 (αS2-CN(1–21)), Lys1-Asn-Thr-Met-Glu-His-Val-Ser(P)2-Glu2-Ser(Ile-Ser(P)7-Glu-NHMe-Thr-Lys)3.

The CPP stabilize calcium and phosphonate ions under neutral and alkaline conditions forming metastable solutions that are supersaturated with respect to the basic calcium phosphate phases (15). Under these conditions, the CPP bind their equivalent weights of calcium and phosphate (16). The CPP are formed in vivo by normal digestion of casein and, because they are relatively resistant to further proteolytic degradation, accumulate in the distal portion of the small intestine (17–21). It has been proposed that this accumulation together with the ability of the peptides to form soluble complexes with calcium phosphate are responsible for the enhanced intestinal calcium absorption that has been observed in vitamin D-deficient animals consuming dietary CPP (17–21). In addition, CPP increase the calcification of embryonic rat bone, and again the mechanism is suggested to be associated with the ability of the peptide to form soluble complexes with calcium and phosphate ions (22). Furthermore, CPP-calcium phosphate complexes have been shown to be antienzymic and to remineralize early stages of enamel caries in animal and human studies (12, 15, 23–25). In summary, the ability to stabilize calcium phosphate and thereby enhance mineral solubility and bioavailability (26) confers upon the CPP the potential to be biological delivery vehicles for calcium and phosphate (27).

As part of our long-term investigation into the structure-function relationships of proteins involved in biomineralization and calcium phosphate stabilization, we have studied the interaction of tryptic phosphopeptides from milk caseins with the amorphous and crystalline phases of calcium phosphate. In this paper, we report our investigations of the calcium and phosphate binding properties of the two major CPP, β-CN(1–25) and αS1-CN(59–79). We demonstrate that the experimentally determined ion activity product of a basic amorphous calcium phosphate (ACP) phase best correlates with the calcium bound by the peptide αS1-CN(59–79) over a range of calcium and phosphate concentrations and sample pH ranging from 7.0 to 9.0. Furthermore, we delineate the regions and residues of these peptides that are responsible for calcium phosphate stabilization. We report the effect of peptide length, residue type, and order of acidic residues on the binding to calcium and calcium phosphate using a library of synthetic analogues. Finally, we describe the ultrastructure of the casein phosphopeptide-calcium phosphate nanocomplex as determined using a range of physicochemical techniques including powder diffraction x-ray crystallography, scanning electron microscopy (SEM), and transmission electron microscopy (TEM).

**EXPERIMENTAL PROCEDURES**

**Preparation of Casein Phosphopeptides**—The casein phosphopeptides β-CN(1–25) and αS1-CN(59–79) were selectively precipitated from a tryptic digest of casein using calcium chloride and ethanol and further purified by union exchange fast protein liquid chromatography and reversed phase HPLC (13). The purity of the peptides was assessed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry, capillary electrophoresis, amino acid composition, and sequence analyses (13, 14). Prior to sequence analysis, the labile phosphoryl residues were converted to S-ethyl cysteinyl residues by β-elimination (13).

**Preparation of Synthetic Peptides**—The peptide Ac-Glu-Ser(P)-Ile-Ser(P)-Glu2-NHMe corresponding to αS1-CN(63–70) was prepared by the use of Boc-Ser(P,PO3)2-OH in the Boc mode of peptide synthesis followed by platinum-catalyzed hydrogenolytic deprotection of the protected Ser(PO3)2-containing peptides (14, 25, 29). The synthetic peptides were capped with an acyl group at the N terminus and a methylamine group at the C terminus. In the case of Ac-Ile-Val-Pro-Asn-Ser(P)-Val-Glu-Glu-NHMe, a homologue of αS1-CN(71–78), a glutamyl residue was substituted for Glu29 to avoid a problematic synthesis associated with a C-terminal glutamate residue. The preparations of the other synthetic analogues have been described previously (29–31). The peptides were purified by reversed phase HPLC, and the purity was confirmed by capillary electrophoresis, amino acid composition, sequence analysis, mass spectrometry, and NMR spectroscopy (13, 14, 23, 28–31).

