Ability of a β -casein phosphopeptide to modulate the precipitation of calcium phosphate by forming amorphous dicalcium phosphate nanoclusters

Carl HOLT*§, N. Magnus WAHLGREN† and Torbjorn DRAKENBERG†‡

*Hannah Research Institute, Ayr KA6 5HL, Scotland, UK., †Physical Chemistry 2, Chemical Centre, University of Lund, S-221 00 Lund, Sweden, and ‡Chemical Laboratory, Technical Research Centre of Finland (VTT), Espoo, Finland

The ability of casein in the form of colloidal-sized casein micelles to modulate the phase separation of calcium phosphate during milk secretion is adapted to produce nanometre-sized particles of calcium phosphate stabilized by a casein phosphopeptide (nanoclusters). The nanoclusters were prepared from an undersaturated solution of salts and the peptide by raising the pH homogeneously from about 5.5 to 6.7 with urea plus urease. Chemical analysis and IR spectroscopy showed that they comprise an amorphous dicalcium phosphate bound to the phosphopeptide. Multinuclear NMR spectroscopy of the cluster solutions showed that the small

ions and free peptide in the solution were in a state of dynamic exchange with the nanoclusters. The peptide is linked to the calcium phosphate through its sequence of phosphorylated residues, but, in a proportion of adsorbed conformational states, the termini retain the conformational freedom of the unbound peptide. The ability of casein to form nanoclusters in milk suggests a more general mechanism for avoiding pathological calcification and regulating calcium flow in tissues and biological fluids exposed to or containing high concentrations of calcium.

INTRODUCTION

In cows' milk, the concentrations of calcium, P_i and casein are approx. 30, 20 and 1 mM respectively and about two thirds of the calcium and half the P_i are bound to the casein [1]. Chemical [1] and physical [2–4] measurements have shown that the colloidal calcium phosphate is present in the form of amorphous nanometre-sized clusters throughout the matrix of the casein micelle. Moreover, milk serum exhibits an invariant ion activity product for a calcium phosphate empirical formula corresponding approximately to that of a dicalcium phosphate ($H/P_i = 1$) [5]. Measurements made *in vitro* [6–8] have shown that individual casein proteins are capable of decreasing the rate of precipitation of calcium phosphate from a moderately supersaturated solution at concentrations of 10^{-6} M or even less, suggesting that the very rapid interaction of the phosphoprotein with calcium phosphate nuclei in such solutions is aided by the open and generally flexible conformation of casein [9]. Notwithstanding the effect of caseins in dilute solution, their action *in vivo* is clearly different, and we have sought a model system where some of the complications of milk itself can be avoided.

MATERIALS AND METHODS

Phosphopeptide

The phosphopeptide sequence is: H_2N -Arg-Glu-Leu-Glu-Glu-Leu-Asn-Val-Pro-Gly-Glu-Ile-Val-Glu-SerP-Leu-SerP-SerP-SerP-Glu-Glu-Ser-Ile-Thr-Arg-COOH. It was prepared essentially by the method of Petersen et al. [10] as modified by Manson and Annan [11], which gave a product comprising about 95% of the above sequence, i.e. residues 1–25 of bovine β -casein, together with about 5% of residues 2–25, as verified by MS, amino acid composition and N-terminal sequence analysis. The impurity was separated from the main component by chromatography on a DEAE-Sephadex A-50 column (2.5 cm $\times$ 25 cm column at a flow rate of 2.2 ml/min) with a pH gradient from 2.6 (10 mM Na_2HPO_4/HCl) to 1.6 (10 mM KCl/HCl). The main peak was

collected, freeze-dried and dissolved in a small volume of water before desalting on a Bio-Rad P2 column (1.5 cm $\times$ 40 cm column, flow rate 22 ml/h).

