Cation-dependent structural features of β -casein-(1–25)

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Complete sequence-specific, proton-resonance assignments have been determined for the calcium phosphate-stabilizing tryptic peptide β -casein-(1–25) containing the phosphorylated sequence motif Ser(*P*)¹⁷-Ser(*P*)-Ser(*P*)-Glu-Glu²¹. Spectra of the peptide have been recorded, in separate experiments, in the presence of excess ammonium ions, sodium ions and calcium ions, and of the dephosphorylated peptide in the presence of excess sodium ions. We observed significant changes to chemical shifts for backbone and side-chain resonances that were dependent upon the nature of the cation present. Medium-range nuclear Overhauser effect (nOe) enhancements, characteristic of small structured regions in the peptide, were observed and also found to be cation dependent. The secondary structure of the peptide was characterized by sequential and medium-range (*i*, *i* + 2/3/4, which denotes an

interaction between residue *i* and residue *i* + 2, *i* + 3 or *i* + 4 in the peptide) nOe connectivities, and H α chemical shifts. Four structured regions were identified in the calcium-bound peptide: residues Arg¹ to Glu⁴ were involved in a loop-type structure, and residues Val⁸ to Glu¹¹, Ser(*P*)¹⁷ to Glu²⁰ and Glu²¹ to Thr²⁴ were implicated in β -turn conformations. Comparison of the patterns of medium-range nOe connectivities in β -casein-(1–25) with those in α _{s1}-casein-(59–79) suggest that the two peptides have distinctly different conformations in the presence of calcium ions, despite having a high degree of sequential and functional similarity.

Key words: anticariogenic peptides, bioactive peptides, α _{s1}-casein-(59–79), ¹H NMR, structure.

INTRODUCTION

Multi-site, hierarchical protein phosphorylation is a prevalent form of protein modification. It has been suggested that this type of modification allows for the regulation of protein activity by expanding the structural repertoire [1]. However, apart from a study of α _{s1}-casein-(59–79), a tryptic-casein peptide containing the fully phosphorylated -Ser(*P*)-Ser(*P*)-Ser(*P*)-Glu-Glu- motif in which structured regions were identified [2], little is known about the conformational preferences of multi-phosphorylated proteins and peptides.

The sequence -SSSEE- has been observed in over 200 protein sequences [3]. The proteins containing this sequence include not only the caseins, but also a variety of growth factors, kinases, immunoglobins and nuclear regulatory proteins. This sequence has the potential to be phosphorylated by the ordered action of a mammary-gland-like kinase that recognizes the motif -S-X-E/S(*P*)-, or to be partially phosphorylated by casein kinase II, which recognizes the sequence -S-X-X-E/S(*P*)-. Furthermore, some of the proteins have acidic residues flanking both sides of the serine cluster (for example -EESSEE-), such that the serine residues may be completely phosphorylated by the combined action of casein kinases I and II, where casein kinase I recognizes the motif -E/S(*P*)-X-X-S- [1].

Multi-phosphorylated proteins also play an important role in the processes of biomineralization. In the development of teeth and bone they act as nucleators of hydroxyapatite and control the growth of the crystals, resulting in a unique crystal morphology characteristic of biological systems. In body fluids such as saliva and milk, phosphorylated proteins maintain metastable solutions that are supersaturated with respect to hydroxyapatite. In milk, calcium phosphate is stabilized in micelles in combination with proteins known as caseins. The caseins are large proteins that overall show substantial amino acid sequence differences between species and act as sources of bioactive peptides and

nutrients for the neonate upon digestion. Overall, the caseins appear to lack any structure in solution, with many side chains exposed to solvent. The calcium-sensitive caseins that are responsible for binding to calcium phosphate are characterized by clusters of acidic residues, including the fully phosphorylated sequence motif SSSEE. This phosphorylated motif is found in the calcium-sensitive bovine caseins α _{s1}-, α _{s2}- and β -casein, and in the tryptic peptides α _{s1}-casein-(59–79) and β -casein-(1–25). The tryptic peptides sequester their own weight in amorphous calcium phosphate to form colloidal complexes [4]. Furthermore, there is evidence that, after digestion, these casein phosphopeptides are found in the soluble part of the intestinal chyme [5,6] and even in the faeces [7] of experimental animals, due to proteolytic resistance of the peptides. The calcium phosphate binding properties of the tryptic casein phosphopeptides, coupled with their ability to form soluble complexes, supports a biological role as calcium carriers.

