Casein Phosphopeptide-Amorphous Calcium Phosphate Nanocomplexes: A Structural Model

*Keith J. Cross, N. Laila Huq, Eric C. Reynolds**

Oral Health CRC, Melbourne Dental School, Bio21 Institute, The University of Melbourne, 720
Swanston Street, Melbourne, Victoria, 3010, Australia.

KEYWORDS. Biomineralisation; phosphoprotein; amorphous calcium phosphate; milk micelles

RUNNING TITLE: Characterisation of Casein Phosphopeptide-Amorphous Calcium Phosphate

*Corresponding author: Eric C. Reynolds. Email: e.reynolds@unimelb.edu.au

FUNDING STATEMENT: Supported by the Australian Government, Department of Industry,
Innovation and Science

ABBREVIATIONS

ACP, amorphous calcium phosphate; CN, casein; CPP, casein phosphopeptides; DQF-COSY, double-quantum filtered correlation spectroscopy; NOESY, nuclear Overhauser effect spectroscopy; Ser(P) andPse, O-phosphoserine; TOCSY, total correlation spectroscopy; Tris, tris(hydroxymethylaminomethane).

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

ABSTRACT

Tryptic digestion of the calcium-sensitive caseins yields casein phosphopeptides (CPP) that contain clusters of phosphorylated seryl residues. The CPP stabilize calcium and phosphate ions through the formation of complexes. The calcium phosphate in these complexes is biologically available for intestinal absorption and remineralisation of subsurface lesions in tooth enamel. We have studied the structure of the complexes formed by the CPP with calcium phosphate using a variety of NMR techniques. Translational diffusion measurements indicated that the β -CN(1-25)-ACP nanocomplex has a hydrodynamic radius of 1.526 ± 0.044 nm at pH 6.0 increasing to 1.923 ± 0.082 nm at pH 9.0. ^1H NMR spectra were well resolved and $^3\text{J}_{\text{H}^{\text{N}}-\text{H}^{\alpha}}$ measurements ranged from a low of 5.5 Hz to a high of 8.1 Hz. TOCSY and NOESY spectra were acquired and sequentially assigned. Experiments described in this paper have allowed the development of a structural model for the β -CN(1-25)-amorphous calcium phosphate nanocomplex.

Bovine milk contains approximately 30 mM calcium and 22 mM inorganic phosphate in solution with most of the calcium (68%) and phosphate (47%) associated with the proteins α_{S1} -, α_{S2} -, β - and κ -casein in casein micelles ^{1, 2}. The α_{S1} -, α_{S2} -, and β -caseins have a number of Ser(*P*) residues in specific phosphorylation-site motifs, such as [-(Ser(*P*))-₃(Glu-)₂], that are essential for their interaction with calcium phosphate ³.

There have been many studies of the ultrastructure of casein micelles using a variety of techniques ^{4, 5} with more recent studies using small-angle X-ray scattering ⁶ and electron microscopy ⁷. Although the structural details are still being elucidated, the casein micelles are believed to be roughly spherical particles with a radius of about 100 nm, dispersed in a continuous phase of water, salt, lactose and whey proteins ⁸. The calcium phosphate isolated after exhaustive hydrazine deproteination 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 nm diameter ⁹. 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 ¹⁰. 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 κ -casein protrudes from the micelle surface forming a so-called 'hairy layer' that sterically stabilizes the complexes ¹¹.

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. The formation of stable casein - calcium phosphate complexes is thought to be part of a general mechanism for

regulating calcium flow in tissues and biological fluids containing high calcium ion concentrations¹¹.

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)¹². The major tryptic CPP are β -CN(1-25) [1] and α_{S1} -CN(59-79) [2] with smaller amounts of α_{S2} -CN(46-70) [3] and α_{S2} -CN(1-21) [4]^{13, 14}. These peptides all contain the cluster sequence motif $-(\text{Ser}(P))_3(\text{Glu})_2$ with three contiguous phosphoserines. This peptide motif is thought to be critical for calcium and calcium phosphate binding by these peptides¹². The sequences of the four major casein tryptic phosphopeptides are shown below with the motif underlined.

[1] Arg¹-Glu-Leu-Glu-Glu-Leu-Asn-Val-Pro-Gly-Glu-Ile-Val-Glu-Ser(P)-Leu-
(Ser(P))₃(Glu)₂Ser-Ile-Thr-Arg²⁵
 β -CN(1-25)

[2] Gln⁵⁹-Met-Glu-Ala-Glu-Ser(P)-Ile-(Ser(P))₃(Glu)₂Ile-Val-Pro-Asn-Ser(P)-Val-Glu-
Gln-Lys⁷⁹
 α_{S1} -CN(59-79)

[3] Asn⁴⁶-Ala-Asn-Glu-Glu-Glu-Tyr-Ser-Ile-Gly-(Ser(P))₃(Glu)₂Ser(P)-Ala-Glu-Val-Ala-
Thr-Glu-Glu-Val-Lys⁷⁰
 α_{S2} -CN(46-70)

[4] Lys¹-Asn-Thr-Met-Glu-His-Val-(Ser(P)-)₃(Glu-)₂Ser-Ile-Ile-Ser(P)-Gln-Glu-Thr-Tyr-Lys²¹
 α_{S2} -CN(1-21)

The CPP stabilize calcium and phosphate ions forming metastable solutions that are supersaturated with respect to the solid calcium phosphate phases¹⁵. Under these conditions, the CPP bind their equivalent weight of calcium and phosphate¹⁶. The CPP are formed *in vivo* by normal digestion of casein and, as they are relatively resistant to further proteolytic degradation, accumulate in the distal portion of the small intestine¹⁷⁻²¹. It has been proposed that this accumulation together with the peptides' ability to form soluble complexes with calcium phosphate are responsible for the enhanced intestinal calcium absorption that has been observed even in vitamin D deficient animals consuming dietary CPP¹⁷⁻²¹. In addition, CPP increase the calcification of *in vitro* cultured embryonic rat bone and again the mechanism is suggested to be associated with the peptide's ability to form soluble complexes with calcium and phosphate ions²². Furthermore, CPP-calcium phosphate complexes have been shown to be anticariogenic and remineralise early stages of enamel caries in animal and human studies^{12, 15, 23-25}. In summary, the ability to kinetically stabilize amorphous calcium phosphate against primary precipitation enhances mineral ion bioavailability²⁶ and confers upon the CPP the potential to be biological delivery vehicles for calcium and phosphate ions²⁷. However, although various examples of CPP-calcium phosphate biological function have been documented, the supramolecular structure of these complexes has yet to be elucidated.

As part of our long-term investigation into the structure-function relationships of proteins involved in biomineralisation and calcium phosphate stabilization, we have studied the solution structures of tryptic phosphopeptides from milk caseins and their interaction with the amorphous

and crystalline phases of calcium phosphate²⁸⁻³⁴. In this paper, we report our ¹H NMR spectroscopic investigations of the β-CN(1-25)–ACP nanocomplex. We present a detailed model of the β-CN(1-25)–ACP nanocomplex consistent with the NMR spectroscopic information presented in this paper and with results presented elsewhere^{29, 30, 32-34}.

