Characterization of Phosphate Sites in Native Ovine, Caprine, and Bovine Casein Micelles and Their Caseinomacropeptides: A Solid-State Phosphorus-31 Nuclear Magnetic Resonance and Sequence and Mass Spectrometric Study

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ABSTRACT

The phosphate sites in native ovine, caprine, and bovine casein micelles have been analyzed using sequence analysis, mass spectrometric analysis, and solid-state ^{31}P nuclear magnetic resonance spectroscopy. Using a combination of S-ethylcysteine derivatization, sequence analysis, and mass spectrometric analysis, the phosphorylation sites of ovine ($SerP_{151}$ and $SerP_{168}$), caprine ($SerP_{151}$ and $SerP_{168}$), and bovine ($SerP_{149}$) caseinomacropeptides have been localized. Various solid-state ^{31}P methods using magic angle spinning have been applied to ascertain the local structure and dynamics of the phosphorylated serine residues and the inorganic calcium phosphates within the micelles. Contributions from the phosphorylated serine residues of κ-CN, located in the C-terminal portion of the molecule, to the mobile constituents of the micelles were assigned by comparison with ^{31}P nuclear magnetic resonance spectra of purified caseinomacropeptides from the various species in the dissolved state. Comparison of the ^{31}P magic angle spinning nuclear magnetic resonance spectra of ovine, caprine, and bovine casein micelles indicates that the micelles from these species are very similar but not identical.

(**Key words**: magic angle spinning nuclear magnetic resonance, casein micelles, phosphorylation, κ-casein)

Abbreviation key: **CMP** = caseinomacropeptide, **HAP** = hydroxyapatite, **MAS** = magic angle spinning, **NMR** = nuclear magnetic resonance, **PTH** = phenylthiohydantoin.

Received December 5, 1995.
Accepted September 6, 1996.
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INTRODUCTION

Despite intensive use of numerous techniques, the exact quaternary structure and dynamic behavior of casein micelles remain largely unresolved. However, experimental results from studies utilizing electron microscopy (25), small angle X-ray and neutron scattering (10, 27), and liquid-state nuclear magnetic resonance (**NMR**) spectroscopy (8, 22, 36) have been interpreted in terms of a commonly accepted submicellar model. In this model, κ-CN is primarily located at the micellar surface, and the flexible C-terminal caseinomacropeptide (**CMP**) protrudes into the solution as "hairs" (6, 23). In addition to the stabilizing effect induced by the κ-CN hairs and potential hydrophobic and electrostatic interactions between proteins, this model involves a calcium phosphate phase, often referred to as colloidal calcium phosphate. Through interactions with phosphorylated serine residues, colloidal calcium phosphate is regarded as fundamental for the integrity and stability of the casein micelle.

Most results concerning the dynamic behavior of the casein micelles and the colloid formation with inorganic calcium phosphates have originated from experiments using liquid-state NMR. This technique, however, has certain limitations when used to investigate the overall structure and dynamics of casein micelles. Motion is restricted within these large colloidal particles, ranging in size from 20 to 600 nm (21), which implies that this method provides information only on mobile micellar components, and resonances from the immobile components within the particles are not detected. To remedy this problem, bovine casein micelles have recently been investigated using solid-state NMR spectroscopy, which is a method well suited for investigating the structure and dynamics of immobilized or partly immobilized matter (33). Thus, application of various ^{31}P NMR methods using magic angle spinning (**MAS**) has proved useful to detect and discriminate signals from

mobile and immobile phosphates as well as organic and inorganic phosphates in native casein micelles. In support of the "hairy" submicellar model, κ-CN exhibited a high degree of freedom of motion, but a major fraction of the SerP residues from the other caseins appeared to be immobilized, most likely by interactions with calcium phosphates or by other interactions associated with formation of micelles. Furthermore, comparison with ^{31}P MAS NMR spectra for a series of inorganic calcium phosphates revealed that the micellar inorganic calcium phosphates exhibit structural similarities to hydroxyapatite (**HAP**).