We are investigating the structure–function relationships of the two multi-phosphorylated tryptic peptides α _{s1}-casein-(59–79) and β -casein-(1–25). Both of these peptides stabilize amorphous calcium phosphate and exhibit anticariogenic activity through their ability to stabilize and localize amorphous calcium phosphate at the tooth surface, thereby inhibiting enamel demineralization and promoting enamel remineralization [8–11]. The tryptic peptides α _{s1}-casein-(59–79) and β -casein-(1–25) contain five and four O-phosphorylated serine residues respectively. The sequences are shown below using the one-letter code, with the Ser(*P*) cluster sequence underlined; the symbol Σ signifies a Ser(*P*).

α _{s1}-casein-(59–79): Q⁵⁹MEAE Σ I Σ Σ Σ Σ EEIVPN Σ VEQK⁷⁹

β -casein-(1–25): R¹ELEELNVPGEIVE Σ L Σ Σ Σ EEISITR²⁵

We have reported previously the structural features of α _{s1}-casein-(59–79) derived from a proton NMR spectroscopic study [2].

Abbreviations used: DQF-COSY, double-quantum-filtered COSY; FTIR, Fourier-transform IR; nOe, nuclear Overhauser effect; rOe, rotating-frame Overhauser effect; ROESY, rotating-frame Overhauser enhancement spectroscopy; TPPI, time proportional phase incrementation.

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Two previous ^1H NMR studies of the β -casein-(1–25) peptide in the presence of calcium ions failed to detect medium- and long-range nuclear Overhauser effect (nOe) enhancements characteristic of structured peptides [12,13]. Tsuda et al. [12] concluded that there was no evidence that the motif V⁸PGE¹¹ was in a β -turn conformation. Wahlgren et al. [13] concluded that β -casein-(1–25) does not contain stable folded structures, but the structure was neither that of a fully extended peptide nor that of a random coil. It was also concluded that the addition of calcium ions decreased the population of extended structure in the Ser(P)¹⁸ to Ile²³ region [13]. Neither of these two earlier studies was able to identify specific structural features of the calcium-bound peptide. In this paper, we present the results of a ^1H NMR spectroscopy study of β -casein-(1–25), and then compare the structural features of β -casein-(1–25) with those of α_{S1} -casein-(59–79) in the presence of calcium ions.

EXPERIMENTAL

The casein phosphopeptides were precipitated selectively from a tryptic digest of casein by the addition of 0.1 M CaCl_2 solution and 50% (v/v) ethanol. Further purification of β -casein-(1–25) was by anion-exchange FPLC and reversed-phase HPLC [9,14]. The purity of the peptide was confirmed by capillary electrophoresis, amino acid composition and sequence analysis, as described previously [14,15].

Five samples were prepared for NMR spectroscopy, each containing 10 mM β -casein-(1–25) peptide in a total sample volume of 650 μl . A sample with 4 mol of Ca^{2+} /mol of peptide was prepared at pH 6.30 in 99.996% $^2\text{H}_2\text{O}$ for a double-quantum-filtered (DQF)-COSY spectrum. The remaining samples were all prepared in 80% H_2O /20% $^2\text{H}_2\text{O}$. The second sample was prepared with 4 mol of Ca^{2+} /mol of peptide at pH 6.55 for NOESY, TOCSY and rotating-frame Overhauser enhancement spectroscopy (ROESY) spectra. The pH of the first and second samples was adjusted by the dropwise addition of 1 M NaOH; these samples will be referred to as calcium β -casein-(1–25). The third sample at pH 6.30 contained no added calcium ions; 1 M NaOH was used to adjust the sample pH. This sample will be referred to as sodium β -casein-(1–25). The fourth sample contained no calcium ions and was adjusted to pH 6.30 by the dropwise addition of ammonium hydroxide, and will be referred to as ammonium β -casein-(1–25). Dephosphorylated β -casein-(1–25) was prepared using mild alkaline (NaOH) conditions [16], and then adjusted to pH 6.3 with HCl.

^1H NMR spectroscopy

NMR spectra were acquired at a temperature of -5°C ; the probe temperatures were calibrated using a sample of neat methanol- $^2\text{H}_4$. Freezing point depression by the solutes allowed spectra to be acquired at -5°C in 80% H_2O solution. Experiments were performed on three spectrometers: a Varian VXR-400 spectrometer operating at a proton frequency of 399.952 MHz, a Bruker AMX-500 spectrometer at 500.139 MHz, and a Varian UNITY^{INOVA} spectrometer at 599.741 MHz. The following spectra were accumulated in the phase-sensitive mode using the States time-proportional phase incrementation (TPPI) method [17] on the Varian spectrometers: DQF-COSY [18,19], NOESY [20,21], TOCSY [homonuclear Hartman–Hahn spectroscopy (HOHAHA)] [22,23] and ROESY (CAMELSPIN) [24,25]. The DQF-COSY spectrum was acquired with 4000 complex points in T_2 (the detection time period) and 800 increments in T_1 (the evolution time period). The spectrum was zero filled to 4000 complex points in T_1 and weighted by a

sinebell window function. NOESY, TOCSY and ROESY spectra were acquired with 1600–2048 complex points in T_2 and 300 increments in T_1 . Linear prediction was used to correct the first five points in T_1 and to extend the T_1 free induction decays by 150 points to avoid truncation artifacts. Spectra were zero-filled to 2048 complex points and weighted by a Hamming window function [26]. Two phase-sensitive NOESY spectra were acquired using TPPI on the Bruker AMX spectrometer.