**Calcium Binding to αS1-CN(59–79), β-CN(1–25), αS2-CN(63–70), and αS2-CN(71–78)**—All calcium binding experiments were performed using a modification of the ultrafiltration method of Marsh (32) without the addition of phosphate. The solutions were buffered using 100 mM Tris-HCl to a pH value of 7.0, 7.5, 8.0, 8.5, or 9.0. Ionic strength was adjusted to 0.16–0.19 using NaCl. The reaction mixtures were incubated at room temperature for 18 h. Subsequently, less than 10% of the total volume was collected as ultrafiltrate by centrifugation at 1000 × g for 15 min using Centrifree MPS-1 Microparticelation cells (Amicon) equipped with YM-3 (3000 molecular weight exclusion limit) or YM-1 (1000 molecular weight exclusion limit) membranes. These membranes were demonstrated not to retain calcium or phosphate ions or ion pairs. Calcium ion concentrations of acidified samples were measured at 422.7 nm by atomic absorption spectroscopy using PerkinElmer instrument model 373, with the addition of 1% LaCl3 to prevent phosphate interference. Calcium concentrations ranged from 0.5 to 21.5 mM for αS1-CN(59–79) and β-CN(1–25), 0.3–5.5 mM Ca2+ for the αS1-CN(63–70) peptide, and 0.05–1.5 mM Ca2+ for the αS1-CN(71–78) peptide. Peptide concentrations of the original reaction mixture after centrifugation to demonstrate no precipitation and of the ultrafiltrate were determined, and the peptide-bound calcium was calculated as the difference between these values. Calcium binding by the peptides was modeled by assuming the number of independent ion-binding sites/peptide (νC), where each site has a dissociation constant (Kd) given by the following equation:

\[
K_d = \frac{[\text{Peptide}][\text{Ca}^{2+}]}{[\text{Peptide}][\text{Ca}^{2+}]} \quad \text{(Eq. 1)}
\]

The dissociation constants for the peptide/calcium complexes (Kd) and the number of calcium ion-binding sites/mol of peptide (νC) were determined by a nonlinear least squares fit to the equation,

\[
[\text{Ca}^{2+}]_{\text{bound}} = \frac{\nu_C[\text{Ca}^{2+}]_{\text{free}}}{K_d + [\text{Ca}^{2+}]_{\text{free}}} \quad \text{(Eq. 2)}
\]

where \( K_d = K_a C \).

**Calcium Phosphate Binding to αS1-CN(59–79) and β-CN(1–25)**—Homopolymers and Analogues—Binding was initiated by adding phosphate to solutions containing 14 mM calcium and 4 mg/ml peptide buffered using 100 mM Tris-HCl to a pH value of 7.0, 7.5, 8.0, 8.5, or 9.0. Sodium chloride was added to bring the ionic strength for each sample to 0.16. For the homopolymers and analogues, binding was determined at pH 7.0 or 9.0. The reaction mixtures were incubated and ultrafiltered as described above for calcium binding. The phosphate concentration ranged from 0 to 9 mM. Phosphate concentration was determined colorimetrically (33), with absorbance measured at 660 nm on a PerkinElmer 552 Spectrophotometer. Calcium and phosphate concentrations in the original solution and the ultrafiltrate were determined. The peptide-bound calcium and peptide-bound phosphate were taken as the difference between the total and free calcium and phosphate, respectively. To confirm that no precipitation had occurred during incubation, the samples were centrifuged at 17,000 × g for 5 min, and the calcium and phosphate concentrations were determined prior to ultrafiltration. Ultrafiltrates were examined by spectrophotometrically at 214 nm, and the samples were contained no peptide. Peptide-bound calcium phosphate is expressed as the number of calcium ions ([Ca2+]b) and phosphate ions ([Pi]b) mol of peptide. The ion activity products for various phases of calcium phosphate were determined from the free calcium and phosphate concentrations and pH using an iterative computational procedure that calculates the ion activity coefficients using the expanded Debye-Hückel equation. This procedure takes into account ion pairs CaHPO4 and H2PO4; and the dissociation of H3PO4 and H2O; and the ionic strength (32, 34). The activity of the CaOH– ion was explicitly assumed to be negligible. Dissociation constants at 37°C were used from the following sources: H2PO4− (35); HPO42− (36); CaH2PO4 and CaHPO4.
The number of phosphate binding sites was determined by nonlinear least squares fits to an equation of the form of Equation 2, in which bound and free phosphate were the variables. The value of \( K \) from these fits can be shown to be approximately given by the equation,

\[
K = \frac{K_{eq}}{K}
\]  
(\text{Eq. 4})

where \( K_p \) is the calcium ion binding constant for the peptide. The number of calcium binding sites was similarly determined using the equation,

\[
[Ca^{2+}]_{\text{bound}} = \frac{v_{Ca}[P]_{\text{free}}}{K} + [Ca^{2+}]_{\text{initial}}
\]  
(\text{Eq. 5})

where \([Ca^{2+}]_{\text{initial}}\) is the amount of calcium bound with no added phosphate.