Preparation of nanoclusters

The initial salt solution comprised 30 mM $Ca(NO_3)_2$, 4 mM $Mg(NO_3)_2$, 10 mM tripotassium citrate, 20 mM KH_2PO_4 and 26 mM KNO_3 , with 1.5 mM NaN_3 as preservative. The phosphopeptide concentration was 1–5 mg/ml and the initial pH was 5.5. A method was required to raise the pH while avoiding precipitation, as occurred if a strongly alkaline solution was added. The problem was solved by generating ammonia homogeneously in the solution from the breakdown of urea by urease. With 5 mg/ml phosphopeptide, 12.7 mM urea was required to raise the pH to 6.7, a typical pH for cows' milk, and this could be accomplished in 4 min with 34 units/ml Sigma crystalline jack-bean urease (type C-3), or in 2 h with approx. 1.6 units/ml. Clusters began to form at about pH 6.0 and exhibited long-term stability at pH 6.7 with peptide concentrations in excess of 3 mg/ml.

X-ray scattering

Low-angle X-ray scattering measurements were performed on Station 2.1 of the Synchrotron Light Source at the CLRC Daresbury Laboratory. X-rays of wavelength 0.154 nm were scattered by a solution of clusters in a thermostatically controlled brass cell with a 1 mm path length and ruby-quality mica windows, thickness 50 μ m. A quadrant detector mounted 3 m from the sample was calibrated in d-spacings over the range 57.8–2.5 nm by using the 3rd–9th order reflections from wet rat tail collagen. Freshly prepared clusters gave a linear Guinier plot in the range $5 \times 10^{-3} \leq s \leq 1.2 \times 10^{-2} \text{ nm}^{-1}$, where s is the scattering wave vector.

§ To whom correspondence should be addressed.

IR spectroscopy

Clusters, prepared as in Figure 1 but with chloride replacing nitrate, were pelleted by centrifugation ($10^5 g$ for 18 h), washed once in water, re-pelleted, then freeze-dried and examined in a KBr pressed disc by IR spectroscopy. Mid-IR spectra were recorded with a Mattson Galaxy 7000 spectrometer at 4 cm^{-1} resolution with a DTGS detector, 32 co-added scans and a triangular apodisation function.

NMR spectroscopy

Proton spectra were recorded at 27°C on a General Electric O 500 spectrometer operating at 500.13 MHz. Spectra were acquired over 8k data points with a spectral width of $\pm 5000\text{ Hz}$. Solvent suppression was achieved by irradiation of the H_2O resonance during the relaxation delay of 30 s. The NMR spectra of ^{31}P , ^{25}Mg and ^{43}Ca were recorded on a Nicolet 360 WB spectrometer operating at 146, 22.05 and 24.35 MHz respectively. ^{31}P spectra were recorded with a normal 1-pulse sequence with a flip angle of 45° , pulse length $20\text{ }\mu\text{s}$ and repetition time of 8 s. A total of 8192 data points was accumulated by using a spectral width of $\pm 500\text{ Hz}$ and with proton decoupling to simplify the spectra. The ^{25}Mg and ^{43}Ca spectra were recorded by using a horizontal solenoid probe, and the effect of acoustic ringing was minimized by using the RIDE pulse sequence. The 90° pulse length was $40\text{ }\mu\text{s}$, and 1024 data points were recorded with a spectral width of $\pm 10\,000\text{ Hz}$. The transients were collected in blocks of 1000 and co-added in double precision. Line broadenings of 50 and 25 Hz were applied to the ^{25}Mg and ^{43}Ca FIDs (free induction decays) respectively, and the linewidth was obtained by fitting a Lorentzian function to the total response.