EXPERIMENTAL PROCEDURES

Materials and Methods

Preparation of casein phosphopeptides. The casein phosphopeptides β-CN(1-25) and α_{S1}-CN(59-79) were selectively precipitated from a tryptic digest of casein using calcium chloride and ethanol and further purified by anion exchange FPLC and reversed phase HPLC¹⁴. The purity of the peptides was assessed by MALDI-TOF mass spectrometry, capillary electrophoresis, amino acid composition and sequence analyses^{13, 14}. Prior to sequence analysis, the labile phosphoserine residues were converted to *S*-ethyl cysteinyl residues by β-elimination¹⁴.

Electrophoresis of cross-linked α_{S1}-CN(59-79). Three 50 μL samples of the peptide α_{S1}-CN(59-79) in distilled water at a concentration of 10 g/L at pH 6.7 were preincubated for 1 hour at room temperature with (a) 0 mM CaCl₂, (b) 60 mM CaCl₂, or (c) 60 mM CaCl₂ and 60 mM K₃PO₄. After the addition of 2 μL of 0.5% v/v glutaraldehyde, the reaction mixtures were incubated for a further 30 min prior to dilution with an equal volume of loading buffer containing 20% glycerol and 0.45 M EDTA. Native polyacrylamide gel electrophoresis was performed

using 1 mm thick 20% gels³⁵ with the Mini-PROTEAN II system (Bio-Rad), at 100 V for 5 hours, at 4 °C. Gels were then washed for 1 hour in 0.25% isopropanol to remove glycine, and stained overnight with “Stains All” (Eastman-Kodak Co.)³⁶.

Preparation of CPP-calcium phosphate. CPP-ACP solutions were prepared as described previously³⁷

NMR Spectroscopy. All NMR spectra were acquired on a Varian^{UNITY}Inova spectrometer operating at a field strength of 599.741 MHz. Samples of β-CN(1-25) were prepared with peptide concentrations ranging from 1.0 mM to 4.5 mM.

Translational diffusion coefficients were determined using the sLED technique³⁸ with CPP-ACP samples dissolved in 99.96% D₂O solution. The diffusion coefficients were determined by fitting the observed signal intensity, *A*, to the following expression

$$A(2t) = A(0) \exp \left[-\gamma^2 D \left(\Delta - \frac{\delta}{3} \right) \delta^2 G^2 \right] \quad [1]$$

where γ is the gyromagnetic ratio for the proton and *G* is the applied gradient magnitude. The magnetic field gradient duration, δ , was fixed at 5 ms, and the diffusion delay Δ was fixed at 50, 100 and 200 ms in successive experiments. The gradient amplitude, *G*, was varied from 0 to a maximum of approximately 30 gauss cm⁻¹. The translational diffusion coefficients, *D*, are related to effective hydrodynamic radii, *a*, by the Stokes-Einstein equation

$$D = \frac{kT}{6\pi a \eta} \quad [2]$$

where k is the Boltzmann constant, T is the absolute temperature, and η is the sample viscosity. In practice, these equations were combined and the sLED data were fitted to an equation of the form

$$A(2t) = A(0) \exp \left[-\alpha G^2 / a \right] \quad [3]$$

The parameter α contains the fundamental constants (γ , k), sample dependent constants (T , η), delays (δ , Δ), and a calibration term converting the applied spectrometer gradients (arbitrary units) to cgs units. The parameter α was determined using the HDO peak as an internal calibrant and assuming a hydrodynamic radius of 1.40 Å for the water molecule. Hydrodynamic radii of the CPP–ACP complexes were calculated using peak amplitude data from a number of resonances and also from peak integrals over selected portions of the NMR spectra.

Solvent exposure of protons in β -CN(1-25)–ACP was studied using the CLEANEX_{PM} pulse sequence³⁹. The sample of β -CN(1-25)–ACP was prepared in 90% H₂O/ 10% D₂O. The only resonances expected to be observed in this experiment were from solvent exposed protons not participating in hydrogen-bonding networks.

Two-dimensional NMR Spectroscopy. Phase-sensitive DQF-COSY, TOCSY, and NOESY spectra were acquired using the States-Haberkorn method⁴⁰. All spectra were acquired with spectral widths of 6000 Hz in F_2 and F_1 . The DQF-COSY spectrum was acquired with 800- t_1 increments each of 2048 complex data points. The spectrum was zero-filled in t_2 and t_1 to yield a final data matrix of 4k by 2k real points on Fourier transformation. TOCSY and NOESY spectra were acquired with 80 to 140 t_1 increments each of 2048 complex data points. Spectra were zero-filled in t_2 and linear prediction used in t_1 to yield final data matrices of 2k by 2k real points on

Fourier transformation. Sine-bell window functions were used in both dimensions of the DQF-COSY spectra, and sine-bell window functions shifted by $\pi/3$ were used in both dimensions for TOCSY and NOESY spectra. The residues were assigned using the standard assignment protocol⁴¹ as modified by Chazin *et al.*⁴².

Molecular modeling of β -CN(1-25)-ACP. For molecular modeling of the β -CN(1-25)-ACP complex, the ACP core particle was simulated as a sphere of randomly located calcium ions, phosphate ions, and water molecules using the Tripos force field and charges from the MMFF94 method (chosen because it gave the correct charge on the phosphate ions). The ACP core particle was modeled with 144 calcium and 96 phosphate ions and 138 water molecules. The ACP core particle was energy minimized with full electrostatics and van der Waal's interactions ramped up from 5% to full value to allow atoms to move past each other to energetically more favourable locations. The oxygen atoms of the water molecules, as well as the calcium and phosphate ions, were constrained to be within 12.7 Å of the centre of the ACP core particle. This constraint was required to prevent destabilization of the ACP core particle in the early stages of the calculation only, once energy minimized this constraint made no contribution to the total system energy.

For modeling of the β -CN(1-25) residues in the β -CN(1-25)-ACP complex, residues -Val⁸-Pro-Gly-Glu¹¹- were constrained in a β -turn. This was based on nOe observations in the calcium complex²⁸, similar chemical shifts for these residues in the calcium and calcium phosphate complexes, and the known propensity for the sequence motif -Pro-Gly- to occur as the second and third residues in type-II β -turns. The type-II β -turn was formed by constraining the ϕ/ψ angles of Pro⁹ and Glu¹⁰ and forcing an H-bond to form between the carbonyl of Val⁸ and the amide proton of Glu¹¹.

Based on previous modeling studies⁴³ and specific surface area measurements of CPP bound to HA surfaces²⁹ further constraints were applied to the polar side-chains of the peptide with the ACP phase. In the early stages of the β -CN(1-25)-ACP modeling process, polar interactions between the peptide and the ACP core were enforced by requiring one oxygen atom of each of the phosphate and carboxylate groups to be in the range 9.0 Å to 16.0 Å from the centre of the ACP particle using a quadratic-well potential with a force constant of 10.1 kcal mole⁻¹ Å⁻¹. The interaction between the ACP particle and the guanidino side-chains were enforced by similarly constraining one hydrogen atom from each of the terminal nitrogen atoms to be in the same range from the ACP particle. Since the initial annealing calculations were performed without electrostatics, the role of the ACP particle in these calculations was primarily to enforce proper van der Waal's constraints on the peptides in the calculation. No constraints were applied to the terminal NH₂ or the side-chain of Asn⁷.