Preparation of CPP-Calcium Phosphate—CPP was dissolved at 10 g/liter in Milli-Q water. 1.6 mM CaCl\(_2\) and 1 mM Na\(_2\)HPO\(_4\) were added slowly by syringe pump (0.5–1.0 mL/min) to the CPP solution in a pH-stat held at pH 9.0 by automatic titration of 5 N NaOH. After 60 min of titration the final calcium and inorganic phosphate concentration were 100 and 60 mM, respectively. The colloidal CPP-calcium phosphate nanocomplexes were then concentrated 5-fold by microfiltration through a 0.1-\(\mu\)m Sartorius filter (polysulphone) in a semi-Sarticon microfiltration system and washed with 3 volumes of Milli-Q water to remove free calcium and phosphate ions. The final CPP-calcium phosphate solutions were then lyophilized.

Preparation of \( \beta-CN(1–25) \)-Calcium Phosphate—The \( \beta-CN(1–25) \) phosphopeptide was dissolved at 1.67 g/liter in Milli-Q water. To the peptide solution, 50 mM CaCl\(_2\) and 21.3 mM Na\(_2\)HPO\(_4\) were added stepwise slowly. The pH was held at 9.0 by automatic titration of 50 mM NaOH. The final \( \beta-CN(1–25) \)-ACP solution was then lyophilized and then redissolved in H\(_2\)O.

Powder Diffraction of CPP-ACP—The powdered samples were mounted in an aluminum holder and were placed in a Sintag pad V x-ray diffractometer, operating with a Cu Ka filter \((\lambda = 1.5406 \text{ Å})\) x-ray source at 45 kV and 40 mA. The x-ray diffraction data were collected with a germanium solid state collector and scanned from 10° to 100° in 2° min \(^{-1} \) (step size 0.03°). Phase identification was accomplished by comparison of the x-ray diffraction data pattern with the JCPDS powder diffraction file, produced by the International Center for Diffraction Data.

SEM and TEM of CPP-ACP—The sample for TEM was prepared from powdered material that was crushed and placed on a 3-mm TEM grid. The TEM grids were covered with a Butvar/Formvar (ProSciTec, Thuringowa Central, Australia) film to support the powder. Transmission electron micrographs were taken on a 200-kV JEOL 2000FX instrument operating at 200 kV.

For SEM, specimens were sprinkled on an aluminum stub covered with adhesive conductive carbon tabs and gold sputter-coated (Gold Sputter coater S150B, Edwards, UK). Observations were made with a field emission SEM instrument (Philips XL 30 FEG, Eindhoven, The Netherlands) operating at 20 kV using the secondary electron mode.

**RESULTS**

Calcium Binding to \( \alpha_{s1}-CN(59–79), \beta-CN(1–25), \) and \( \alpha_{s2}-CN(63–70) \) and \( \alpha_{s2}-CN(71–78) \) in Figures 1 and 2 show the calcium-binding curves for (a) \( \alpha_{s1}-CN(59–79) \) and (b) \( \alpha_{s1}-CN(63–70) \) with the bound calcium/mole of peptide \([Ca^{2+}]_{\text{bound}}\) being plotted as a function of free calcium ion concentration. Table I summarizes the values of the dissociation constants and the number of calcium ion-binding sites/peptide from fitting the data for \( \alpha_{s1}-CN(59–79), \beta-CN(1–25), \) and \( \alpha_{s1}-CN(63–70) \) to Equation 2 using a nonlinear least squares algorithm. In the case of the C-terminal region \( \alpha_{s1}-CN(71–78) \) homologue containing only a single phosphoserine, no calcium binding was observed.

**FIG. 1. Calcium ion binding by casein phosphopeptides.** Titration of peptides with calcium ions. Bound calcium is plotted as a function of the free calcium ion concentration. Units of bound calcium are calcium ions bound/mole of peptide. (a) \( \alpha_{s1}-CN(59–79), \) (b) \( \beta-CN(1–25), \) and (c) Ac-Glu-Ser(P)-Ile-Ser(P)-Ser(P)-Ser(P)-Glu-Glu-NHMe, the synthetic octapeptide corresponding to \( \alpha_{s1}-CN(63–70) \). The solid curves are nonlinear least squares fits of the data to Equation 2.