Spectra recorded on the Varian spectrometers in H_2O solution were digitally filtered with a 20-pole tan Butterworth filter having a null width of 140 Hz before Fourier transformation to eliminate the water resonance and its dispersive contribution to the spectra [27]. Data processing was carried out using Varian's VnmrSGI program running on an SGI Indigo² workstation equipped with 192 MB of RAM. Data acquired on the Bruker spectrometer were processed using Bruker's UXNMR program.

The resonances were assigned using the standard sequential assignment procedure [28,29]. Secondary chemical shifts were determined by subtracting the random-coil shifts of Wishart et al. [30] from the chemical shifts determined in the present study.

RESULTS AND DISCUSSION

The spin systems of a number of amino acyl residues of β -casein-(1–25) could be assigned from inspection of the DQF-COSY and 80 ms TOCSY spectra. These spin systems included Arg¹ and Arg²⁵ (the latter having an amide proton resonance), Pro⁹, Gly¹⁰ and Thr²⁴. The relatively low-frequency NH and H α shifts for the single unphosphorylated serine residue helped to distinguish it from the phosphoserine residues. This assignment was confirmed during the sequential assignment process. Finally, Asn⁷ could be readily assigned; the H α of the asparagine was the highest-frequency H α resonance observed for this peptide. Using these starting points, a sequential assignment was completed by following the standard sequential assignment procedure [28,29]. In practice, the spectra were very similar to each other and the assignment of one spectrum provided a guide to the assignment of later spectra. The dehydroalanine residues, present in the dephosphorylated peptide, do not have H α protons, and thus these residues could not be sequentially assigned using ^1H NMR spectra.

Table 1 summarizes the secondary chemical shifts of β -casein-(1–25) in the presence of sodium ions, ammonium ions and calcium ions, and of dephosphorylated β -casein-(1–25) in the presence of sodium ions. Figures 1 and 2 show the fingerprint regions from a 300 ms NOESY and a 125 ms ROESY spectrum respectively. Sequential and medium-range connectivities are identified. All rotating-frame Overhauser effects (rOes) are positive, i.e. the cross-peaks have the opposite phase to the diagonal peaks. The secondary shifts of the amide protons are summarized in Figure 3(A) and the H α secondary shifts are summarized in Figure 3(B). Since the random-coil shifts for phosphorylated amino acyl residues are not available, we have used the value corresponding to a serine residue. For comparison purposes, the secondary shifts for β -casein-(1–25) in the absence of calcium reported by Wahlgren et al. [13] are also summarized in Figure 3. To facilitate the comparison of the secondary shifts, shown graphically in Figure 3, we calculated the correlation coefficients between the amide secondary shifts and between the H α secondary shifts for the different cations [31].

Non-sequential nOes were observed in three of the spectra: in the presence of sodium ions, ammonium ions and calcium ions. The number of sequential and non-sequential nOes, their intensity, and the identity of the interacting atoms depended upon

Table 1 Secondary proton chemical shifts at 5 °C and pH 6.30 of dephosphorylated β -casein-(1–25) in the presence of sodium ions, and of β -casein-(1–25) in the presence of sodium, ammonium or calcium ions

Secondary shifts, separated by semi-colons, are in the order (from left to right): dephosphorylated; sodium; ammonium; calcium. Values separated by a comma represent secondary shifts of geminal protons. Asterisks (*) denote resonances that are not assigned or missing.