In this study, we have employed sequence and mass spectrometric analysis as well as solid-state ^{31}P MAS NMR spectroscopy to perform a comparative examination of the phosphate sites in ovine, caprine, and bovine caseins. Mercier (12) has previously shown that bovine κ-CN contains at least one phosphorylated serine residue ($SerP_{149}$) in the caseinomacropeptide region. Two phosphorylation sites ($SerP_{151}$ and $SerP_{168}$) have been localized in caprine CMP (13), and, because of the sequence homology between sheep and goat, ovine CMP also possibly contains two SerP in the corresponding positions. To attain further knowledge of the specific phosphorylation sites of caprine, ovine, and bovine CMP, we have applied S-ethylcysteine derivatization, sequence analysis, and mass spectrometric analysis to localize these sites. This strategy has recently been applied successfully in studies of the milk proteins PP3 (29) and osteopontin (28). To characterize and compare the structure and dynamics associated with the phosphate sites of native micelles from the three species, we performed a series of ^{31}P single-pulse, cross-polarization (18), and spin-echo MAS experiments. Furthermore, to support our assignment of ^{31}P resonances in the individual ^{31}P MAS NMR spectra to κ-CN, CMP from the different species were purified and analyzed by liquid-state ^{31}P NMR.

MATERIALS AND METHODS

Materials

Rennet extract containing approximately 30% pepsin was from Chr. Hansen Laboratories (Copenhagen, Denmark), and aprotinin was from Merck (Damstadt, Germany). Superdex 75 HR 10/30 was from Pharmacia (Uppsala, Sweden). Endoproteinase Glu-C (EC 3.4.21.19) and thermolysin (EC 3.4.24.2) were obtained from Boehringer Mannheim (Mannheim, Germany) and Sigma Chemical Co. (St. Louis, MO), respectively. Vydac C_{18} (10 μm) was from The Separations Group (Hesperia, CA). Reagents used for sequencing were from Applied Biosystems (Foster City, CA). All other chemicals used were of analytical grade. Caprine milk was provided by The National Institute of Animal Science (Foulum, Denmark) and from Ørums sogn udviklingsmejeri (Hjallerup, Denmark). Ovine and bovine milks (Black and White Danish Dairy Cattle, heterozygous for κ-CN) were delivered from Aggersborggaard (Løgstør, Denmark) and MD Foods Research and Development Center (Brabrand, Denmark), respectively.

Preparation of Casein Micelles and CMP

Raw milk samples were skimmed (2600 × *g* for 10 min at 4°C), and native casein micelles were prepared by ultracentrifugation as described (33). Briefly, aprotinin (final concentration 3 mg/ml) was added to prevent plasmin-induced proteolysis. Native casein micelles were prepared by centrifugation at 120,000 × *g* for 1.5 h at 5°C in a preparative ultracentrifuge (Beckman L8-M; Beckman Instruments, Inc., Fullerton, CA). The pellets were resuspended in water and centrifuged. Prior to the NMR experiments, aprotinin was added to the casein micelles (pellets) and packed in the rotor. The CMP was purified from acid-precipitated whole casein treated with rennet, followed by TCA precipitation as described (11). The filtrates were dialyzed against 50 m*M* ammonium bicarbonate, lyophilized, and applied to a Superdex 75 HR 10/30 column eluted with 50 m*M* ammonium bicarbonate at a flow rate of 0.5 ml/min. Peptides were detected in the effluent by recording the absorbance at 226 nm. The identity of the purified CMP from the various species was confirmed by amino acid sequence analysis performed by automated Edman degradation on a model 477 sequencer (Applied Biosystems) with on-line phenylthiohydantoin (**PTH**) analysis (Applied Biosystems 120A HPLC).

NMR Spectroscopy

All experiments were performed at ambient temperatures on a Varian XL-300 spectrometer (Varian Associates, Inc., Palo Alto, CA; operating at 121.4 MHz for ^{31}P) equipped with a home-built MAS probe (7) using 7-mm (outer diameter), partially stabilized zirconia (220-μl volume) rotors with spinning speeds of ca. 5 kHz for solid samples. The experiments, which were performed essentially as described (33), used radio frequency field strengths of 40 kHz for ^{1}H pulses and decoupling and 30 kHz for ^{31}P pulses and ^{1}H-^{31}P Hartmann-Hahn match in cross-polarization experiments. The experimental details are given in

the figure legends. The ^{31}P chemical shifts are expressed in parts per million relative to external 85% H_3PO_4. Deconvolutions of the ^{31}P single-pulse, cross-polarization, and spin-echo MAS spectra were performed on a SUN Sparc 10/50 computer (Sun Microsystems, Inc., Mountain View, CA) using the Varian VNMR software package.