RESULTS AND DISCUSSION

The system devised was one in which concentrations of Ca, Mg, P_i and citrate were made comparable with typical values in cows' milk (Table 1), but with a pH of only 5.5. To eliminate the hydrophobic interactions between polypeptide chains, a hydro-

philic phosphopeptide was used. At 5 mg/ml, this peptide provides approximately the same concentration of phosphoserine residues as in cows' milk. In the absence of phosphopeptide, raising the pH homogeneously above 6 induced the formation of a calcium phosphate–citrate precipitate, but in the presence of 3 mg/ml of phosphopeptide or more the solution retained its initial homogeneity and apparent clarity (Figure 1a). However, almost exactly half the P_i was no longer ultrafilterable, and the free Ca^{2+} concentration was decreased from about 7.0 mM at pH 5.5 to 2.0 mM at pH 6.7 (Figure 1b), demonstrating that a complex had formed. Ultrafiltrate Ca, Mg, citrate, P_i and the free Ca^{2+} concentrations at pH 6.7 were very close to representative values for milk ultrafiltrate (Table 1) [5].

From the compositional data on the colloid, H/P_i was found to be 0.98 ± 0.02 , confirming the essentially dicalcic nature of the complex. Although the classical amorphous calcium phosphate is predominantly tricalcic [12], more acidic amorphous calcium phosphates have been prepared in purely inorganic systems at about neutral pH [13,14], though none so acidic and stable as this.

The formation of clusters was detected as an increase in the intensity of scattered X-rays above pH 6. A Guinier plot of freshly prepared clusters brought up to pH in 4 min gave an apparent radius of gyration of 2.4 nm. In other experiments, the time to reach pH was varied from 2 min to 2 h, and the size of the complex, measured immediately after the final pH was reached, increased linearly with $\log(\text{reaction time})$, as shown in Figure 2.

The IR spectrum (Figure 3) showed the amorphous nature of the clusters, since the degeneracy of the broad phosphate asymmetric stretch at about 1100 cm^{-1} and the phosphate bending mode at about 550 cm^{-1} were not lifted by site symmetry or factor group splitting, found in all the known crystalline calcium orthophosphates [15].

One-dimensional ^1H -NMR spectra were recorded as a function of pH during the formation of the nanoclusters (Figure 4). Apart from additional resonances from citrate (two doublets at 2.72 and 2.54 p.p.m.) and the internal dioxan standard (3.77 p.p.m.), the spectrum shown at pH 5.5 is very similar to the spectrum of the free peptide in the presence of 22 mM Ca^{2+} , for which a complete spectral assignment has been made [16]. In solution, the peptide exhibits a high degree of conformational flexibility, though certain short sequences exhibit a preference for regular secondary structure [16]. As the pH rose, some resonances broadened strongly, and effectively could not be detected at pH 6.7 (Figure 4). The resonances at 4.45–4.65 p.p.m. showed such behaviour, and these correspond to the C^α protons of the SerP residues and Ser-22. From this, it was concluded that all the peptide chains are either bound to the nanoclusters (bound lifetime $> 100\text{ ms}$) or are broadened by exchange with the nanoclusters (lifetime $< 10\text{ ms}$). In contrast, the δ -H resonances (3.20 and 3.24 p.p.m.) from the two terminal arginyl residues were broadened hardly at all, but the intensity was decreased by about 25%. Other resonances exhibited an intermediate behaviour, with both a decrease in intensity and greater broadening. From this, it may be suggested that the binding of the peptide to the calcium phosphate always involves the phosphate centre, lying in the middle of the peptide, giving it the same correlation time as the particle. In the bound state, a proportion of the peptide is strongly bound, such that the whole peptide adopts the correlation time of the particle. The remainder is more loosely bound, in that the termini retain the conformational freedom that they have when free in solution.

This model of the structure of the complexes was supported by ^{31}P -NMR measurements, which showed a narrow signal at a chemical shift of 1.95 p.p.m. (relative to 85% orthophosphoric

Table 1 Comparison of total and ultrafiltrate concentrations (mM) of salts and casein phosphate (P_i) in skim-milk and an artificial cluster solution prepared by a reaction time of 2 h.

Abbreviation: MWCO, molecular-mass cut-off.