Residue	Chemical shift (p.p.b.)				
	NH	α H	β H	γ H	Others
Arg ¹		–250; –250; –241; –320	–10, 70; –30, 80; 21, 89; 170, 20	110, 30; 0, 70; 147, 67; 90, 10	(δ CH ₂): 90, 130; 100, 140; 112, 152; 40, 80; (ϵ NH ₂): 735, 55; 710, 30; 782, 102; 720, 40
Glu ²	652; 610; 673; 600	120; 140; 144; 70	–25, 71; –30, 70; –21, 71; –100, –170	46, –14; 60, 15; 64, 4; –40, –40	
Leu ³	929; 890; 970; 890	–10; –20; 0; –70	131, –29; 70, –90; 70, –50; 90, –70	88, 0; 0, 0; 90, 0; 30, 0	(δ CH ₃): 290, 170; 270, 150; 240, 120; 190, 110
Glu ⁴	350; 310; 384; 320	32; 30; 38; –40	6, –40; 0, –60; 4, –106; 50, –20	9, –51; 10, –50; 12, –48; 0, 60	
Glu ⁵	273; 220; 307; 260	53; 50; 64; –20	–18, –33; –30, –40; –16, –26; –80, –120	–18, –78; –20, –80; –20, –80; –80, –140	
Leu ⁶	420; 400; 461; 440	10; 20; –5; –30	131, –29; 50, –110; 20, –50; –50, –140	30, 0; 70, 0; 50, 0; –30, 0	(δ CH ₃): 234, 156; 230, 110; 220, 180; 150, 100
Asn ⁷	254; 230; 320; 250	104; 100; 104; 30	193, –187; 160, –170; 159, –116; 50, –210		(δ NH ₂): 27, 68; –10, 70; 55, 80; 10, 60
Val ⁸	230; 220; 277; 250	290; 310; 331; 250	34, 0; 30, 0; 40, 0; –40, 0	127, 30; 140, 0; 130, –10; 70, –40	
Pro ⁹		91; 80; 102; 20	512, –225; 500, –220; 523, –211; 450, –270	327, 187; 300, 160; 330, 190; 260, 120	(δ CH ₂): 295, 233; 250, 230; 304, 261; 300, 255
Gly ¹⁰	225; 180; 267; 190	372; 240; 279; 240	–206, 0; –80, 0; –84, 0; –80, 0		
Glu ¹¹	–111; –110; –70; –110	77; 70; 90; 0	–34, 44; –50, 0; 70, –40; 10, –10	–24, –84; –20, –80; –50, –40; 50, –70	
Ile ¹²	480; 460; 545; 500	90; 90; 100; 30	146, 0; 140, 0; 160, 0; 80, 0	95, 302; 80, 300; 100, 310; 40, 320	(δ CH ₃): 182, 0; 170, 0; 180, 0; 110, 0; (γ CH ₃): 124; 120; 130; 60
Val ¹³	440; 430; 530; 470	60; 50; 70; –10	–16, 0; –20, 0; –10, 0; 160, 0	127, –13; 120, –20; 141, 1; 70, –70	
Glu ¹⁴	184; 270; 344; 270	39; 130; 123; 40	–22, –24; –30, –10; –12, –15; –80, –80	–9, –69; 20, –40; 16, –44; –50, –110	
Ser(P) ¹⁵	*; 460; 621; 590	*; 200; 196; 100	*; *, 270, 220; 226, 160; 150, 70		
Leu ¹⁶	640; 570; 697; 670	–70; 150; 148; 90	120, –40; 80, –80; 50, –33; 60, –100	66, 0; 100, 0; 80, 0; 100, 0	(δ CH ₃): 234, 156; 210, 90; 240, 190; 160, 100
Ser(P) ¹⁷	*; 450; 621; 580	*; 240; 224; 200	*; *, 350, 230; 329, 209; 260, 140		
Ser(P) ¹⁸	*; 510; 729; 730	*; 260; 250; 170	*; *, 340, 220; 314, 194; 240, 120		
Ser(P) ¹⁹	*; 470; 678; 620	*; 200; 187; 110	*; *, 350, 230; 323, 203; 240, 120		
Glu ²⁰	87; –20; 160; 80	42; 100; 56; 20	–9, –30; 60, 40; 10, –21; 10, –100	–29, –89; 90, 30; –2, –10; –310, –370	
Glu ²¹	87; 30; 220; 140	42; 40; 45; –20	–9, –30; 60, –50; –2, –112; –10, –100	–29, –89; –50, –10; 2, –30; –330, –360	
Ser ²²	220; 140; 257; 210	107; 80; 95; 30	60, –60; 60, –60; 75, 56; 0, –120		
Ile ²³	310; 180; 325; 270	190; 210; 227; 150	225, 0; 230, 0; 250, 0; 170, 0	107, 279; 110, 270; 115, 290; 120, 290	(δ CH ₃): 182, 0; 220, 0; 180, 0; 120, 0; (γ CH ₃): 179; 170; 198; 190
Thr ²⁴	250; 190; 281; 220	–4; –20; –2; –70	–22, 0; –20, 0; –11, 0; –80, 0	30, 0; 30, 0; 40, 0; 30, 0	
Arg ²⁵	–30; –100; –19; –70	–105; –110; –99; –170	–90, 18; 30, 10; 44, 20; –20, –50	90, 10; 80, 0; 100, 20; 80, 0	(δ CH ₂): 63, 103; 60, 100; 69, 109; 0, 40; (ϵ NH ₂): 673, –7; 630, –50; 705, 25; 630, –50

Figure 1 Fingerprint region from 300 ms NOESY of β -casein-(1–25) in the presence of calcium ions at 600 MHz

Sequential nOes are connected by a solid line, and the intra-residue cross-peaks are labelled by residue. Medium-range nOes are circled.