**TABLE I**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>pH</th>
<th>( v_{Ca} )</th>
<th>( K_d )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \alpha_{s1}-Casein(63–70) )</td>
<td>8.00</td>
<td>3.52 ± 0.13</td>
<td>2.71 ± 0.18</td>
</tr>
<tr>
<td>( \alpha_{s1}-Casein(59–79) )</td>
<td>8.00</td>
<td>6.84 ± 0.45</td>
<td>5.07 ± 0.61</td>
</tr>
<tr>
<td>( \beta-Casein(1–25) )</td>
<td>8.00</td>
<td>4.64 ± 0.17</td>
<td>2.28 ± 0.25</td>
</tr>
</tbody>
</table>

\( v_{Ca} = \) number of calcium-binding sites/molecule of peptide.

Means ± S.D. (mM).

**Calcium Phosphate Binding to Amorphous Calcium Phosphate**—Fig. 2A shows a plot of bound phosphate \([P]_{\text{bound}}\) versus free phosphate \( (P)_\text{free}\). Nonlinear least squares fits were performed to determine the number of mol of calcium and phosphate bound and the effective dissociation constant \( K \). The fits to Equation 5 showed a strong correlation between the value of \([Ca^{2+}]_{\text{bound}}\) and the value of \( K \). If \([Ca^{2+}]_{\text{bound}}\) was not constrained, it adopted unreasonable values, and the value of \( K \) determined from fits to the phosphate data disagreed with those derived from the calcium data. Fixing \([Ca^{2+}]_{\text{bound}}\) at the experimental value of the bound calcium ion concentration at zero phosphate gave values of \( K \) in reasonable agreement with those derived from the phosphate data. The number of calcium ion-binding sites/mol of peptide \( v_{Ca} \) was not sensitive to the value of \([Ca^{2+}]_{\text{bound}}\). The values reported in Table II are appropriately weighted averages of the experimental parameters determined from repeated measurements. Fig. 2C, a plot of \([Ca^{2+}]_{\text{bound}}\) versus \([P]_{\text{bound}}\) for the samples at different pH, reveals a linear relationship between bound calcium and bound phosphate. This demonstrates that the binding of calcium ions and phosphate ions is co-dependent. The number of calcium phosphate binding sites/mol of phosphate shows a strong correlation with the length of the peptide as shown in Fig. 5.
of [Ca\(^{2+}\)]\(_{\text{bound}}\) versus [Pi]\(_{\text{bound}}\) increases as the peptides become shorter (Table II).

**Determination of the Calcium Phosphate Phase Stabilized by α\(_{\text{51}}\)-CN(59–79)**—To determine the calcium phosphate phase bound by α\(_{\text{51}}\)-CN(59–79), the binding of calcium phosphate was related to the ion activity products of the various biologically relevant calcium phosphate phases. The bound calcium/mol of peptide at various pH values was plotted as a function of the ion activity products of the following biologically relevant phases: DCPD (Ca\(^{2+}\)/(HPO\(_{4}\)^{2–})(H\(^{+}\)/(OH\(^{–}\))) (Fig. 3A) (42), OCP (Ca\(^{2+}\)(_4(H\(^{+}\)/(PO\(_{4}\)^{3–})(OH\(^{–}\))) (Fig. 3B), HA (Ca\(^{2+}\)(_5(PO\(_{4}\)^{3–})(OH\(^{–}\))) (Fig. 3C), and a basic ACP (Ca\(^{2+}\)(_3(PO\(_{4}\)^{3–})(OH\(^{–}\)))\(_z\), where \(z = 0.0877\) (Fig. 3D). Only the ACP phase produced a function that was independent of pH (Fig. 3D), indicating that this was the phase stabilized by α\(_{\text{51}}\)-CN(59–79). Two types of ACP were considered, acidic ACP phases represented by (Ca\(^{2+}\)(_z(PO\(_{4}\)^{3–})(OH\(^{–}\)))\(_z\), and basic ACP phases represented by (Ca\(^{2+}\)(_3(PO\(_{4}\)^{3–})(OH\(^{–}\)))\(_z\). These formulae are capable of representing a wide range of calcium phosphate compositions ranging from HA (\(z = \frac{1}{3}\)) in the basic range, to tricalcium phosphate (\(z = 0\)), OCP (\(z = \frac{2}{3}\)) and DCPD (\(z = 2\)) in the acidic range. To determine the optimum stoichiometry, ion activity products were calculated at a number of points in the interval \(z = 0.00\) to \(z = 0.20\). At each value of \(z\), the ion activity product was plotted as a function of the number of calcium-binding sites, and a quartic polynomial was fitted to the data. The correlation coefficients from these fits were then plotted against \(z\), and a quadratic polynomial fitted through these data points to determine the optimal value of \(z\). Fig. 4 shows the variation in the correlation coefficient as a function of \(z\) for the amorphous phases.