Ion	Total		Ultrafiltrate			
	Cluster	Milk	Cluster 100 000 MWCO	Cluster 10 000 MWCO	Milk 10 000 MWCO	
Ca	33.7	30.3	11.05	8.74	10.2	
Mg	4.47	5.2	3.4	3.1	3.4	
Citrate	10.0	9.8	8.6	8.5	9.2	
P_i	22.5	21.4	12.0*	11.3*	12.4	
P_o	6.4	6.4	1.42†	0†	0	
pH	6.68	6.75				
Ca^{2+}	2.00	2.00				

* Approximate agreement of the two values suggests that the clusters permeate the 100 000 MWCO membrane only slightly.

† Ultrafiltration of a phosphopeptide solution prepared in 30 mM phosphate buffer, pH 6.7, showed that the peptide (molecular mass 3125 Da) behaved as if it was considerably extended compared with a globular protein. It was completely retained by membranes of $\leq 10\,000$ MWCO, partially permeated membranes of 30 000 and 50 000 MWCO, and freely permeated the 100 000 MWCO membrane.

Figure 1 Formation of nanoclusters by homogeneously raising the pH of a salt plus peptide solution

(a) Variation in the turbidity of calcium phosphate solutions as the pH is raised homogeneously from 5.5 to 6.7. Increasing the concentration of phosphopeptide from 1 to 5 mg/ml (values shown to right of graphs) suppressed the formation of a precipitate of calcium phosphate/citrate. (b) The pH (○, left axis) and free Ca^{2+} concentration (●, right axis) in the same salt solution as that used in (a) and containing 5 mg/ml phosphopeptide, after addition of 1.6 units/ml urease.

Figure 2 Radii of gyration of clusters shown as a function of the reaction time in min over the range 2–120 min

acid) arising from the free P_i , superimposed on which was a much broader signal (–5 to 10 p.p.m.) due to the nanoclusters (Figure 5, top). Other relatively sharp signals (3.30, 3.09 and 2.81 p.p.m.) arise from the four phosphoserine residues in the free peptide, estimated to comprise 25% of the total amount of peptide, in accordance with the ultrafiltration result (Table 1). On addition of sufficient EDTA to dissolve the clusters, the narrow P_i signal doubled in intensity (Figure 5, bottom), in good agreement with the partition of P_i found by ultrafiltration (Table 1). At the same time, the line width decreased from 7.5 to 2.5 Hz, the latter value being typical of pure phosphate solutions and the former value being consistent with broadening of the free signal mainly due to exchange with the clusters. Over the temperature range 10–65 °C the line width of the P_i signal increased only from 5.7 to 8.5 Hz, a rise comparable with that observed in milk (Figure 6), indicating that the exchange of the P_i remained slow throughout this temperature range (lifetime of the free $\text{P}_i \geq 30$ ms at 65 °C). The dynamic nature of the clusters was further demonstrated in their ^{43}Ca - and ^{25}Mg -NMR spectra (Figure 6). These showed only one resonance arising from the free metal ion.

Figure 3 Mid-IR spectrum of freeze-dried clusters at a concentration of 1% in a KBr pressed disc

Assuming C_{3v} symmetry for the HPO_4 and PO_4 species, the components around 1100 cm^{-1} are the ν_6 (E) P–O stretch at 1094 cm^{-1} , the ν_3 (A) P–O(H) stretch at about 890 cm^{-1} and the ν_2 (A) P–O stretch at 1001 cm^{-1} and the components around 550 cm^{-1} are the ν_4 (A) and ν_7 (E) O–P–O bends; the weak ν_5 (E) P–O–H bend expected at about 1230 cm^{-1} may overlap the stronger amide III band of the peptide at 1248 cm^{-1} . Wavenumber is given in cm^{-1} .

Nevertheless, exchange broadened the signal from a few Hz to 160–300 Hz. On increasing the temperature from 10 to 65 °C, the line width of the ^{43}Ca signal increased from 160 to 280 Hz, which compares closely with the behaviour observed for native casein micelles [17]. From this, the lifetime of the ion in the continuous phase is estimated to be at least 1 ms at 65 °C. The line width of the ^{25}Mg signal was also about 1 ms, but it showed little, if any, dependence on temperature.