The model of the β -CN(1-25)-ACP complex was built in several stages. In the first step, the constraints were applied to a single peptide constrained, as described above, to lie on the surface of the ACP sphere. Constraints corresponding to d_{NN} nOes were applied in the form of a quartic-well potential with lower bound distances of 1.8 Å and upper bound distances of 2.7 Å and a force constant of 20 kcal mole⁻¹ Å⁻¹. The upper bounds for the Leu¹⁶-Pse¹⁷ and Ser²²-Ile²³ constraints were increased to 4.5 Å because the shorter constraints were systematically violated in the early models. Torsional constraints were implemented with a force constant of 0.06 kcal mole⁻¹ degree⁻². Simulated annealing was used with the system heated to a temperature of 700 K for 5 ps, and then cooled to 20 K over a period of 2 ps. This cycle was repeated 50 times to generate 50 structures. The twenty lowest energy structures were then taken and individually energy minimized. In the second stage, six peptides identified as (chains

1
2
3 A-E) having the lowest energy were arranged around the ACP sphere. The peptides were then
4
5 allowed to relax through a molecular dynamics simulation at 300 K for 50 ps, to remove bad
6
7 van der Waal's contacts, subject to the constraints previously applied to the single peptide. The
8
9 peptide moieties were then fixed and the ACP core was energy minimized, using both
10
11 dynamics and energy minimization, while the van der Waal's and electrostatic contributions to
12
13 the energy were gradually ramped up to their full values. The peptides were once again energy
14
15 minimized subject to the constraints. These calculations were performed using the Tripos force
16
17 field with charges calculated by the MMFF94 method.
18
19

20
21
22 During the course of the energy minimization, it was noted that water molecules were
23
24 escaping from the ACP core and forming H-bonds between the peptide moieties. To ensure that
25
26 the H-bonding potential of the peptides were satisfied, the CPP-ACP particle was solvated
27
28 using a droplet model with a total of 1239 water molecules. The solvent molecules were
29
30 constrained to lie within a sphere of radius 24 Å from the nanocomplex centre by a quartic-well
31
32 potential with a force constant of 7 kcal mole⁻¹ Å⁻¹ to prevent evaporation of solvent molecules.
33
34 The energy minimization was carried out using the full MMFF94s force field including
35
36 electrostatic interactions and a dielectric constant of unity. Energy minimization was continued
37
38 until the average force on the atoms fell below 0.05 kcal mole⁻¹ Å⁻¹.
39
40
41
42

43
44 To ensure that the energy-minimized structure was not trapped in a local minimum, a 60 ps
45
46 dynamics simulation at 300 K was performed with a dynamics step size of 0.75 fs. Three
47
48 conformations having low potential energy were selected at 8.910 ps, 40.068 ps, and 56.028 ps
49
50 respectively, and energy minimized until the average force on the atoms fell below 0.05 kcal
51
52 mole⁻¹ Å⁻¹. To confirm that the peptide constraints were not distorting the final structures the
53
54
55
56
57
58
59
60

force constants for torsional constraints and nOe distance constraints were set to zero and the structure from the third model was energy minimized for a further 100 iterations.

The program NMRCLUST⁴⁴ was used to investigate the conformational properties of the β -casein(1-25) peptides bound to the ACP core particle. Secondary structural features of the peptides conformations were identified using STRIDE⁴⁵.

RESULTS

Electrophoresis of cross-linked α_{S1} -CN(59-79). Incubation of α_{S1} -CN(59-79) with glutaraldehyde in the presence of either calcium alone or calcium and phosphate resulted in the cross-linking of the α_{S1} -CN(59-79) peptides. Fig. 1 shows the migration of glutaraldehyde cross-linked α_{S1} -CN(59-79) in a 20% native gel. In the absence of calcium, or in the presence of phosphate only (not shown), the largest aggregate observed was a dimer. In the presence of either calcium or calcium phosphate, the largest multimer observed was a hexamer.

NMR Spectroscopy Studies β -CN(1-25)–ACP. The decay of the echo amplitude due to translational diffusion of β -CN(1-25)–ACP complex was measured using the sLED technique³⁸. At a concentration of 1.0 mM β -CN(1-25), the decay of the echo amplitude was single exponential (Fig. 2). The Stokes-Einstein equation, [2], relating the translational diffusion coefficient, the hydrodynamic radius, the sample viscosity, and the temperature was used to estimate the relative sizes of water molecules (hydrodynamic radius ~ 1.40 Å) and the complexes.

The hydrodynamic radius of the carrier particles was most accurately determined by the experiments recorded with a diffusion delay, Δ , of 200 ms due to the greater dynamic range for this experiment. The behaviour of the signals recorded at shorter diffusion delay times were consistent with the value determined at the longer diffusion delay time. An estimated hydrodynamic radius of 1.526 ± 0.044 nm was determined for the β -CN(1-25)-ACP complex at pH 6.0 increasing to 1.923 ± 0.082 nm at pH 9.0. The degeneracy of the HDO resonance and the $H\alpha$ resonance of Asn⁷ resulted in a bi-exponential decay of the calibration resonance even at the lower sample concentration and was responsible for much of the error associated with the estimate of the hydrodynamic radius. At a higher concentration of 4.5 mM β -CN(1-25), the decay of the echo amplitude was multi-exponential (Fig. 2b) indicating aggregation of the complexes.

The CLEANEX-PM³⁹ experiment was used to identify the solvent exposed protons of the peptide moieties in the β -CN(1-25)-ACP complex by transfer of magnetization from the solvent water to the peptide. The only resonances observed in this experiment were in the $H\alpha$ region and were assigned to the $H\delta$ protons of Pro⁹, the $H\alpha$ proton of Val⁸, the $H\alpha$ proton of either Ile¹² or Val¹³ or possible both, and a glutamate $H\alpha$ proton (Fig. 3). The signals were observed to increase in intensity as the mixing period increased from 50 ms to 300 ms in 50 ms steps (Fig. 3) ruling out an artifactual response.

One and Two-dimensional NMR Spectroscopy of β -CN(1-25)-ACP. In contrast to the spectra of β -CN(1-25) complexed with calcium alone²⁸, the NMR spectra of β -CN(1-25)-ACP were markedly sharper with well-resolved $^3J_{H^N-H^\alpha}$ splittings observed in the amide region (Fig. 4). The

amide resonances were well dispersed. The measured $^3J_{H^N-H^\alpha}$ ranged from a low of 5.5 Hz to a high of 8.1 Hz with no significant tendency for runs of low or high values to occur in adjacent residues. The observation of $^3J_{H^N-H^\alpha}$ coupling constants that differed significantly from the random-coil value of about 6 to 7 Hz clearly indicated that the peptide adopts a preferred conformation when bound to the ACP phase.

