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A multinuclear, high-resolution NMR study of bovine casein micelles and submicelles *

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High-resolution, natural abundance $^{13}C\{^1H\}$ (100.5 MHz), $^{31}P\{^1H\}$ (161.8 MHz) and ^{1} H (400.0 MHz) NMR spectroscopy was used to identify the calcium-binding sites of bovine casein and to ascertain the dynamic state of amino acid residues within the casein submicelles (in 125 mM KCl, pD = 7.4) and micelles (in 15 mM $CaCl_2$/80 mM KCl, pD = 7.2). The presence of numerous, well-resolved peaks in the tentatively assigned ^{13}C-NMR spectra of submicelles (90 Å radius) and micelles (500 Å radius) suggests considerable segmental motion of both side chain and backbone carbons. The partly resolved ^{31}P-NMR spectra concur with this. Upon Ca^{2+} addition, the phosphoserine βCH_2 resonance (65.8 ppm vs DSS) shifts upfield by 0.2 ppm and is broadened almost beyond detection; a general upfield shift (up to 0.3 ppm) is also observed for the ^{31}P-NMR peaks. The T_1 values of the αCH envelope for submicelles and micelles are essentially identical corresponding to a correlation time of 8 ns for isotropic rotation of the caseins. Significant changes in the ^{31}P T_1 values accompany micelle formation. Data are consistent with a loose and mobile casein structure, with phosphoserines being the predominant calcium-binding sites.

1. Introduction

Caseins (α_{s1}-, α_{s2}-, β- and κ-) are a group of phosphoproteins that comprise approx. 83% of the total proteins (typically 3.5% (w/w)) in bovine milk [1,2]. Their monomer molecular masses are 23.6 kDa (α_{s1}-), 25.2 kDa (α_{s2}-), 24.0 kDa (β-) and 19.0 kDa (κ-), and their secondary structures have been considered to be mostly in the form of random coil [1,2]. Recent spectroscopic evidence has shown that caseins contain a significant amount of periodic structure (approx. 40%) attributed to β-turns [3]. Caseins exhibit a strong tendency for self-association and for association with each other that is markedly influenced by pH, ionic strength, temperature, calcium concentration and protein genetic variant [1,4–6].

Caseins occur in milk as micelles, i.e., spherical, loosely packed, colloidal complexes of protein and salts. Their unusual stability and flexibility are essential for their biological role, i.e., the efficient transport and delivery of protein, calcium and

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Abbreviations: SAXS, small-angle X-ray scattering; DSS, sodium 2,2-dimethyl-2-silapentane-5-sulfonate, $(CH_3)_3Si(CH_2)_3SO_3^-$ Na^+; HMPA, hexamethylphosphoramide, $((CH_3)_2N)_3P(O)$; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid; ACN, acetonitrile; DD, dipole-dipole interactions; CSA, chemical shift anisotropy; SerP, phosphoserine; TEM, transmission electron microscopy. Reference to brand or firm names does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

phosphorus to the neonate under a variety of dietary and environmental conditions. Electron microscopy has suggested a size distribution for micelles from 200 to 6000 Å with an average diameter of 1400 Å [1,4]. Removal of calcium is thought to result in the dissociation of the micellar structure into non-colloidal spherical protein complexes called submicelles [1,4]. Casein submicelles have a molecular mass of approx. 280 kDa [4,7,8] and they consist of α_{s1}-, α_{s2}-, β- and κ-caseins in the approximate ratio of 4 : 1 : 4 : 1 [1,4]. Estimates of their diameter from electron microscopy data range between 80 and 200 Å; gel filtration has suggested an upper limit of 94 Å for their Stokes radius [1,4]. The addition of calcium causes the reconstitution of micelles from submicelles: the mostly hydrophobically associated caseins (submicellar form) further self-associate via calcium-protein side chain salt bridges to colloidal micelles with average radii of 650 Å, as determined by electron microscopy [1,4].