Purification and Characterization of Peptides Containing Phosphoserine

The CMP from the different species were subjected to endoproteinase Glu-C digestion using a ratio of enzyme to substrate of 1:50 (wt/wt) in 0.1 *M* ammonium bicarbonate, pH 8.1, at 37°C for 3 h. Separation of peptides was carried out by reverse-phase HPLC and detected in the effluent by measuring the absorbance at 226 nm. Peptides containing SerP and ThrP were detected by short-time hydrolysis (110°C in 6 *M* HCl, 0.05% phenol for 3 h) and subsequent amino acid analysis essentially as described (28). Some of the peptides containing SerP from the bovine CMP digest were further cleaved with 5 μg of thermolysin in 400 μl 0.1 *M* pyridine and acetate, pH 6.5, and 5 m*M* $CaCl_2$ at 55°C for 3 h. Peptides containing SerP were treated with ethanethiol to convert SerP into S-ethylcysteine (16) and identified by amino acid sequence analysis performed on a protein sequencer (ABI 477A/120A; Applied Biosystems Inc.) as described. The PTH-S-ethylcysteine was localized after its release in the corresponding cycle and eluted just before the diphenylthiourea peak in the system used. Mass spectra were acquired using a matrix-assisted laser desorption ionization (MALDI) mass spectrometer (Bruker BIFLEX; Bruker-Franzen, Bremen, Germany) that was equipped with a reflector and a nitrogen ultraviolet laser at 337 nm. Samples (2 μl) dissolved in 0.1% trifluoroacetic acid were mixed with 2 μl of α-cyano-4-hydroxycinnamic acid (15 g/L), and 0.9 μl were applied to the target. Thirty to 100 calibrated mass spectra were averaged. Theoretical peptide masses were calculated using the GPMAW program (Lighthouse Data, Odense, Denmark).

RESULTS AND DISCUSSION

Localization of Phosphorylation Sites in Ovine, Caprine, and Bovine CMP

The HPLC chromatograms of the endoproteinase Glu-C digests from purified ovine, caprine, and bovine CMP are presented in Figure 1. To reveal the