Figure 2 Fingerprint region from 125 ms ROESY of β -casein-(1–25) in the presence of calcium ions at 500 MHz

Sequential rOes are connected by a solid line, and the intra-residue cross-peaks are labelled by residue. Medium-range rOes are circled.

the nature of the cation present in solution. The observed nOes are summarized in Figure 4. Figure 5 shows the dependence of the d_{NN} nOe intensity on the cation present.

In a previous ^1H NMR study of β -casein-(1–25), Tsuda et al. [12] was unable to complete the sequential assignment of the N-terminal residues. The chemical shifts reported for the NH resonances of Glu² and Leu³ [12] differed by a significant amount from the values found in the present study. Furthermore, titration of calcium ions into the sample was reported by these authors to

have little effect on chemical shifts (changes of less than 0.03 p.p.m.) [12]. These authors [12] also reported that the chemical shifts for most H_α and side-chain resonances were within 0.08 p.p.m. of the random-coil values reported by Bundi and Wüthrich [32]. Our analysis of their results indicates that substantial deviations from random-coil values did in fact occur for a number of residues. Finally, Tsuda et al. [12] were unable to observe any non-sequential nOes, and therefore concluded that β -casein-(1–25) had no preferred conformation and that, in

Figure 3 Comparison of secondary chemical shifts for β -casein-(1–25) as a function of the counter-ion for (A) amide-proton resonances and (B) H α -proton resonances

The shading of the bars indicates the counter-ion present according to the key. 'Wahlgren et al.' is ref. [13].

particular, there was no NMR evidence supporting the existence of a β -turn in the sequence -Val⁸-Pro⁹-Gly¹⁰-Glu¹¹-.

Wahlgren et al. [13], in their ¹H NMR study of β -casein-(1–25), were able to complete the sequential assignment of β -casein-(1–25), and reported chemical shifts similar to those in Table 1. However, in their study calcium titration had little effect on chemical shifts, with only five NH resonances changing by an amount ≥ 0.10 p.p.m.; one H α resonance changed by 0.05 p.p.m., and side-chain resonances changed by less than 0.02 p.p.m. No non-sequential nOes were observed using either the calcium-free or the calcium-bound samples. These authors concluded that there was no evidence for a stable, folded conformation of β -casein-(1–25) [13]. However, based on their reported changes in chemical shifts and decreased intensities of the d_{αN} nOe peaks in the phosphoserine region, an increased tendency to α -helical or turn-type structure was indicated for residues Ser¹⁸ to Glu²⁰ when calcium was complexed with the phosphoserine cluster motif.

Chemical shifts

The results of the present study show that, independently of the nature of the cation, many residues of β -casein-(1–25) displayed

significant secondary chemical shifts of both amide and H α proton resonances (Figure 3). Using the chemical shift index method of Wishart et al. [30], the data suggest that these large secondary shifts were not the result of regular secondary structure. In α -helix and β -sheet structures, the direction of the amide secondary shift tends to be opposite to that of the H α secondary shift. Nevertheless, these large secondary shifts are difficult to reconcile with the assertion in previous reports [12,13] that β -casein-(1–25) adopts a random-coil or extended structure.

Chemical shift is dependent on the electron density at the observed nucleus. Consequently, it is sensitive to a number of influences, such as changes in secondary structure and electrostatic interactions between metal ions and, for example, carbonyl oxygen atoms. These effects may be difficult to disentangle, but comparison of the changes in chemical shifts obtained in the presence of different cations allows certain broad conclusions to be drawn. First, comparison of the shifts observed in dephosphorylated β -casein-(1–25) with those of the peptide in the presence of excess sodium ions revealed substantial differences in H α shifts from about Pro⁹ to Glu²⁰ and differences in amide shifts from Glu¹⁴ to Thr²⁴, not just in residues adjacent to the -Ser(P)-Ser(P)-Ser(P)-Glu-Glu motif. There is little evidence that sodium β -casein-(1–25) adopts a preferred conformation; the absence of medium- or long-range nOes suggests a fairly flexible

Figure 4 Observed nOes for β -casein-(1–25) illustrating their counter-ion dependence

(A) Dephosphorylated β -casein-(1–25); (B) β -casein-(1–25) in the presence of sodium ions; (C) β -casein-(1–25) in the presence of ammonium ions; (D) β -casein-(1–25) in the presence of calcium ions. Thinner lines for d_{NN} nOes indicate low-intensity peaks. Broken lines indicate peaks observed in ROESY spectra.