Many properties of the artificial clusters appear to be similar to properties of the micellar calcium phosphate nanoclusters isolated from native casein micelles [1]. Thus, micellar calcium

Figure 4 ^1H -NMR spectra of (top) clusters prepared with a reaction time of 2 h to reach a final pH of 6.7 and (bottom) the same solution at pH 5.5 before urease was added

phosphate clusters were found to be similar in size to the artificial clusters (results not shown). The IR spectra are very similar (cf. Figure 3 and Figure 5.17 of [16]), as are the exchange rates of the free P_i and Ca^{2+} ions (cf. Figure 6 (this paper) with Figure 5 of [15]). In overall composition and solubility, the artificial clusters resemble the native ones (Table 1), and they comprise an amorphous dicalcium phosphate in which a proportion of the HPO_4^{2-} ions are substituted by the RPO_4^{2-} moieties of the phosphopeptide, possibly at the surface sites.

The mammary gland is one of the most active organs known to mammalian biology in terms of the transport of calcium [18] and, not surprisingly, there is an associated pathology, in which *corpora amylacea* granules form in the cisterns and ducts [19]. Highly phosphorylated phosphoproteins are widely distributed and relatively abundant in mineralizing tissues (both calcium phosphate- and calcium carbonate-forming), but their role is obscure (reviewed in [20]). In saliva, for example, where the chemistry of the fluid is known at a level of detail comparable with that for milk, proline-rich phosphoproteins are found which show a number of similarities of sequence to β -casein [9]. They are thought to be involved in some aspect of tooth mineralization. It has been suggested [9,20] that the generally open and flexible conformation in solution of a number of the most important highly phosphorylated phosphoproteins of mineralizing tissues

Figure 5 The ^{31}P NMR spectrum of nanoclusters at pH 6.7 (top) and after adding sufficient EDTA at pH 6.7 to dissolve the calcium phosphate particles (bottom)

Figure 6 Variation in ^{43}Ca and ^{25}Mg (◆, ■, left axis) and $^{31}\text{P}_i$ (right axis: ●, cluster solution; ○, $^{31}\text{P}_{\text{nat}}$: data for native casein micelle [17]) linewidths with temperature

facilitates a rapid interaction with calcium phosphate nuclei during the precipitation process. The term *rheomorph* [from the Greek *rheos* (stream) and *morphe* (form)] has been coined [9] to describe proteins with an open and flexible conformation in preference to random coil, which perhaps implies a lack of

biological function. Thus, many of the phosphoproteins isolated from mineralizing tissues have been shown to act *in vitro* to decrease the rate and extent of precipitation of calcium phosphate from solution at concentrations of 10^{-6} M or less [20]. However, it has also been postulated that some highly acidic but unphosphorylated rheomorphic proteins (chromogranins) [21–23] found in synaptic vesicles containing catecholamines at a concentration as high as 6 M might also be important in maintaining the stability of the vesicular fluid [9]. In the light of the results reported here on casein, it may be conjectured that even rheomorphic proteins cannot act quickly enough to control precipitation at μ M concentrations in even the moderately supersaturated solutions used for the assays *in vitro*. In one such assay [6], the rate of nucleation was estimated to be about $10^{19} \text{ ml}^{-1} \cdot \text{s}^{-1}$, and inhibition of nucleation was found to be detectable at phosphoprotein concentrations $\geq 10^{-7}$ M. The catalytic-centre activity of the protein molecules responsible for inhibiting nucleation (i. e. about 10^6 – 10^7 s^{-1}) is therefore about the same as those for the fastest enzymes known to science, such as carbonic anhydrase and peroxidase.