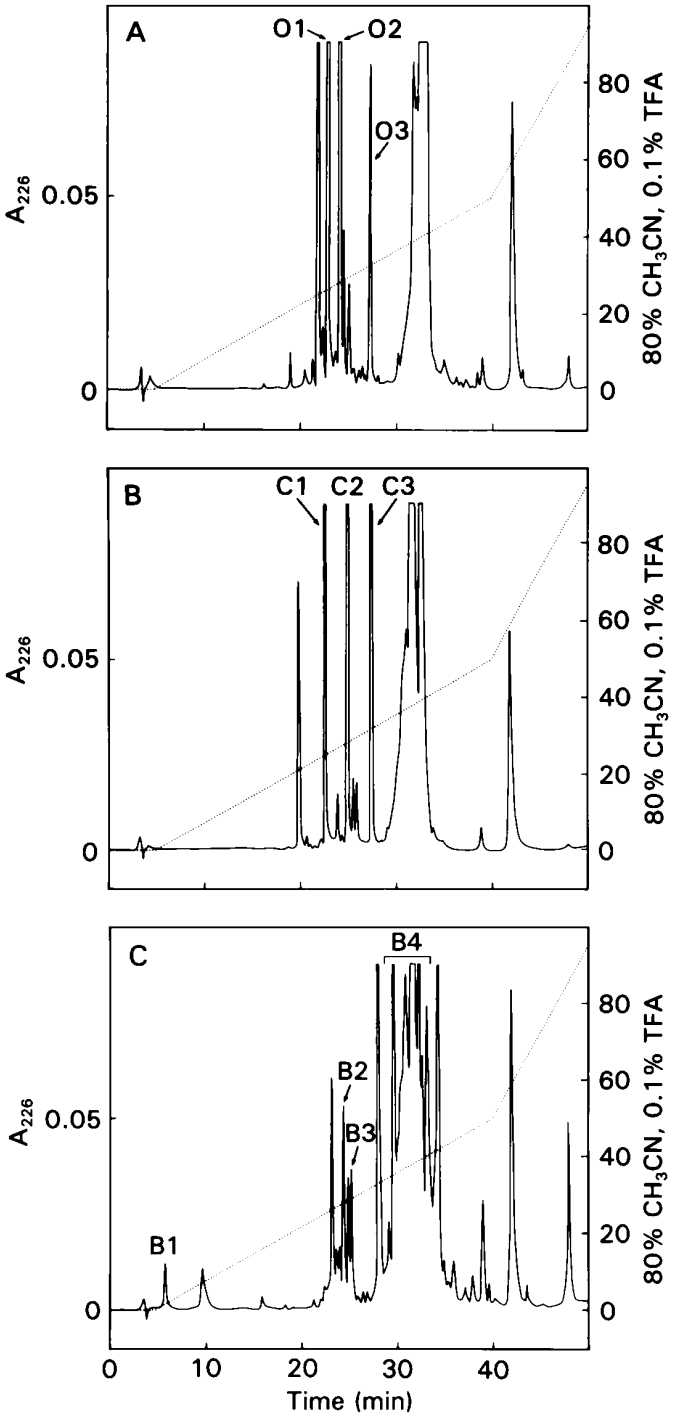

Figure 1. Reverse-phase HPLC separation of peptides obtained from caseinomacropeptides (CMP) by digestion with endoproteinase Glu-C of ovine CMP (A), caprine CMP (B), and bovine CMP (C). Peptides were eluted with a gradient of 80% acetonitrile in 0.1% trifluoroacetic acid (TFA; dotted line) on a (10-μm) Vydac C_{18} column (4 × 250 mm; The Separations Group, Hesparia, CA). The column was operated at 40°C with a flow rate of 0.85 ml/min. Peptides were detected in the effluent by recording the absorbance at 226 nm (A_{226}; solid line). Fractions containing phosphate (B_1 to B_4, C_1 to C_3, and O_1 to O_3) were identified by amino acid analysis (see Materials and Methods).

presence of peptides containing SerP or ThrP, aliquots from each peak were subjected to short-time acid hydrolysis followed by amino acid analysis (28). No ThrP was detected in any of the analyzed species. For ovine and caprine CMP, SerP was primarily present in three peaks designated O_1 to O_3 and C_1 to C_3, respectively. The bovine phosphorylation profile showed the presence of SerP in three peaks (B_1 to B_3) as well as some amount in the unresolved peaks (B_4), which were pooled and subdigested with thermolysin. The peptides containing SerP were analyzed by sequencing samples treated with ethanethiol combined with mass spectrometric analysis of underivatized samples; the results are summarized in Table 1. By ethanethiol treatment, SerP is quantatively converted into S-ethylcysteine, which yields a stable PTH-derivative during Edman degradation. In the sequence analysis of fractions that had been treated with ethanthiol, PTH-S-ethylcysteine was identified in the cycles corresponding to SerP, except when a proline residue was positioned C-terminal to a SerP, which seemed to impair the conversion (31). In all cases for which mass spectra were obtained, the mass difference was approximately 80 Da relative to the calculated peptide mass, thereby confirming the attachment of a phosphate group. In agreement with the reported phosphorylation pattern of caprine CMP (13) and, as expected for ovine CMP (15), two phosphorylation sites, $SerP_{151}$ and $SerP_{168}$, were localized in both species. In accordance with earlier studies (14, 32), we were only able to identify a single phosphorylation site, $SerP_{149}$, in the bovine counterpart employing the outlined strategy. However, bovine κ-CN has been reported to display heterogeneity with respect to both glycosylation and phosphorylation (17, 19, 35). At least 10 components, differing in carbohydrate or phosphate content, were obtained by ion-exchange chromatography of the whole κ-CN variant B (35). Based on phosphate analysis, two of these components appeared to be multiphosphorylated without any carbohydrates attached. Recently, the heterogeneity of bovine κ-CN variant A from a single cow has been investigated by electrospray ionization-mass spectrometry (17). Mono-, di-, and triphosphorylated forms were found in the ratio 78:20:2. According to the sequence motif for mammary gland kinase (12), Figure 2 reveals 2, 3, and 3 potential serine phosphorylation sites in unglycosylated bovine, ovine, and caprine CMP, respectively. Because no peaks containing ThrP were detected and because

TABLE 1. Localization of phosphorylation sites in bovine, ovine, and caprine caseinomacropeptides by sequence analysis and mass spectrometric analysis.