Analysis of the line widths indicated that they were similar for all residues and hence the dynamics of residues were similar. Furthermore there was no evidence for particular residues having markedly different dynamics from neighbouring residues due to interactions with the calcium phosphate core particles. The resonances of the phosphoseryl residues could be readily assigned from TOCSY and NOESY spectra. Sequential d_{NN} and $d_{\alpha N}$ nOes were observed allowing the spectra to be sequentially assigned. Table 1 summarized the coupling constants and the 1H NMR assignments obtained.

A number of NOESY spectra were acquired with mixing times up to 250 ms. Fig. 5 shows the finger print region and the amide region of a NOESY spectrum acquired with a mixing time of 250 ms. All NOESY cross-peaks were explicable as intramolecular cross-peaks. Since no signals were observed in spectra acquired at 300 ms, this absence of medium- and long-range intramolecular nOes indicated that the peptides adopted an extended conformation. The observed nOes and $^3J_{H^N-H^\alpha}$ coupling constants, summarized in Fig. 6, were used to build a model of the peptide in the β -casein(1-25)–ACP nanocomplex.

Molecular modeling of the β -casein(1-25)–ACP nanocomplex. The model was built in several stages. At the first step, each peptide was assigned a unique chain label A-E. This was used to follow the progress of the chains during the dynamics runs. Three molecular models of the β -

1
2
3 CN(1-25)-ACP nanocomplex were constructed. In the final dynamics run the first was annealed
4
5 for 8.910 ps, the second for 40.068 ps, and the third for 56.028 ps prior to energy minimization.
6
7
8 The models of the β -CN(1-25)-ACP nanocomplex are both chemically and stereochemically
9
10 reasonable as can be seen from the statistics presented in Table 2. Inspection of the
11
12 Ramachandran plot, for the third model, shows that the majority of the ϕ/ψ angles are in either
13
14 the 'core' or 'allowed' regions and that only a few residues are in the 'forbidden' region: Glu⁵
15
16 with four examples in the 'forbidden' region is particularly noteworthy. All models satisfy the
17
18 NMR torsion constraints, while the NMR distance constraints are reasonably well satisfied with no
19
20 pattern of systematic violations. The peptide backbones of model 3 and the structure resulting
21
22 from the unrestrained minimization could be superimposed with an RMSD of 0.226 Å, confirming
23
24 that the NMR constraints were not significantly distorting the peptide conformation.
25
26
27
28

29
30 Analysis of the peptide secondary structure in model 3 using STRIDE⁴⁵ showed that the
31
32 peptides (chains A-E) adopt secondary structures consisting of a series of β -turns. Type IV β -
33
34 turns were identified for Glu⁴-Asn⁷ and Ser(*P*)¹⁹-Ser²² in all chains, a type VIII β -turn was
35
36 identified for Glu¹⁴-Ser(*P*)¹⁷ in all chains except B and E, and a type IV β -turn was identified for
37
38 Leu³-Leu⁶ in chains B, E, and F. The region from Val⁸ to Ile¹² does not adopt a single
39
40 conformation across the different chains: the A-chain has a type II β -turn for Val⁸-Glu¹¹, a type
41
42 IV β -turn for Pro⁹-Ile¹² followed by a type VIII β -turn for Glu¹¹-Glu¹⁴, in chains B, D, and E
43
44 there is a type IV β -turn for Val⁸-Glu¹¹, in chain F a short 3_{10} -helix connects Pro⁹-Glu¹¹, while in
45
46 chain C no secondary structure assignment was made.
47
48
49
50

51
52 Fig. 7 shows the model of β -CN(1-25)-ACP based on the constraints previously summarized
53
54 in Fig. 6. The model consists of an ACP core of radius 1.27 nm consisting of 96 phosphate ions,
55
56
57
58
59
60

144 calcium ions and 132 water molecules (six water molecules escaped from the core during modeling). This core is surrounded by six β -CN(1-25) peptides. Binding energies were calculated by ignoring the solvent molecules, using a distance-dependent dielectric of $r/2$, and an electrostatic cutoff distance of 8 Å. The structure is predicted to be stable with a net average binding energy of -1265 kcal/(mole of peptide). The average interaction between the ACP core and the peptides was -1270 ± 430 kcal/(mole of peptide). The peptide-peptide interactions were overall repulsive with an average value of 54 ± 210 kcal/(mole of peptide). However in solution, these repulsive interactions would be shielded by the polar solvent and counter ions. It is probable that individual peptide chains can be inserted with random orientations into the β -CN(1-25)–ACP nanocomplex since the packing of peptides was determined by strong, electrostatic peptide–ACP interactions rather than the weaker, van der Waal’s peptide-peptide interactions.

The model was subjected to a series of analyses. The interface was defined as the set of atoms that belong to either the ACP core or the peptides that were within 2.5 Å of atoms belonging to the peptides or the core respectively. Analysis of the interface revealed that each peptide contributed, on average, 41 atoms to the interface forming contacts with a similar number of core atoms. These atoms interacted through either electrostatic or H-bonding that make a considerable contribution to the overall stability of the complex as modeled. Four of the six unconstrained side-chains of Asn⁷ formed H-bonds with core atoms, while in three of the peptides, an H-bond was formed between the terminal amide of Arg¹ to the carbonyl oxygen of the Asn⁷ side-chain. In all cases where there was an electrostatic interaction between an oxygen atom and a calcium ion from the core, the oxygen atoms were associated with either the side-chains or the C-terminal

carboxyl group; the backbone carbonyl groups did not form part of the interface. Fig. 8 shows one of the peptides and the atoms of the ACP core within 2.5 Å of the peptide.

The conformations of individual peptides within the models were also analysed. Superimposing the heavy backbone atoms of the peptides from any of the models showed that the peptide adopts a reasonably well-defined conformation. The root mean squared deviation of the backbone atoms of the peptides of the third model from their average position was 2.149 Å. However, when the conformations of the peptides from all three models were compared and clustered (using NMRCLUST⁴⁴) a total of six conformational clusters were found with backbone RMSDs ranging from 0.578 Å to 0.846 Å. Each cluster consisting of the corresponding peptide chains from the each of the three models: that is the three A chains form one cluster, the three B chains another, and so on. The modeling suggested that the peptide adopted a conformation close to the ensemble average conformation but with minor changes to better accommodate differences in the mineral surface with which the peptide is interacting. This interpretation of the modeling result is supported by the observation that when the heavy atoms of the peptides are superimposed, the polar residues make the largest contributions to the calculated heavy-atom RMSD. Of the thirteen residues that make an above average contribution to the calculated heavy-atom RMSD only Ile²³ is non-polar. Furthermore the only polar residues that make below average contributions to the heavy-atom RMSD are Asn⁷, Glu²⁰, Ser²², and Thr²⁴ (predominantly the neutral, polar residues). This is consistent with the charged, polar residues making specific interactions with features of the ACP core that vary with the location of one peptide chain to that of another.