**Calcium Phosphate Binding to Homologues and Analogues**—In addition to β-CN(1–25) and α\(_{\text{51}}\)-CN(59–79), the calcium phosphate binding characteristics of a series of synthetic homologues and analogues were investigated as shown in Table II. The aim of this investigation was to delineate the residues important for calcium phosphate stabilization and to investigate the importance of phosphorylation, peptide length, residue type, and order. The synthetic peptides, Ile-Val-Pro-Asn-Ser(P)-Glu-Glu and Gln-Met-Glu-Ala-Glu, corresponding to the C and N termini of α\(_{\text{51}}\)-CN(59–79), respectively, did not bind calcium or stabilize calcium phosphate. The importance of the multiphosphorylated motif Glu-Ser(P)-Ile/Leu-Ser(P)\(_3\)-Glu\(_2\) was further investigated using a series of analogues. A plot of \(v_{\text{Ca}}\) versus the number of residues in the peptide is shown in Fig. 5, which produced a linear correlation coefficient of 0.9956.
Casein Phosphopeptide-Amorphous Calcium Phosphate

Table II
Calcium and phosphate binding characteristics of CPP and analogues

<table>
<thead>
<tr>
<th>Peptide</th>
<th>pH</th>
<th>Charge</th>
<th>$v_{Ca}^a$</th>
<th>$v_{Pi}^c$</th>
<th>$K_d$</th>
<th>Calcium:Pi</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha_{s_1}$-Casein(59–79)</td>
<td>7.0</td>
<td>13.5</td>
<td>16.86 ± 0.35</td>
<td>11.22 ± 0.56</td>
<td>1.63 ± 0.17</td>
<td>1.50 ± 0.11</td>
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<tr>
<td>7.5</td>
<td>13.85</td>
<td>23.2 ± 1.6</td>
<td>15.8 ± 1.1</td>
<td>0.74 ± 0.10</td>
<td>1.46 ± 0.20</td>
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</tr>
<tr>
<td>8.0</td>
<td>13.99</td>
<td>21.73 ± 0.68</td>
<td>14.26 ± 0.38</td>
<td>0.257 ± 0.021</td>
<td>1.524 ± 0.088</td>
<td></td>
</tr>
<tr>
<td>8.5</td>
<td>14.10</td>
<td>22.3 ± 2.3</td>
<td>14.3 ± 1.6</td>
<td>0.064 ± 0.020</td>
<td>1.56 ± 0.33</td>
<td></td>
</tr>
<tr>
<td>9.0</td>
<td>14.29</td>
<td>21.43 ± 0.39</td>
<td>14.65 ± 0.79</td>
<td>0.072 ± 0.016</td>
<td>1.52 ± 0.11</td>
<td></td>
</tr>
<tr>
<td>$\alpha_{s_1}$-Casein(59–79) N74D</td>
<td>7.0</td>
<td>-14.20</td>
<td>15.3 ± 1.5</td>
<td>10.32 ± 0.56</td>
<td>1.48 ± 0.22</td>
<td></td>
</tr>
<tr>
<td>9.0</td>
<td>-15.27</td>
<td>22.35 ± 0.33</td>
<td>14.25 ± 0.57</td>
<td>1.569 ± 0.086</td>
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</tr>
<tr>
<td>$\alpha_{s_1}$-Casein(63–70)</td>
<td>9.0</td>
<td>-11.00</td>
<td>9.64 ± 0.26</td>
<td>5.60 ± 0.14</td>
<td>0.132 ± 0.015</td>
<td>1.721 ± 0.089</td>
</tr>
<tr>
<td>(Ala-Ser(P)4-Ala-Glu)</td>
<td>9.0</td>
<td>-10.99</td>
<td>7.85 ± 0.22</td>
<td>5.11 ± 0.15</td>
<td>1.538 ± 0.090</td>
<td></td>
</tr>
<tr>
<td>$\alpha_{s_1}$-Casein(59–63)</td>
<td>9.0</td>
<td>-2.00</td>
<td>Precipitated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\alpha_{s_1}$-Casein(71–78)</td>
<td>9.0</td>
<td>-2.99</td>
<td>Precipitated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\beta$-Casein(1–25)</td>
<td>7.0</td>
<td>-12.61</td>
<td>15.65 ± 0.16</td>
<td>10.55 ± 0.98</td>
<td>1.48 ± 0.15</td>
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<tr>
<td>9.0</td>
<td>-13.26</td>
<td>24.76 ± 0.63</td>
<td>16.67 ± 0.47</td>
<td>1.485 ± 0.079</td>
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<tr>
<td>$\beta$-Casein(14–21)</td>
<td>9.0</td>
<td>-11.00</td>
<td>12.01 ± 0.30</td>
<td>6.35 ± 0.16</td>
<td>0.084 ± 0.024</td>
<td>1.891 ± 0.095</td>
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<td>Ser(P)2-Glu</td>
<td>9.0</td>
<td>-8.00</td>
<td>8.76 ± 0.19</td>
<td>4.004 ± 0.083</td>
<td>1.824 ± 0.071</td>
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<td>Thr(P)2-Glu</td>
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<td>-7.99</td>
<td>4.90 ± 0.10</td>
<td>2.69 ± 0.16</td>
<td>1.82 ± 0.16</td>
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<td>Ser-Thr(P)2-Glu</td>
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<td>1.861 ± 0.051</td>
<td>1.104 ± 0.061</td>
<td>1.69 ± 0.14</td>
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<td>1.16 ± 0.12</td>
<td>1.47 ± 0.22</td>
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<td>2.045 ± 0.029</td>
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<td>Asp-Ser(P)2-Glu</td>
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<td>-7.00</td>
<td>1.917 ± 0.088</td>
<td>0.992 ± 0.052</td>
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<td>(Ala-Ser(P)1-Ala-Glu)</td>
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<td>-9.00</td>
<td>4.948 ± 0.049</td>
<td>2.899 ± 0.065</td>
<td>1.670 ± 0.037</td>
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</tr>
<tr>
<td>(Ala-Ser(P)2-Ala-Glu)</td>
<td>9.0</td>
<td>-7.00</td>
<td>4.948 ± 0.049</td>
<td>2.899 ± 0.065</td>
<td>1.670 ± 0.037</td>
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</tr>
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<td>(Ala-Ser(P)3-Ala-Glu)</td>
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<td>-5.00</td>
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<tr>
<td>Al-Ser-Ala-Glu</td>
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$^a$ Charges calculated using the $pK_a$ parameters listed in Bundi and Withrich (46) and Bienkiewicz and Lumb (47).