Peak[1]	Observed sequence[2]	Molecular mass		Peptide identification[4]
		Observed	Calculated[3]	
Bovine				
B_1	A148–E151	. . .[5]	482.4	A***S***PE[6]
B_2	A138–E147	1481.2	1483.5	AVESTVATLEA***S***PE[6]
	S141–E151	. . .	1228.2	STVATLED***S***PE[7]
B_3	A148–E158	1234.1	1234.2	A***S***PEVIESPPE[6]
B_4	L146–P151	. . .	768.7	LED***S***PE[7]
Ovine				
O_1	T161–V171	1231.2	1230.2	TNTAQVT***S***TEV
	T163–V171	. . .	1015.0	TAQVT***S***TEV
O_2	A140–E153	1480.7	1481.5	AVVNAVDNPEA***S***SE
O_3	A140–P159	2138.1	2137.2	AVVNAVDNPEA***S***SESIASAPE
Caprine				
C_1	T161–V171	1229.9	1230.2	TNTAQVT***S***TEV
	T163–V171	. . .	1015.0	TAQVT***S***TEV
C_2	A140–A150	1525.0	1525.5	AIVNTVDNPEA***S***SE
	P148–E153	. . .	698.6	PEA***S***SE
C_3	A140–D146	2172.6	2171.2	AIVNTVDNPEA***S***SESIASASE

[1]Peak numbers are as shown in Figure 1.

[2]Only phosphopeptides in the respective peaks are presented.

[3]Calculated mean masses including one phosphorylated serine residue.

[4]Identified peptide from caseinomacropeptide. Phosphorylated serine residues are shown in bold and italics.

[5]No ions corresponding to the calculated mass could be observed in the mass spectra.

[6]Derived from κ-CN variant B.

[7]Derived from κ-CN variant A.

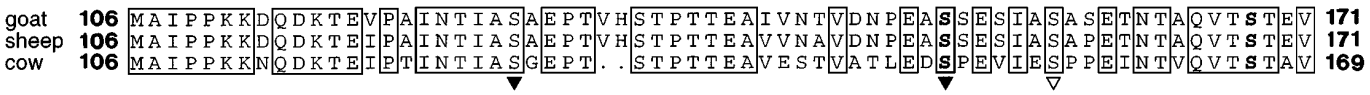

Figure 2. Comparison of the phosphorylation sites in ovine, caprine, and bovine caseinomacropeptides (CMP). The deduced amino acid sequences of bovine variant A (26), ovine (5), and caprine (2) CMP were aligned using the PileUp program from the GCG program package (20) and presented using Alscript (1). Boxes indicate identical residues in all three species, and introduced gaps are indicated by dots. Identified phosphorylated serines in bovine CMP (14), caprine CMP (13), and ovine CMP (this study) are in boldface type. Potential sites of serine phosphorylation localized in the recognition motif of mammary gland kinase (Ser/-X-Glu/Asp/SerP), and the recognition motif of casein kinase II (Ser-X-X-Glu) are shown by filled and open triangles, respectively.

threonine residues were seldom phosphorylated in the caseins (12), Ser_{127} was the only other potential phosphorylation site. However, no peptide containing Ser_{127} was detected by phosphate analysis, and sequence analysis of bovine CMP showed an expected yield of PTH-Ser in the cycle corresponding to Ser_{127}, thereby confirming that this serine was not phosphorylated. We have recently discovered the phosphorylation of serine residues located in another sequence motif, Ser-X-X-Glu (30). This motif corresponds to the recognition sequence of casein kinase II. Analysis of the bovine CMP shows the presence of one serine residue (Ser-155) located in a recognition motif for casein kinase II. Mass spectrometry and sequence analysis of the peptide Ala_{148} to Glu_{158} showed that Ser_{155} was not phosphorylated in bovine CMP (Table 1). Only Ser_{149} in bovine CMP, purified from bulk milk containing both the κ-CN A and B variants, was found conclusively to be phosphorylated. The existence of other reported phosphorylation sites could be ascribed to microheterogeneity in the level of phosphorylation and glycosylation that was dependent on the lactation stage and season or simply because of differences between individuals.