Analysis of the residues that contributed most to the RMSD of the backbone atoms from the average conformation revealed that the residue that deviated most was Gly¹⁰. Residue Gly¹⁰

showed ϕ/ψ isomerism with two populations having approximate ϕ/ψ angles of $100^\circ/30^\circ$ and $-100^\circ/-75^\circ$ respectively. This was observed despite the use of constraints on the ϕ/ψ angle of this residue to force a β -turn between Val⁸ and Glu¹¹. Inspection of two representative chains indicated that both structures contain a similar set of H-bonds that served to stabilize a β -turn with different backbone conformations at Gly¹⁰. Further scrutiny of the STRIDE⁴⁵ analysis of model 3, revealed a variety of secondary structural features for Val⁸ to Glu¹¹ consistent with this region of the peptide backbone having some flexibility.

The intrapeptide, proton-proton distances from the models were measured to account for the absence of non-sequential nOes in the ¹H NMR spectra. For example, the distance from Leu³ H α to Val⁸ H β ranged from 2.726 Å to 9.927 Å in the peptides of model 3. After taking into account the R⁻⁶ dependence of the nOe on the interatomic distance, averaging over the six peptides in model 3 yielded an effective distance of 3.67 Å for these two protons. A nOe that is readily observed between two atoms at 2.726 Å would be much harder to observe at the longer average distance. Calculations using intrapeptide, proton-proton distances from model 3 confirmed that the distance dependence of the nOe effect biases against the observation of non-sequential nOes relative to the observed sequential nOes. Consequently, the absence of non-sequential nOes in the ¹H NMR spectra is consistent with the ability of the β -casein(1-25) peptide to adapt to differences in the mineral surface.

DISCUSSION

NMR spectroscopy reveals differences between the structures of β -CN(1-25) complexed with calcium and with ACP. In contrast to previous studies⁴⁶, all residues in the complexes of β -CN(1-25)–ACP were assigned. Previously the H α resonances of the phosphoseryl residues were reported to be not observable based on the inspection of one-dimensional spectra of β -CN(1-25)–ACP, with the explanation they had been broadened due to their rigid attachment to the slowly tumbling amorphous calcium phosphate core particle. However in the calcium ion complexes, these resonances have higher frequencies than the other H α protons, and the resonances are observable in a narrow window between the water resonance and the other H α resonances. Our assignment of the two-dimensional spectra in the current study shows that in the complex with calcium phosphate, these resonances move to significantly lower frequencies and the resonances now overlap the H α resonances of the other residues.

In contrast to the spectra of β -CN(1-25) complexed with calcium alone²⁸, the NMR spectra of β -CN(1-25)–ACP revealed only nOes corresponding to sequential d_{NN}, d _{α N}, and d _{β N} connectivities. No NOESY cross-peaks were observed at longer mixing times, that is for mixing times of 300 ms or greater, including the intraresidue and sequential cross-peaks noted at shorter mixing times. This observation suggests that relaxation of the nuclear spins by the spin-diffusion mechanism is efficient. The absence of medium- and long-range nOes might be explained if the long-range neighbouring protons were disordered, the amplitude of the many possibly medium- and long-range nOes from a particular proton falling below the threshold required for observation. This is consistent with a model of the CPP–ACP particle in which strong peptide-ACP interactions (i.e. electrostatic) are responsible for holding the complex together. The weaker, non-specific peptide-peptide interactions (i.e. van der Waal's forces) allow the peptides to be packed, but do not force the peptides into a specific regular arrangement.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

The CPP-ACP complexes pose experimental challenges during the acquisition of structural data. One of the key features of the CPP-ACP complexes is the ease with which large aggregates form. Due to aggregation we have noted that solutions form clear, thixotropic gels at sufficiently high complex concentrations. We have used a wide variety of techniques in an approach to determine particle sizes for these complexes. Even simple techniques such as the use of microfilters with a known pore size can give contradictory results, as CPP-ACP at low concentrations move freely through 0.2 μm filters but are retained at higher concentrations. We have applied a variety of light-scattering techniques to CPP-ACP solutions and have obtained characteristic dimensions ranging from 3-300 nm. These experiments are not discussed in detail because we believe the results relate to aggregates; however, they do emphasize the need to ensure that measurements relate to the monomeric species. Hence, a structural model of the $\beta\text{-CN}(1\text{-}25)\text{-ACP}$ complex provides insight into the architecture of the CPP-ACP complex incorporating several different peptides.

The $\beta\text{-CN}(1\text{-}25)\text{-ACP}$ nanocomplex model is consistent with a range of experimental observations. In attempting to model the $\beta\text{-CN}(1\text{-}25)\text{-ACP}$ complex, it was necessary to satisfy a number of constraints from a wide variety of experimental sources. Some of the constraints, for example the distance and torsion constraints from the NMR experiments, were used directly to provide numeric constraints on the peptides in our model. Other constraints, for example the size of the complex and the number of calcium and phosphate ions bound per peptide, were incorporated into our model. Finally, there were a number of experimental observations, for example, the rheological behaviour of concentrated solutions of the $\beta\text{-CN}(1\text{-}25)\text{-ACP}$ complex

that cannot be used to constrain the modeling, but should be explicable in terms of features of the final model.

Glutaraldehyde cross-linking of the peptides in the α_{S1} -CN(59-79)-ACP complex suggested that up to six peptides are involved in the formation of each nanocomplex. We have assumed that the β -CN(1-25)-ACP complex has a similar structure. Specific surface area, binding data, and hydrodynamic radius measurements are consistent with this assumption²⁹.

We have very little information about the ACP core. Our chemical binding studies indicated that there are a maximum of 14 and 16 phosphate ions in the complexes formed by α_{S1} -CN(59-79) and β -CN(1-25) respectively³⁰. Hence assuming there are six peptides per complex then there are a maximum of 96 phosphate ions per β -CN(1-25)-ACP complex. For simplicity, the ACP is treated as having the composition $\text{Ca}_3(\text{PO}_4)_2$. The actual non-stoichiometric composition³² implies an additional 5 calcium ions and associated hydroxyl ions for the β -CN(1-25)-ACP complex. In addition to these known components of the ACP core, there is evidence, from the behaviour of CPP-ACP under heat treatment at or slightly above 100 °C, that there is water in the calcium phosphate phase. Heat treatment tends to produce material with less bio-available calcium and phosphate and X-ray powder diffraction patterns indicating some partial crystallinity with conversion to a disordered apatitic phase³². We have chosen to model the ACP core with 138 water molecules, however, the dimensions of the core particle were not very sensitive to the number of water molecules included in the model. The predicted radius of the ACP core is then about 1.27 nm, in good agreement with the electron microscopy observations of ACP from casein micelles^{9, 47}.

Two observations suggest that the ACP core particles in milk are coated by the caseins and that the caseins do not form an integral part of the ACP core. First, the caseins can be removed

by chemical treatments such as soaking in a solution of hydrazine^{9, 47}. Second, the CLEANEX-PM experiment reported above, indicated that some protons are solvent exposed. These observations are consistent with the protein not being embedded in the mineral phase.

The calcium phosphate binding studies performed with the CPP and analogues revealed a strong correlation between peptide length and the number of bound calcium and phosphate ions³². This observation suggests that the entire length of the peptides is involved in interactions with the amorphous calcium phosphate phase, not just the ‘calcium-binding motif’ -(Ser(*P*)-)₃(Glu-)₂. This is consistent with the highly polar nature of the CPP peptides, which contain many negatively charged residues such as glutamyl and phosphoseryl residues outside of the so-called ‘calcium-binding motif’.