$^b$ $v_{Ca}$ = number Ca$^{2+}$-binding sites/mol of peptide.

$^c$ $v_{Pi}$ = number Pi-binding sites/mol of peptide.

$^d$ Mean ± S.D. (mm).

$^e$ E = Glu, I = Ile, O = Thr(P).

Fig. 2. pH dependence of ion activity products of calcium phosphate phases and peptide-bound calcium.

The number of mol of bound calcium at various pH values plotted as a function of the activity product of the DCPD phase (Ca$^{2+}$)($HPO_4^{2-}$) (A), the OCP phase (Ca$^{2+}$)($PO_4^{3-}$)($H^+$) (B), the HA phase (Ca$^{2+}$)($H_2PO_4^{-}$)($OH^-$) (C), and the basic ACP phase (Ca$^{2+}$)$_{0.0877}$($PO_4^{3-}$)$_2$($OH^-$)$_{0.0774}$ (D). The curves in A–C are interpolated curves using the method of Stineman (42) provided as an aid to visualization and to emphasize that a single function does not relate the bound calcium to the particular ion activity product. The curve in D is a quartic polynomial fit to all the data points.
The substitution of any of the Ser(P) residues by Ser, Glu, or Asp residues in the peptide Ser(P)3-Glu or the substitution of Thr(P) residues by Thr in the Thr(P)3-Glu peptide substantially reduced the peptides ability to stabilize calcium phosphate. Further, the substitution of Ser(P) by Thr(P) was also associated with a reduced ability to stabilize calcium phosphate. Three contiguous phosphoseryl residues were necessary for full stabilization of calcium phosphate, because the (Ala-Ser(P))4 peptide, although containing the excess calcium bound substantially bound 6.84 ± 0.45 mol of Ca2+/mol of peptide, whereas the (Ala-Thr(P))4 peptide bound 3.52 ± 0.13 mol of Ca2+/mol of peptide. All of the measurements were performed at pH 8. The number of calcium ion-binding sites (νCa) appears to be determined by charge for the longer peptides, the amount of calcium bound being sufficient to nearly cancel the intrinsic negative charge of the peptides.