structure. However, the large secondary shifts observed in sodium β -casein-(1–25) are similar to those observed in calcium β -casein-(1–25), which clearly adopts a preferred conformation, with ϕ/ψ angles in the α_R region of the Ramachandran plot. The peptide in the presence of sodium and ammonium ions appears to be conformationally labile, with a preference for the α_R region of the Ramachandran plot. The change in chemical shifts for residues remote from the phosphorylated motif suggests that these residues interact with the motif residues because of the non-random-coil dynamic structure of the peptide. Secondly, there are significant differences between the secondary shifts recorded in the presence of sodium or ammonia counter-ions and those recorded in the presence of calcium counter-ions. This suggests that there is something qualitatively different about the in-

teraction of β -casein-(1–25) with calcium ions compared with its interaction with sodium or ammonium ions.

nOes

Figure 5 shows expansions of the amide regions of two β -casein-(1–25) NOESY spectra collected under similar conditions, but with either sodium or calcium as counter-ions. A few, weak d_{NN} nOes were observed in the spectra of β -casein-(1–25) recorded with either sodium (Figure 5A) or ammonia counter-ions, or of dephosphorylated β -casein-(1–25) with sodium counter-ions. However, in the presence of calcium counter-ions there was a dramatic increase in both the number and the relative intensity of the d_{NN} nOes (Figure 5B). These observations are consistent with

Figure 5 Expansions of the amide regions from NOESY spectra of β -casein-(1–25) buffered using NaOH (A), and with added CaCl_2 and buffered using NaOH (B)

Note the dramatic increase in intensity of the d_{NN} cross-peaks in spectrum (B). The diagonal peaks are scaled to the same absolute values in each spectrum.

a co-operative nature of calcium ion binding by β -casein-(1–25). The phosphoserine groups are essential for calcium ion binding to occur, but the results of the present study suggest that calcium ion binding involves changes to the β -casein-(1–25) backbone conformation, allowing residues at sites remote from the phosphoserine residues to also interact with the calcium ions. Comparison of our data in Figure 5 with those of Wahlgren et al. [13] indicates that, apart from changes in chemical shift, these authors

[13] did not observe major changes in the number or intensity of the d_{NN} nOes. In the present study, only a few, isolated non-sequential nOes were observed for β -casein-(1–25) with either sodium or ammonium counter-ions. However, in the presence of calcium ions a number of non-sequential nOes were observed. Coupled with the dramatic increase in the intensity of d_{NN} nOes, this suggests that the distribution of conformations of β -casein-(1–25) is weighted towards conformations with many of the amino acyl residues in the α_{R} region of the Ramachandran plot. This would be consistent with either a helical structure or a structure containing turns and loops. The absence of a pattern of overlapped $d_{\text{NN}}(i, i+4)$ (i.e. interaction between residues i and $i+4$ in the peptide), $d_{\text{NN}}(i, i+3)$, $d_{\text{NN}}(i, i+2)$ or $d_{\text{NN}}(i, i+1)$ connectivities is not consistent with rigid helical structures [28], a conclusion that supports findings from earlier CD, ORD and NMR studies of the intact caseins and related phosphoproteins [33–36].

The identification of tight turns by qualitative NMR data of the type presented in Figure 4 is problematic [28]. A list of accepted criteria [37] for turn formation includes: (a) d_{NN} nOe connectivities connecting some or all of the residues in the turn; (b) a $d_{\text{NN}}(i+2, i+4)$ nOe connectivity; (c) appropriate $^3J_{\text{HN}\alpha}$ coupling constants; (d) evidence of hydrogen bond formation involving the amide proton of residue 4 of the turn, e.g. slow exchange of the amide proton with solvent or a low temperature coefficient for the NH resonance. The unambiguous identification of $d_{\text{NN}}(i+2, i+4)$ nOes depends on the signals occurring in spectrally uncluttered regions. Furthermore, because small peptides exist in solution as a mixture of interconverting conformers, the observation of appropriate coupling constants and low temperature coefficients for the NH resonance of the $i+4$ residue cannot always be made.

From the current NMR data on β -casein-(1–25), four structured regions have been identified: the N-terminus comprising residues Arg¹ to Glu⁴; residues Val⁸ to Glu¹¹; the multi-phosphorylated region comprising residues Ser(P)¹⁷ to Glu²⁰; and the C-terminus comprising residues Glu²¹ to Thr²⁴.

The first structured region of β -casein-(1–25) extends from Arg¹ to Glu⁴. Unusually large secondary chemical shifts of the amide resonances, such as those associated with residues Glu² and Leu³, strongly suggest the formation of some secondary structural feature near the N-terminus of the molecule. NOESY spectra failed to reveal any cross-peaks associated with these N-terminal residues. However, a ROESY spectrum recorded with a mixing time of 125 ms at a field strength of 500 MHz showed a weak $d_{\text{NN}}(1,4)$ peak. This is consistent with the formation of a loop-type structure located at the N-terminus of the molecule.