Solid-State ^{31}P MAS NMR of Native Ovine, Caprine, and Bovine Casein Micelles

A series of different ^{31}P MAS NMR spectra of ovine, caprine, and bovine casein micelles is shown in Figure 3 along with the spectra of bovine casein micelles for comparison. The single-pulse (Figure 3A), spin-echo (Figure 3B), and cross-polarization (Figure 3C) MAS NMR spectra display resonances from all phosphate groups, mobile phosphate groups, and immobile phosphate groups in the casein micelle, respectively.

The ^{31}P MAS spectrum of bovine casein micelles (Figure 3A, right column) shows a narrow resonance at 1.5 ppm and a somewhat broader resonance at 3.0 ppm, which both reside on a very broad resonance (appears as a hump) centered at ca. 2 to 3 ppm. The two resolved resonances and the hump originate from mobile and immobile micellar constituents, respectively, as evidenced by the spin-echo (Figure 3B, right column) and cross-polarization (Figure 3C, right column) MAS spectra probing these components selectively. The ^{31}P MAS spectra were deconvoluted (shown below the experimental spectra) and assigned using this spectral information and comparison with ^{31}P MAS spectra of α_{s1}-CN, α_{s2}-CN, β-CN, CMP and a series of inorganic calcium phosphates as well as mineralized bone tissue (33). The resonance at 1.5 ppm has been assigned to mobile SerP of κ-CN located in the C-terminal part of the molecule, and the resonance at 3.0 ppm has been assigned to micellar inorganic calcium phosphate in a mobile state exhibiting structural similarities to HAP. This interpretation supports a model in which κ-CN is mainly located at the micellar surface, and the CMP protrudes into the solutions as "hairs" (6, 23). In our earlier study (33), the resonance at 1.5 ppm appeared as two narrow resonances. However, in later experiments, only a single peak was obtained. The reason for this variation is at present not clear. The three broad resonances at 1.6, 2.9, and 3.7 ppm of the deconvolution, which together formed the broad hump, were assigned to immobilized organic SerP residues, primarily from α_{s1}-CN, α_{s2}-CN, and β-CN (1.6 ppm) and immobile inorganic phosphates (2.9 and 3.7 ppm). Casein micelles are affected by pH and temperature in a number of ways (3, 24). The pH of the casein micelle suspension was in the range 6.5 to 6.8, thus corresponding to that of normal milk. As the temperature was lowered to 4°C (normal storage temperature), the caseins dissociated from the casein micelles. To examine the influence of temperature on our results, we obtained spectra of 1) bovine bulk milk stored at 4°C and ultracentrifuged at 20°C and 2) fresh bovine milk to which aprotinin (final concentration 3 mg/L) was added immediately after milking and then stored at room temperature for 2 h prior to ultracentrifugation at 20°C. Deconvolutions of the im-