The calculated dimensions of the β-CN(1-25)-ACP are consistent also with the previously unexplained observation of a secondary maximum in the X-ray and neutron diffraction studies at a scattering vector of $Q \sim 1.6 \text{ nm}^{-1}$ ⁴⁸.

Our model of the CPP-ACP complex is fundamentally different from that proposed by Holt *et al.*⁴⁸. The values derived by Holt *et al.*⁴⁸ suggest an ACP core with a radius of about 2.30 nm coated by 48 peptides. The thickness of the peptide coat, 17.4 Å, implies that each peptide occupies a volume of about 4.69 nm³, compared to a van der Waal’s volume of about 1.06 nm³ for the β-CN(1-25) peptide. The peptide chains can only fill this volume if a substantial proportion of each peptide is in rapid motion forming an ‘entropic brush’⁴⁹. Such a description is inconsistent with the authors’ own description of the peptide coat as being dense. Furthermore, it is unclear why such a polar peptide should be in rapid motion when attached to a highly polar calcium phosphate surface.

The model of the β -CN(1-25)-ACP complex also provides some insight into the rheological behaviour of the complexes. A large, hydrophobic patch on the surface of the complex is revealed in the model. This patch contains Pro⁹, Ile¹², Val¹³ and a glutamate residue, Glu⁵. These residues were not constrained by our modeling procedure, and the prediction that these residues should be solvent exposed is consistent with the CLEANEX-PM experimental results. Hydrophobic interactions between patches on different complexes would be predicted to mediate the aggregation of these complexes: this prediction is consistent with the observed solution behaviour of these complexes.

The observation of ϕ/ψ isomerism at Gly¹⁰ may explain the doubling of the ¹H NMR transitions that was previously ascribed to *cis/trans* isomerism of Pro⁹ in the β -casein(1-25) calcium complex^{28, 50, 51}.

CONCLUSIONS

The ability of glutaraldehyde to cross-link up to six α_{S1} -CN(59-79) molecules in the presence of either calcium ions alone or calcium and phosphate ions, combined with earlier results³² suggests a nanocomplex having the formula [α_{S1} -CN(59-79)-(ACP)₇]₆. The corresponding β -CN(1-25)-ACP nanocomplex has the formula [β -CN(1-25)-(ACP)₈]₆.

A molecular model of the β -CN(1-25)-ACP nanocomplex based on NMR observations combined with a variety of earlier results suggests that the dominant interactions within the complex are between the ACP core and the peptides with weaker interactions between the peptides. The model demonstrates chemically reasonable interactions between the ACP core and the peptides and is consistent with a wide variety of experimental observations. The model predicts the occurrence of 'patches' containing lipophilic residues at the surface of the

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

nanocomplex, these patches may account for the observed tendency toward aggregation of the nanocomplexes. Because such lipophilic patches would allow interactions between the nanocomplexes and biological membranes, this feature may play an important role in the biological function of these peptides as biological delivery vehicles for calcium and phosphate.

ACKNOWLEDGMENTS

We thank Ms Fiona Webber for technical assistance. This work was supported by the Australian Government, Department of Industry, Innovation and Science.

REFERENCES

- (1) Walstra, P., and Jenness, R. (1984) *Dairy chemistry and physics*, John Wiley and Sons, New York, N.Y.
- (2) Van Hooydonk, A. C. M., Boerrigter, I. J., and Hagedoorn, H. G. (1986) pH-induced physicochemical changes of casein micelles in milk and their effect on renneting. 2. Effect of pH on renneting of milk, *Neth. Milk Dairy J.* 40, 297-313.
- (3) Holt, C., and Sawyer, L. (1988) Primary and predicted secondary structures of the caseins in relation to their biological functions, *Protein Eng.* 2, 251-259.
- (4) Dalgleish, D. G., and Corredig, M. (2012) The structure of the casein micelle of milk and its changes during processing, *Annu Rev Food Sci Technol* 3, 449-467.
- (5) Holt, C., Carver, J. A., Ecroyd, H., and Thorn, D. C. (2013) Invited review: Caseins and the casein micelle: their biological functions, structures, and behavior in foods, *J. Dairy Sci.* 96, 6127-6146.
- (6) Ingham, B., Erlangga, G. D., Smialowska, A., Kirby, N. M., Wang, C., Matia-Merino, L., Haverkamp, R. G., and Carr, A. J. (2015) Solving the mystery of the internal structure of casein micelles, *Soft Matter* 11, 2723-2725.
- (7) Tercinier, L., Ye, A., Anema, S. G., Singh, A., and Singh, H. (2014) Interactions of casein micelles with calcium phosphate particles, *J Agric Food Chem* 62, 5983-5992.
- (8) Schmidt, D. G. (1982) Association of caseins and casein micelle structure., *Dev. Dairy Chem. I*, 61-86.

(9) McGann, T. C., Buchheim, W., Kearney, R. D., and Richardson, T. (1983) Composition and ultrastructure of calcium phosphate-citrate complexes in bovine milk systems, *Biochim. Biophys. Acta* 760, 415-420.

(10) de Kruif, C. G. (1999) Casein micelle interactions, *Int. Dairy J.* 9, 183-188.

(11) Holt, C., and Horne, D. S. (1996) The hairy casein micelle: evolution of the concept and its implications for dairy technology, *Neth. Milk Dairy J.* 50, 85-111.

(12) Reynolds, E. C., Black, C. L., Cai, F., Cross, K. J., Eakins, D., Huq, N. L., Morgan, M. V., Nowicki, A., Perich, J. W., Riley, P. F., Shen, P., Talbo, G., and Webber, F. (1999) Advances in enamel remineralization: casein phosphopeptide-amorphous calcium phosphate, *J. Clin. Dent.* 10, 86-88.

(13) Adamson, N. J., Riley, P. F., and Reynolds, E. C. (1993) The analysis of multiple phosphoserine-containing casein peptides using capillary zone electrophoresis., *J. Chromatogr.* 646, 391-396.

(14) Reynolds, E. C., Riley, P. F., and Adamson, N. J. (1994) A selective precipitation procedure for the purification of multiple-phosphoserine containing peptides and their identification., *Anal. Biochem.* 217, 277-284.

(15) Reynolds, E. C., Cain, C. J., Webber, F. L., Black, C. L., Riley, P. F., Johnson, I. H., and Perich, J. W. (1995) Anticariogenicity of calcium phosphate complexes of tryptic casein phosphopeptides in the rat, *J. Dent. Res.* 74, 1272-1279.

(16) Reeves, R. E., and Latour, N. (1958) Calcium phosphate sequestering phosphopeptide from casein., *Science* 128, 472.

(17) Mykkanen, H. M., and Wasserman, R. H. (1980) Enhanced absorption of calcium by casein phosphopeptides in rachitic and normal chicks., *J. Nutr.* 110, 2141-2148.