The present results suggest that a short phosphopeptide at a concentration of about 10^{-3} M can act to form a stable population of calcium phosphate nanoclusters, thereby preventing uncontrolled precipitation from even a grossly supersaturated solution. Moreover, the ability to vary the size of the clusters through the rate of increase of pH argues against the formation of a fixed stoichiometric complex of calcium phosphate with the phosphopeptide and in favour of the idea that the phosphopeptide stabilizes the precipitation process shortly after the nucleation of the solid phase. The solution of nanoclusters containing 5 mg/ml phosphopeptide was stable to steam sterilization and could be stored for months at ambient temperature without appreciable change in appearance.

E.M. Little and J. Leaver are thanked for preparing and characterizing the phosphopeptide and the clusters. S. Slawson, the Director and other staff of the CLRC

Daresbury Laboratory, and X.L. Qi of the Hannah Research Institute, are thanked for help in measuring the size of nanoclusters by small-angle X-ray scattering. The research was supported in part by the Scottish Office Agriculture Environment and Fisheries Department.

REFERENCES

- Holt, C. (1992) *Adv. Protein Chem.* **43**, 63–151
- Lyster, R. L. J., Mann, S., Parker, S. B. and Williams, R. J. P. (1984) *Biochim. Biophys. Acta* **801**, 315–317
- Knoop, A., Knoop, E. and Wiechen, A. (1979) *J. Dairy Res.* **46**, 347–350
- Holt, C. and Hukins, D. W. L. (1991) *Int. Dairy J.* **1**, 151–165
- Holt, C. (1985) *Dev. Dairy Chem.* **3**, 143–181
- van Kemenade, M. J. J. M. (1988) Ph.D. Thesis, University of Utrecht
- van Kemenade, M. J. J. M. and de Bruyn, P. L. (1989) *Colloids Surf.* **36**, 359–368
- van Kemenade, M. J. J. M. and de Bruyn, P. L. (1989) *J. Colloid Interface Sci.* **129**, 1–14
- Holt, C. and Sawyer, L. (1993) *J. Chem. Soc. Faraday Trans.* **89**, 2683–2692
- Petersen, R. F., Nauman, L. W. and McMeekin, T. L. (1958) *J. Am. Chem. Soc.* **80**, 95–97
- Manson, W. and Annan, W. D. (1971) *Arch. Biochem. Biophys.* **145**, 16–26
- Betts, F. and Posner, A. S. (1974) *Trans. Am. Crystallogr. Assoc.* **10**, 73–84
- Holt, C., van Kemenade, M. J. J. M., Nelson, L. S., Jr., Harries, J. E., Bailey, R. T., Hukins, D. W. L., Hasnain, S. S. and de Bruyn, P. L. (1989) *Mater. Res. Bull.* **23**, 55–62
- Christoffersen, J., Christoffersen, M. R., Kibalczyk, W. and Andersen, F. A. (1989) *J. Cryst. Growth* **94**, 767–777
- Elliot, J. C. (1994) *Structure and Chemistry of the Apatites and Other Calcium Orthophosphates*, Elsevier, Amsterdam
- Wahlgren, N. M., Léonil, J., Dejmek, P. and Drakenberg, T. (1993) *Biochim. Biophys. Acta* **1202**, 121–128
- Wahlgren, N. M., Dejmek, P. and Drakenberg, T. (1990) *J. Dairy Res.* **57**, 355–364
- Bauman, D. E. and Elliot, J. M. (1983) in *Biochemistry of Lactation* (Mepharm, T. B., ed.), pp. 437–468, Elsevier, Amsterdam
- Brooker, B. E. (1978) *Cell Tissue Res.* **191**, 525–538
- Holt, C. and van Kemenade, M. J. J. M. (1989) in *Calcified Tissue* (Hukins, D. W. L., ed.), pp. 21–33, Macmillan, London
- Simon, J.-P. and Aunis, D. (1989) *Biochem. J.* **262**, 1–13
- Smith, A. D. and Winkler, H. (1967) *Biochem. J.* **103**, 483–492
- Winkler, H. (1976) *Neuroscience (Oxford)* **1**, 65–80