The charge on the peptide α51-CN(63–70) is only slightly lower than that on the other two peptides, and yet the amount of calcium bound is significantly lower than would be expected based on charge neutralization. A structural explanation appears to be the most plausible for the markedly lower than expected calcium binding ability of the shorter peptide. Inspection of calcium-binding proteins whose structures have been deposited in the Protein Data Bank suggests that calcium ions

DISCUSSION

Calcium Binding—As shown in Table I, α51-CN(59–79) maximally bound 6.84 ± 0.45 mol of Ca2+/mol of peptide, whereas β-CN(1–25) bound 4.64 ± 0.17 mol of Ca2+/mol of peptide. The shorter peptide α51-CN(63–70) bound 3.52 ± 0.13 mol of Ca2+/mol of peptide. All of the measurements were performed at pH 8. The number of calcium ion-binding sites (νCa) appears to be determined by charge for the longer peptides, the amount of calcium bound being sufficient to nearly cancel the intrinsic negative charge of the peptides.

The charge on the peptide α51-CN(63–70) is only slightly lower than that on the other two peptides, and yet the amount of calcium bound is significantly lower than would be expected based on charge neutralization. A structural explanation appears to be the most plausible for the markedly lower than expected calcium binding ability of the shorter peptide. Inspection of calcium-binding proteins whose structures have been deposited in the Protein Data Bank suggests that calcium ions

phosphate bound by the CPP and their analogues increased as the pH increased. The calcium phosphate phase that was found to bind to α51-CN(59–79) was a basic ACP phase of the composition, Ca3.0877(PO4)2(OH)0.1754.

X-ray Powder Diffraction of CPP-ACP—A number of samples of CPP-ACP and β-CN(1–25)-ACP, were studied using x-ray powder diffraction. An understanding of the degree of the crystallinity of the CPP-ACP complex can be obtained from the line broadening of the peak intensity spectrum for the polycrystalline powder. A sample spectrum is shown in Fig. 6A. The absence of sharp features in these spectra was consistent with an amorphous calcium phosphate phase associated with the CPP and the individual peptides. A few peaks in the spectrum of the β-CN(1–25)-ACP complex were observed and assigned to traces of crystalline NaCl and KCl contaminating the sample. Heat treatment of CPP-ACP produces material with less bio-available calcium and phosphate, and x-ray powder diffraction patterns indicated some partial crystallinity with conversion to a disordered apatitic phase upon heat treatment, as shown in Fig. 6B.

SEM and TEM Characterization of CPP-ACP—Neither SEM nor TEM showed any crystallites in the CPP-ACP samples. Selected area electron diffraction over the particles also did not show any ring pattern characteristic of crystal structure. The SEM and TEM images of CPP-ACP and the selected area diffraction pattern therefore confirm the amorphous nature of the complex (Fig. 7).
usually have between six and eight oxygen atoms in their first coordination sphere. The structure of the calcium-binding sites is based on distorted octahedral geometries with "small bite" ligands, such as carboxylate groups, contributing two oxygen atoms at some vertices of the octahedra. Water molecules usually account for only one or two oxygen atoms/binding site. Apart from interactions with side chain oxygen atoms, calcium ions also interact with oxygen atoms from the backbone carbonyl moieties. The structural constraints implied by the multiple interactions between the shorter peptide and the calcium ions that are required to balance the peptide charge are likely to be impossible to satisfy, resulting in the lower calcium binding ability of the shorter peptide. The structural explanation is consistent with our previous reports of calcium ion-dependent, structural features in the \(^1\)H NMR spectra of \(_{(59-79)}\) and \(\beta\)-CN(1–25) complexed with calcium (43, 44). We have shown that these peptides adopt structures consisting of loops and turns and that the specific structure adopted depends on the peptide sequence outside of the calcium-binding motif Ser(P)3-Glu2.

**Calcium Phosphate Binding**—In the presence of phosphate ions, the CPP and their analogues bound additional calcium (over and above that bound in the absence of phosphate). The linear dependence of the excess calcium bound versus the bound phosphate, independent of pH, implies that a specific phase of calcium phosphate was being bound (Fig. 2C). The amount of calcium and phosphate bound by the CPP and their analogues increased as the pH increased.