- (18) Lee, S. L., and Veis, A. (1980) Cooperativity in calcium ion binding to repetitive, carboxylate-serylphosphate polypeptides and the relationship of this property to dentin mineralization, *Int. J. Pept. Protein Res.* 16, 231-232.
- (19) Lee, Y. S., Noguchi, T., and Naito, H. (1983) Intestinal absorption of calcium in rats given diets containing casein or amino acid mixture: the role of casein phosphopeptides, *Br. J. Nutr.* 49, 67-76.
- (20) Sato, R., Noguchi, T., and Naito, H. (1986) Casein phosphopeptide (CPP) enhances calcium absorption from the ligated segment of rat small intestine, *J. Nutr. Sci. Vitaminol.* 32, 67-76.
- (21) Meisel, H., and Fristar, H. (1988) Chemical characterization of a caseinophosphopeptide isolated from *in vivo* digests of a casein diet., *Biol. Chem. Hoppe Seyler* 369, 1275-1279.
- (22) Gerber, H. W., and Jost, R. (1986) Casein phosphopeptides: their effect on calcification of in vitro cultured embryonic rat bone., *Calcif. Tiss. Int* 38, 350-357.
- (23) Reynolds, E. C. (1991) Anticariogenic phosphopeptides. United States Patent 5,015,628.
- (24) Shen, P., Cai, F., Nowicki, A., Vincent, J., and Reynolds, E. C. (2001) Remineralization of enamel subsurface lesions by sugar-free chewing gum containing casein phosphopeptide-amorphous calcium phosphate, *J. Dent. Res.* 80, 2066-2070.
- (25) Reynolds, E. C., Cai, F., Shen, P., and Walker, G. D. (2003) Retention in plaque and remineralization of enamel lesions by various forms, *J. Dent. Res.* 82, 206-211.
- (26) Kitts, D. D., Yuan, Y. V., Nagasawa, T., and Moriyama, Y. (1992) Effect of casein, casein phosphopeptides and calcium intake on ileal ⁴⁵Ca disappearance and temporal systolic blood pressure in spontaneously hypertensive rats, *Br. J. Nutr.* 68, 765-781.

- (27) Ferraretto, A., Signorile, A., Gravaghi, C., Fiorilli, A., and Tettamanti, G. (2001) Casein phosphopeptides influence calcium uptake by cultured human intestinal HT-29 tumor cells, *J. Nutr.* *131*, 1655-1661.
- (28) Cross, K. J., Huq, N. L., Bicknell, W., and Reynolds, E. C. (2001) Cation-dependent structural features of beta-casein-(1-25), *Biochem. J.* *356*, 277-286.
- (29) Cross, K. J., Huq, N. L., O'Brien-Simpson, N. M., Perich, J. W., Attard, T. J., and Reynolds, E. C. (2007) The role of multiphosphorylated peptides in mineralized tissue regeneration, *Int. J. Pept. Res. Ther.* *13*, 479-495.
- (30) Cross, K. J., Huq, N. L., Palamara, J. E., Perich, J. W., and Reynolds, E. C. (2005) Physicochemical characterization of casein phosphopeptide-amorphous calcium phosphate nanocomplexes, *J. Biol. Chem.* *280*, 15362-15369.
- (31) Cross, K. J., Huq, N. L., Stanton, D., Sum, M., and Reynolds, E. C. (2003) NMR spectroscopy and mass spectrometry studies of a novel calcium, phosphate and fluoride delivery vehicle - the multiphosphorylated peptide alpha(SI)-Casein(59-79) complexed with amorphous calcium fluoride phosphate, *J. Dent. Res.* *82*, 92.
- (32) Cross, K. J., Huq, N. L., Stanton, D. P., Sum, M., and Reynolds, E. C. (2004) NMR studies of a novel calcium, phosphate and fluoride delivery vehicle - α_{S1} -Casein(59-79) complexed with amorphous calcium fluoride phosphate, *Biomaterials* *25*, 5061-5069.
- (33) Huq, N. L., Cross, K. J., and Reynolds, E. C. (1995) A ^1H NMR Study of the casein phosphopeptide α_{S1} -Casein(59-79), *Biochim. Biophys. Acta* *1247*, 201-208.
- (34) Huq, N. L., Cross, K. J., and Reynolds, E. C. (2003) Nascent helix in the multiphosphorylated peptide alphaS2-casein(2-20), *J. Pept. Sci.* *9*, 386-392.

- (35) Davis, B. J. (1964) Disc electrophoresis. II. Method and application to human serum proteins, *Ann. N. Y. Acad. Sci.* 121, 404-427.
- (36) Green, M. R., Pastewska, J. V., and Peacock, A. C. (1973) Differential staining of phosphoproteins on polyacrylamide gels with a cationic carbo-cyanine dye, *Anal. Biochem.* 56, 43-51.
- (37) Reynolds, E. C. (2013) Stabilized calcium phosphate complexes. United States Patent 8,609,071.
- (38) Altieri, A. S., Hinton, D. P., and Byrd, R. A. (1995) Association of biomolecular systems via pulsed field gradient NMR self-diffusion measurements., *J. Am. Chem. Soc.* 117, 7566-7567.
- (39) Hwang, T. L., Mori, S., Shaka, A. J., and Vanzijl, P. C. M. (1997) Application of phase-modulated clean chemical exchange spectroscopy (cleanex-pm) to detect water-protein proton exchange and intermolecular noes, *J. Am. Chem. Soc.* 119, 6203-6204.
- (40) States, D. J., Haberkorn, R. A., and Ruben, D. J. (1982) A two-dimensional nuclear Overhauser experiment with pure absorption phase in four quadrants, *J. Magn. Reson.* 48, 286-292.
- (41) Wüthrich, K. (1986) *NMR of Proteins and Nucleic Acids*, Wiley and Sons, New York.
- (42) Chazin, W. J., and Wright, P. E. (1987) A modified strategy for identification of ¹H spin systems in proteins., *Biopolymers* 29, 973-977.
- (43) Huq, N. L., Cross, K. J., and Reynolds, E. C. (2000) Molecular modelling of a multi-phosphorylated sequence motif bound to hydroxyapatite surfaces, *J. Mol. Model.* 6, 35-47.

- (44) Kelley, L. A., Gardner, P. G., and Sutcliffe, M. J. (1996) An automated approach for clustering an ensemble of NMR-derived protein structures into conformationally-related subfamilies., *Protein Eng.* 9, 1063-1065.
- (45) Frishman, D., and Argos, P. (1995) Knowledge-based protein secondary structure assignment, *Proteins: Struct., Funct., Genet.* 23, 566-579.
- (46) Holt, C., Wahlgren, N. M., and Drakenberg, T. (1996) Ability of a beta-casein phosphopeptide to modulate the precipitation of calcium phosphate by forming amorphous dicalcium phosphate nanoclusters, *Biochem. J.* 314, 1035-1039.
- (47) McGann, T. C., Kearney, R. D., Buchheim, W., Posner, A. S., Betts, F., and Blumenthal, N. C. (1983) Amorphous calcium phosphate in casein micelles of bovine milk, *Calcif. Tiss. Int* 35, 821-833.
- (48) Holt, C., Timmins, P. A., Errington, N., and Leaver, J. (1998) A core-shell model of calcium phosphate nanoclusters stabilized by beta-casein phosphopeptides, derived from sedimentation equilibrium and small-angle x-ray and neutron-scattering measurements, *Eur. J. Biochem.* 252, 73-78.
- (49) Bright, J. N., Woolf, T. B., and Hoh, J. H. (2001) Predicting properties of intrinsically unstructured proteins, *Prog. Biophys. Mol. Biol.* 76, 131-173.
- (50) Tsuda, S., Niki, R., Kuwata, T., Tanaka, I., and Hikichi, K. (1991) Proton NMR study of casein phosphopeptide (1-25): Assignment and conformation, *Magn. Reson. Chem.* 28, 1097-1102.
- (51) Wahlgren, N. M., Léonil, J., Dejmek, P., and Drakenberg, T. (1993) Two-dimensional nuclear magnetic resonance study of the β -casein peptide 1-25: Resonance assignments and secondary structure., *Biochim. Biophys. Acta* 1202, 121-128.