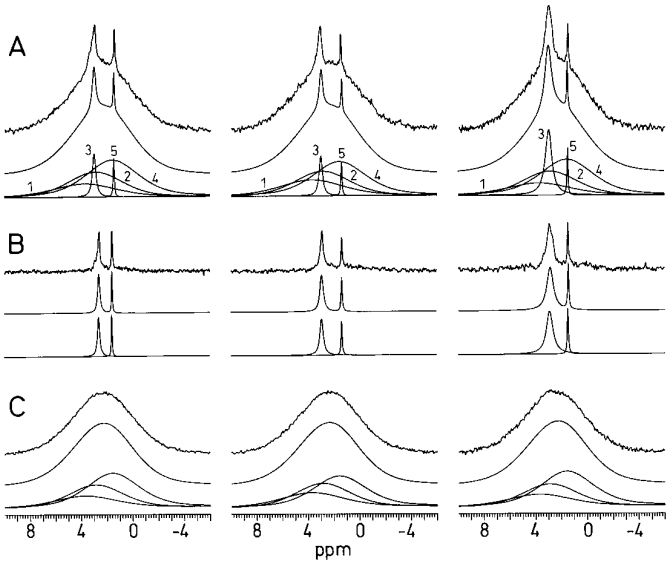

Figure 3. Experimental (top) and deconvoluted (bottom) ^{31}P magic angle spinning (MAS) nuclear magnetic resonance (NMR) spectra of native ovine (left column), caprine (center column), and bovine casein micelles (right column). The experimental spectra were obtained using single-pulse [224 transients, 32-s recycle delay, and spinning speed (υ_r) of 5 kHz] (A), spin-echo [1024 transients, 4-s recycle delay, υ_r = 5 kHz, and spin-echo time (τ) of 10 ms in the $90° - \tau - 180° - \tau$ pulse sequence] (B), and cross-polarization [1024 transients, 4-s recycle delay, υ_r of 5 kHz, and 1.5-ms contact time] (C). For the deconvoluted spectra, numbers indicate resonances assigned to immobile inorganic (numbers 1 and 2), mobile inorganic (number 3), immobile organic (number 4), and mobile organic (number 5) components. From the relative intensities of the deconvoluted signals, the following ratios were estimated: organic (numbers 4 and 5) to inorganic (numbers 1, 2, and 3) components: 0.89 (sheep), 0.78 (goat), and 0.79 (cow); mobile inorganic (number 3) to immobile (numbers 1, 2, and 4) components: 0.045 (sheep), 0.041 (goat), and 0.13 (cow). The ratios between the phosphate groups of mobile κ-casein (number 5) and all other phosphate signals (numbers 1, 2, 3, and 4) were 0.013 (sheep), 0.011 (goat), and 0.013 (cow).

mobile components were identical when casein micelles were harvested at 5 or 20°C and when milk was stored at 4 or 20°C.

The existence of mobile and immobile colloidal calcium phosphate supports a model proposed by van Dijk (34) in which inorganic phosphates are present partly as stable calcium phosphate complexes immobilized by the colloidal formation and partly as unstable complexes that potentially have more freedom of motion.

The single-pulse MAS ^{31}P NMR spectra of native ovine (Figure 3A, left column) and caprine casein micelles (Figure 3A, center column) display two well-resolved resonances superimposed on a broad resonance, which clearly resembled the corresponding resonances of bovine casein micelles in line positions and line widths. Similarly, the ^{31}P spin-echo (Figure 3B) and cross-polarization (Figure 3C) MAS spectra for the micelles of the three species were almost identical, indicating both structural and dynamic similarities. Because the ^{31}P cross-polarization MAS spectra that selectively display resonances from the immobile components were largely indistinguishable for the three types of micelles, the micelles were assumed to contain phosphates in environments that were structurally and morphologically similar to those of bovine micelles. Thus, parameters (chemical shift, line width, and line shape) that were identical to those used for the deconvolution of the immobile components in the bovine spectrum were employed as input for the deconvolutions of the ^{31}P MAS spectra of ovine and caprine micelles. Despite obvious similarities, the resonance assigned to HAP in a mobile state (ca. 3.0 ppm) was smaller for caprine and ovine micelles (line width, ~35 Hz) than for bovine micelles (line width ~70 Hz), for both intensity and line width. The narrow resonances at 1.4 ppm were assigned to SerP groups of ovine and caprine CMP. To verify this assignment, Figure 4 contains ^{31}P liquid-state NMR spectra of purified CMP. However, the resonances from ovine, caprine, and bovine CMP were not identical to the resonances at 1.4 and 1.5 ppm in the spectra of the corresponding casein micelles. Because the dissolved CMP represents a model system, discrepancies in the chemical shift, line width, and number of resonances might be ascribed to an environmental change of the SerP residues when present in the buffer than when present in the native casein micelle.