The number of calcium-binding sites in the calcium phosphate complexes (\(n_{\text{Ca}}\)) correlates strongly with the number of aminoacyl residues in the peptide used to stabilize the CPP-ACP complex. This is consistent with a model of the CPP-ACP complexes in which the phosphorylated sequence motif, Ser(P)-Ser(P)-Ser(P)-Glu-Glu, is required to initialize binding to the calcium phosphate phase. Once the peptide is bound to the ACP phase, weaker interactions allow the remaining polar residues to interact with the ACP core particle, thus accounting for the correlation between peptide length and the number of mol of calcium and phosphate bound by these peptides. Given that the entire length of the peptide interacts with the calcium phosphate phase and the stringent steric requirements associated with the formation of calcium ion-binding sites, it is unlikely that peptides separately bind calcium ions that are not associated with the calcium phosphate phase.

**Characterization of the Bound Calcium Phosphate Phase**—Fig. 4 shows plots of the calcium bound by the peptide \(\beta\)-CN(1–25) versus ion activity products for the four relevant calcium phosphate phases: HA, DCPD, OCP, and a basic amorphous calcium phosphate having the composition \(\text{Ca}_{3+z}(\text{PO}_4)_2(\text{OH})_{2z}\), where \(z = 0.0877\). The calcium phosphate phase stabilized by \(\beta\)-CN(1–25) is identified by the functional dependence of bound calcium (or phosphate) on the ion activity product independent of sample pH. Inspection of Fig. 4 shows that the amount of calcium bound is not a pH-independent function of the ion activity products for the phases HA, DCPD, or OCP. Fig. 4 shows that the bound calcium best correlates with a basic amorphous calcium phosphate phase \(\text{Ca}_{3+z}(\text{PO}_4)_2(\text{OH})_{2z}\), with \(z = 0.0877 \pm 0.0022\). The stabilization of a basic calcium phosphate phase is consistent with the observation that these complexes form over a pH range from ~5.0 to 9.0.

The ratio of \(\nu_{\text{Ca}}:\nu_{\text{Pi}}\) was found to increase as the length of the peptide decreased (Table II). One possible explanation for this observation would be that the shorter peptides bind both a calcium phosphate phase and independent calcium ions. However, as discussed above, the stringent requirements associated with the formation of calcium ion-binding sites makes this an unlikely explanation.

These separate observations can be rationalized by proposing a model of the ACP core that consists of two calcium phosphate phases: a calcium-poor phase with a Ca:P ratio of 1.5, such as \(\text{Ca}_6(\text{PO}_4)_2\), forming the core of the ACP particles as suggested by Meyer and Eanes (45), and a calcium-rich phase with a Ca:P ratio of 2.0, such as \(\text{Ca}_9(\text{PO}_4)(\text{OH})_3\), that is in contact with the peptide. As the peptides become shorter, a given number of peptides are able to fully cover a smaller surface area; hence the size of the complex formed decreases as the peptides become shorter. The thickness of the \(\text{Ca}_6(\text{PO}_4)(\text{OH})_3\) interface with peptide is proposed to remain constant as the complexes shrink in size, whereas the size of the relatively more soluble \(\text{Ca}_9(\text{PO}_4)_2\) core decreases in size, resulting in a steady increase in the ratio of \(\nu_{\text{Ca}}:\nu_{\text{Pi}}\) as the peptides decrease in length. It is interesting that substitution of Ser(P) with Thr(P) reduced calcium phosphate stabilization activity. This together with possible steric hindrance in phosphorylation of Thr residues may help explain why to date all of the proteins known to stabilize calcium phosphate contain clusters of Ser(P) residues.

The ion product that best correlates with the total bound calcium by \(\alpha_{57}\)-CN(59–79) in a pH-independent manner is that of a basic amorphous calcium phosphate phase having the approximate composition \(\text{Ca}_{3.0877}(\text{PO}_4)_2(\text{OH})_{0.1754}\). Given that each \(\alpha\)-CN(59–79) maximally binds 14 phosphate ions, the composition of the \(\alpha_{57}\)-CN(59–79)-ACP complex is approximately \((\alpha_{57}\)-CN(59–79)-ACP\()_n\), where \(n\) is a small integer.

Extensive studies using a library of synthetic homologues and analogues have revealed that the Ser(P)-Glu motif found in most of the CPP is a specific sequence of acidic residues that has a strong affinity for calcium phosphate. However, the conformational preferences of the intact major peptides enable stabilization of the maximum amount of ACP.
Acknowledgment—We thank Fiona Webber for technical assistance.

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Physicochemical Characterization of Casein Phosphopeptide-Amorphous Calcium Phosphate Nanocomplexes
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