The second structured region of the peptide β -casein-(1–25) extends from Val⁸ to Glu¹¹. The H α resonance of Val⁸ was shifted significantly from the random-coil value. The NH resonance of Glu¹¹ was also shifted towards lower frequencies in the presence of calcium. Using the parameters of Wilmot and Thornton [38,39], the sequence VPGE is predicted to have high type II β -turn potential. The existence of a β -turn in this region would be expected to give rise to a $d_{\text{NN}}(9,11)$ nOe. Unfortunately, doubling of the cross-peaks associated with Asn⁷, Val⁸ and Gly¹⁰ due to *cis-trans* isomerism about the Pro⁹ bond resulted in excessive congestion in the spectral region where a $d_{\text{NN}}(9,11)$ nOe might be expected. Inspection of models of various types of β -turn indicates that medium-range nOes between $\delta\delta'$ H of Pro⁹ and the NH of Glu¹¹ might be observable in type I' and type II' β -turns. We are unable to provide an explanation for the observation of both $d_{\text{NN}}(9,11)$ and $d_{\text{NN}}(9,11)$ nOes with near equal intensities that is consistent with the expectation that nOes are only observable up to distances of less than 4.5–5 Å. Nevertheless,

our experimental observation of $d_{\alpha\text{N}}(9,11)$ and $d_{\delta\text{N}}(9,11)$ nOes is consistent with Val⁸–Gly¹¹ being in a tight turn.

The third structured region involves the -Ser(P)-Ser(P)-Ser(P)-Glu-Glu- motif common to the two peptides α_{s1} -casein-(59–79) and β -casein-(1–25). The chemical shifts of the H α and NH proton resonances of residues -Ser(P)-Ser(P)-Ser(P)-Glu-Glu- displayed a wide range of values, and the Glu residue resonances were shifted from the random-coil values. In the fingerprint region of β -casein-(1–25) (Figure 1), a medium-range nOe between the H α proton of Ser(P)¹⁸ and the amide proton of Glu²⁰ was observed both in the presence of calcium ions at 400 MHz and in the presence of sodium ions at 500 MHz. This nOe was not observable between the corresponding residues in the spectra of α_{s1} -casein-(59–79). One interpretation is that there are two or more conformations of this cluster sequence -Ser(P)-Ser(P)-Ser(P)-Glu-Glu- and that the conformation adopted is sensitive to neighbouring residues in the sequence. The other interpretation is that spectral congestion has obscured the nOes. Exhaustive inspection of the relevant regions of the spectra did not support this latter explanation. In the α_{s1} -casein-(59–79) peptide, the region comprising residues Glu⁶¹ to Ser(P)⁶⁷ appears to involve a loop structure [2], whereas Ser(P)¹⁸ to Glu²⁰ appears to be involved in a turn in β -casein-(1–25).

The fourth structured region, at the C-terminus, was defined on the basis of the perturbed H α resonance of Thr²⁴ and the medium-range nOes between the H α and $\beta\beta'$ H of Ser²² and the NH of Thr²⁴ observed at 400 MHz. This structured region was calcium dependent, as the Ser²² β and the Thr²⁴ γ H resonances were shifted towards lower frequencies in the presence of calcium. The medium-range nOes H α and $\beta\beta'$ H of Ser²² to the NH of Thr²⁴ have not been reported previously. Wahlgren et al. [40] reported a continuous stretch of weak $d_{\text{NN}}(i, i+2)$ nOe cross-peaks in the region between Ser(P)¹⁹ and Asn²⁷ in the larger peptide β -casein-(1–41) in the absence of Ca²⁺. However, these nOe cross-peaks were not observed by these authors in the presence of Ca²⁺. A β -turn in this region is interesting, in that the Wilmot and Thornton [38,39] algorithm does not predict any turns for this sequence; indeed, the predicted turn propensities fall substantially below the threshold levels at which turns are predicted. Because of the substantial changes to side-chain chemical shifts as a function of calcium ion concentration, we believe that the turn is formed as a consequence of calcium ion binding to the Ser²² and Thr²⁴ side chains, thereby stabilizing the turn.

The medium-range $d_{\alpha\text{N}}(18,20)$ and $d_{\alpha\text{N}}(22,24)$ nOe connectivities and the observed pattern of d_{NN} nOes in these regions are consistent with the existence of β -turns. The observation of relatively intense $d_{\text{NN}}(i+2, i+3)$ nOes is consistent with type I turns where the corresponding amide protons are separated by about 2.6 Å [28].