- 1
2
3
4 (52) Cornell, W. D., Cieplak, P., Bayly, C. I., Gould, I. R., Merz, K. M., Jr., Ferguson, D. M.,
5
6 Spellmeyer, D. C., Fox, T., Caldwell, J. W., and Kollman, P. A. (1995) A second
7
8 generation force field for the simulation of proteins, nucleic acids, and organic
9
10 molecules., *J. Am. Chem. Soc.* 117, 5179-5197.
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Table 1: $^3J_{H^N-H}^a$ coupling constants and proton chemical shifts for β -casein(1-25) – ACP nanocomplex at 25° C and pH 6.00.

Residue	$^3J_{H^N-H}^a$	NH	H β	H β	H γ	H δ	γ CH ₃	NH ₂
Arg ¹	-	-	4.01	1.89, 1.89	1.65, 1.65	3.23, 3.23		
Glu ²	6.2	8.62	4.23	1.95, 1.95	2.26, 2.26			
Leu ³		8.48	4.25	1.62, 1.62	1.62	0.90, 0.90		
Glu ⁴		8.45	4.24	1.96, 1.96	2.24, 2.24			
Glu ⁵		8.51	4.29	1.95, 1.95	2.22, 2.22			
Leu ⁶	6.6	8.25	4.35	1.59, 1.59	1.59	0.88, 0.88		
Asn ⁷		8.44	4.60	2.70, 2.79				
Val ⁸	8.1	8.15	4.40	2.06	0.92, 0.92			
Pro ⁹	-	-	4.44	2.32, 2.29	2.01, 1.96	3.87, 3.71		
Gly ¹⁰		8.40	4.00, 3.87					
Glu ¹¹		8.19	4.28	1.91, 1.91	2.21, 2.21			
Ile ¹²	8.1	8.31	4.17	1.82	1.47, 1.17	0.84	0.84	
Val ¹³		8.36	4.15	-	0.90, 0.90			
Glu ¹⁴	7.0	8.53	4.34	2.02, 1.91	2.24, 2.24			
Pse ¹⁵	6.2	8.69	4.53	4.03, 4.03				
Leu ¹⁶		8.44	4.47	1.62, 1.62	1.62	0.88, 0.88		
Pse ¹⁷	6.2	8.72	4.59	4.12, 4.12				
Pse ¹⁸	5.9	8.79	4.61	4.12, 4.12				
Pse ¹⁹	5.5	8.76	4.54	4.12, 4.12				
Glu ²⁰		8.37	4.27	2.01, 2.01	2.29, 2.29			
Glu ²¹		8.41	4.25	2.01, 2.01	2.27, 2.27			

Ser ²²		8.37	4.44	3.86, 3.86			
Ile ²³	7.7	8.09	4.26	1.90	1.45, 1.19	0.89	0.89
Thr ²⁴	7.3	8.24	4.33	4.18	1.19		
Arg ²⁵	7.7	8.00	4.18	1.83, 1.72	1.58, 1.58	3.16, 3.16	7.22

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Table 2: Summary of local geometric properties and NMR constraints for the β -casein(1-25) – ACP model.

Model Property	Value	RMSD
Bond lengths	Tabulated. ⁵²	$\pm 0.017 \text{ \AA}$
Bond angles	Tabulated. ⁵²	$\pm 3.02^\circ$
Peptide linkages	180°	$\pm 11.5^\circ$
NMR range constraints	See text.	$\pm 0.22 \text{ \AA}$
NMR torsion constraints	See text.	$\pm 2.95^\circ$

FIGURE LEGENDS

Figure 1: Native gel showing the migration of α_{S1} -CN(59-79) complexed with calcium and then cross-linked with glutaraldehyde. Lane (a) α_{S1} -CN(59-79) alone, lane (b) α_{S1} -CN(59-79) complexed with calcium ions, and lane (c) α_{S1} -CN(59-79) complexed with calcium and phosphate ions.

Figure 2: Representative sLED data, the signals are integrals over the methyl resonances, for the β -CN(1-25)–ACP complex. The solid curve and associated data points are from a sample having a peptide concentration of 1.0 mM, the solid curve represents the fit to equation [3]. The dashed curve represents the fit to the first six data points acquired for a sample having a peptide concentration of 4.5 mM, note the deviation between the experiment and theory at higher gradient strengths indicative of the formation of aggregates.

Figure 3: CLEANEX-PM spectrum of β -CN(1-25)–ACP complex showing resonances of solvent exposed H α protons of Val⁸, Ile¹² or Val¹³, and a glutamate residue, and the solvent exposed H δ proton of Pro⁹.

Figure 4: Comparison of amide spectral regions of [A] β -casein(1-25) calcium ion complex, and [B] β -casein(1-25) – ACP nanocomplex.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Figure 5: (A): Amide region of NOESY recorded with a mixing time of 250 ms of the β -CN(1-25)–ACP complex showing sequential d_{NN} nOe connectivities, (B) finger-print region from same NOESY spectrum.

Figure 6: Diagrammatic summary of observed nOes and $^3J_{H^N-H^{\alpha}}$ couplings in the β -CN(1-25)–ACP complex.

Figure 7: Model of β -CN(1-25)–ACP complex using constraints summarized in the text: (A) the ACP core is shown as CPK space-filling atoms while the peptide backbones are shown in ribbon form. Each chain is 'rainbow' colour coded with shades of red toward the *C terminus*. (B) the Connolly solvent accessible surface of one of the peptides has been coloured by the hydrophilicity of the peptide and the residues associated with hydrophobic regions are labeled.

Figure 8: Detail of the β -CN(1-25)–ACP complex showing one of the peptides and the core atoms forming the interface with the peptide. Interface core atoms are shown as colour-coded spheres: oxygen atoms are red; calcium atoms are orange; and hydrogen atoms are teal. Polar contacts at 2.5 Å or less are identified by blue, dashed lines and all contacts at 3.0 Å or less by green, dashed lines.

FIGURE 1

FIGURE 2

FIGURE 3

FIGURE 4

FIGURE 5

FIGURE 6

FIGURE 7

FIGURE 8

TABLE OF CONTENTS GRAPHIC