To obtain a quantitative assessment of the various micellar components, the ^{31}P MAS NMR spectra of the three types of micelles were deconvoluted, and the resulting intensities were used to estimate the ratios of organic to inorganic and mobile to immobile phosphates within the micelles (included in the legend of Figure 3). For all three species, this procedure led to a very similar ratio between organic and inorganic phosphates that was close to the range 0.77 to 0.83 reported previously (4). Evaluation of the amount of mobile inorganic phosphate using the deconvoluted signal intensities reveals that the ovine and caprine micelles contained a smaller amount than did bovine micelles. Furthermore, as judged from a calculated ratio between mobile phosphate groups of the κ-CN and the sum of all other phosphate resonances and in contrast to the phosphorylation patterns, κ-CN from all species apparently harbors the same amount of mobile SerP groups. However, the intensities of the ^{31}P resonances from the organic components depend both on the concentration and the extent of phos-

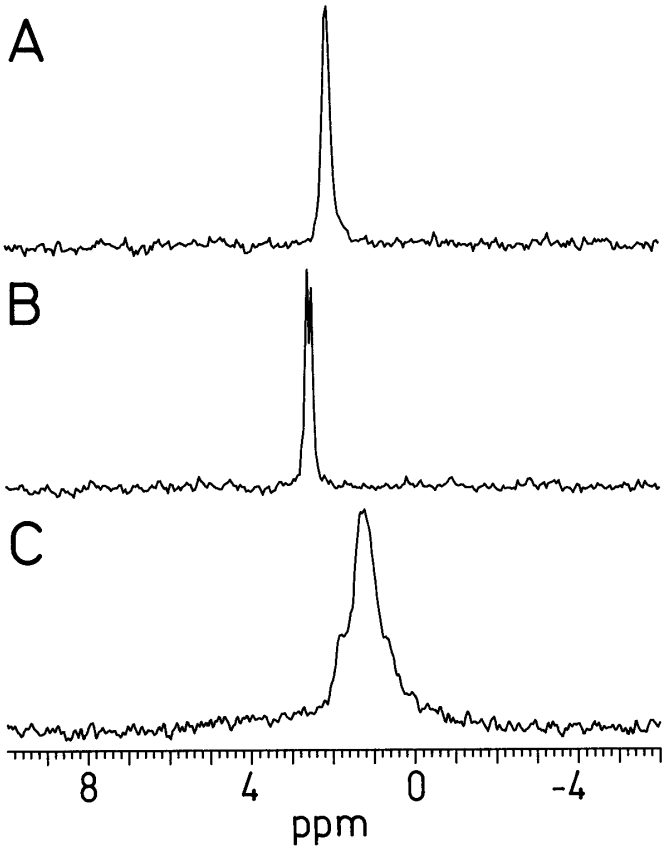

Figure 4. The ^{31}P nuclear magnetic resonance (NMR) spectra of ovine (A), caprine (B), and bovine (C) caseinomacropeptides dissolved in cacodylate buffer (pH 6.9). The spectra were recorded using single-pulse excitation, 4-s recycle delay, and 2048 (A and B) or 20,000 (C) transients.

phorylation. Hence, the discrepancy between the NMR data and the number of phosphorylation sites might be ascribed to differences in the composition of the micelles (e.g., caprine micelles are very low in the α_s components), the degree of phosphorylation of the individual caseins (21), and the possibility that part of the κ-CN might be in an immobilized state. Similarly, the smaller amount of mobile inorganic phosphate (e.g., HAP) in ovine and caprine micelles might reflect differences in the size of the micelles between the species (21).

We conclude that the casein micelles from the three species are alike in dynamic behavior of the micelles and the colloid formation with inorganic calcium phosphate. It has been possible to differentiate and, to a certain degree, quantitate the signals from mobile and immobile as well as organic and inorganic phosphates in native casein micelles. This study emphasizes that MAS ^{31}P NMR represents a useful method for obtaining information about the structure and dynamics of organic and inorganic phosphates in biological macromolecules with regions of restricted motion, and the potential use of this method to dairy products has recently been reported (9).

ACKNOWLEDGMENTS

This work is part of the FØTEK program funded by the Danish Government and the Danish Dairy Board. This research was also supported by grants from Aarhus University Research Foundation, Carlsbergfondet, the Danish Natural Science Research Council, and the Danish Technical Research Council. We thank Hanne A. Due, Roy E. Guldberg, and Lise Møller for technical assistance. Special thanks are expressed to John Hermansen, the National Institute of Animal Science, Tjele, Denmark; Regitse Johnsen, Aggersborggaard, Løgstør, Denmark; Ørums sogn udviklingsmejeri, Hjallerup, Denmark; and Andreas Madsen, MD Foods Research and Development Center, Brabrand, Denmark, for providing the different milk samples.

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