The results presented in this paper differ significantly from those reported in earlier ¹H NMR studies of β -casein-(1–25) [12,13]. In the present study the secondary shifts of the NH and H α resonances of most residues of β -casein-(1–25) showed substantial changes in the presence of calcium ions (Figure 3), compared with no change [12] or changes only to residues in the phosphoserine cluster motif [13] in the other studies. Significant calcium-induced changes were also observed in secondary shifts of side-chain resonances, in contrast with no significant change (< 0.03 p.p.m.) in the earlier studies [12,13]. Further, in contrast with these earlier studies, we observed only a few, weak d_{NN} connectivities in sodium β -casein-(1–25). Consequently we report here a substantial change in the number and intensity of d_{NN} connectivities in β -casein-(1–25) in the presence of calcium ions. Finally, we also observed medium-range nOes and rOes in

calcium β -casein-(1–25) that were not observed in the earlier studies [12,13].

There are two obvious differences between the conditions employed in the earlier studies [12,13] and those of the present study. First, the earlier studies were performed at pH 6.0, whereas the present study was performed at pH 6.3. Secondly, the earlier studies were performed using peptide prepared according to the method of Manson and Annan [41] using barium ion precipitation, whereas our peptide was prepared using calcium ion precipitation [14].

The difference in pH values used in the studies would have had some effect on the calcium ion binding capacity of the peptide, as the pH value of 6.3 used in the present study is closer to the pK_a values of the phosphoserine residues [42]. The greater calcium ion binding capacity of the peptide in the present study therefore may help to explain why earlier investigations did not observe the calcium-induced changes reported here. However, there is ¹H NMR evidence that the related peptides α_{s1} -casein-(59–79) and α_{s2} -casein-(2–21), which also contain the Ser(P)-Ser(P)-Ser(P)-Glu-Glu- motif, display medium-range nOes in the presence of calcium ions even at pH 6.0 [2,43]. Furthermore, a difference in calcium ion binding capacity at the two different pH values used would fail to explain the observations of extensive d_{NN} connectivities in the sodium β -casein-(1–25) samples in the earlier studies [12,13]. The sodium β -casein-(1–25) samples in these earlier studies appear to be more structured than in the present study. The secondary chemical shifts reported for the 'calcium-free' β -casein-(1–25) by Wahlgren et al. [13] correlate best with our calcium β -casein-(1–25) sample secondary chemical shifts. These observations suggest that some of the calcium ion binding sites may have already been occupied in the earlier studies, accounting for the lack of change in side-chain chemical shifts as calcium was added, and for the small number of residues showing chemical shift changes [12,13]. It is possible that the barium ions used to prepare β -casein-(1–25) in the earlier studies [12,13] were occupying some of the binding sites of the peptide, as barium ions are difficult to remove completely from casein phosphopeptide preparations even under acidic conditions ([44]; P. Riley and E. C. Reynolds, unpublished work).

A Fourier-transform IR (FTIR) spectroscopy study of bovine casein micelles formed in the presence of calcium ions and of submicelles formed in the presence of sodium or potassium ions alone showed that the casein backbone structure experienced a shift to higher-energy structures in the presence of calcium ions when compared with the structures present without calcium in the submicelles [45]. Our NMR results are therefore consistent with these observations.

In conclusion, we have shown that the ¹H chemical shifts of peptide resonances in β -casein-(1–25) are dependent upon the nature of the cation present in solution. Furthermore, significant cation-dependent changes in chemical shifts occurred for all resonances, not just the amide and H α resonances as observed previously [12,13]. We have shown that both the sequential and non-sequential nOes depend on the nature of the cation present. The changes in nOes suggest that β -casein-(1–25) has a fairly flexible structure in solution in the presence of sodium or ammonium ions. However, the similarities in chemical shifts (Figure 3) suggest that the structure of β -casein-(1–25) adopted in the presence of sodium or ammonium ions is probably fluctuating around the structure adopted when calcium is present.

The observation of a substantial increase in the intensity of the d_{NN} nOes on the addition of calcium ions to the β -casein-(1–25) solution, combined with a lack of nOes characteristic of α -helical structure, is consistent with the peptide adopting a structure consisting of loops or turns with backbone ϕ angles in the α_R

region. The observation of isolated medium-range nOes in the spectra of the calcium-bound species has enabled us to locate three tight turns and a loop.

Our results are fully consistent with previous FTIR experiments on casein micelles and submicelles, and their interpretation by Curley et al. [45] that suggests that the degree to which caseins adopt a specific conformation is dependent upon the cation present in the micelle. Furthermore, the increased tendency towards a structure consisting of turns and loops in the presence of calcium ions is consistent with the observation of higher-energy structures in the FTIR spectra of casein micelles and submicelles.

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