The exact supramolecular structure of the casein micelle has remained controversial despite the large number of proposed models [1,4]. Recent small-angle X-ray scattering (SAXS) data [8] have suggested that submicelles (90 Å radius) consist of a loosely packed hydrophobic core surrounded by an even more loosely packed hydrophilic region that is related to trapped water and/or fast-moving protein side chains. Deuterium NMR relaxation measurements have indicated the existence of 'bound' water with nanosecond mobility within the casein micelle that may be associated with discrete casein submicelles within the micellar structure [9], a notion also supported by scattering studies [7,8]; the amount of the so-determined bound water is only a fraction of the hydrodynamically trapped water detected by SAXS [8,9]. On the other hand, very little is known with regard to the mobility of protein groups within either the submicelles or the colloidal micelles.

NMR is well-suited to the study of static and dynamic aspects of protein structure [10]; however, only three ^{1}H-NMR studies of casein micelles have been reported so far [11–13]. ^{13}C-NMR offers several advantages over ^{1}H-NMR, in addition to the superior resolution: the spectra are less sensitive to impurities, easier to assign and allow a direct observation of the protein backbone [10,14, 15]. ^{31}P-NMR can be used to investigate protein phosphorylated sites [16,17] which, for the caseins, are particularly important since they are generally thought to be primarily involved in the binding of calcium [1,4].

In view of the established structural importance of calcium and the emerging possibility of fast motion within the casein submicelles and micelles, high-resolution ^{13}C-, ^{31}P- and ^{1}H-NMR experiments were undertaken in order to gain further insights into the structure and the dynamics of bovine casein submicelles and reconstituted micelles.

2. Materials and methods

2.1. Materials

All reagents used were of analytical grade or 'ACS certified' from Baker, Fisher, Sigma and Aldrich. Deuterium oxide (99.8 atom% D) was obtained from Sigma (St. Louis, MO).

2 l of fresh, uncooled milk was obtained from the pooled whole milk of Jersey cows. The animals were in mid-lactation, in good health, and were part of a commercial herd. Phenylmethylsulfonyl fluoride (0.1 g/l) was added immediately to retard proteolysis. The milk was transported to the laboratory and skimmed twice by centrifugation at 4000 × g for 10 min at room temperature. Skim milk (500 ml) was diluted with an equal volume of distilled water and warmed to 37°C. Casein was precipitated by careful addition of 1 N HCl to pH 4.6. The precipitate was homogenized with a Polytron ST-10 homogenizer at low speed and dissolved by addition of NaOH to yield a solution of pH 7.0. The casein was reprecipitated, washed, and then resuspended. The sodium caseinate was subsequently cooled to 4°C, centrifuged at 100 000 × g for 30 min to remove residual fat, and then freeze-dried. The integrity of the sample was confirmed by SDS-PAGE. Alkaline polyacrylamide gel electrophoresis in the presence of urea together with standard caseins of known genetic variance [18] showed that the sample consisted of α_{s1}-BB, β-AA and κ-AA caseins.

The lyophilized casein, when dissolved in 125 mM KCl in D_2O (sample pD 7.4), is in the submicellar form (clear sample) whereas, upon addition of 15 mM $CaCl_2$, at 125 mM total ionic strength (sample pD 7.2), a 'milky' micelle sample is obtained. * Although phosphate and citrate ions occur naturally in casein micelles and are important for maintaining their structure, we did not include them in the solutions in order to facilitate spectral assignments to avoid competition for calcium binding with the protein, and to forestall precipitation of salts during the gathering of NMR data.

We have noticed that protein-bound phospholipid(s), normally present in casein preparations [20] and detectable by ^{31}P-NMR, favor markedly the formation of micelles, presumably through hydrophobic interactions. Special care (centrifugation at $100\,000 \times g$) was taken to exclude such non-protein material from our sample, in order to simplify spectral assignments. As a result, casein micelles, as judged by turbidity, were unstable below 20 °C; all experiments were conducted above 25 °C to favor micelle formation.

2.2. NMR measurements

Proton-decoupled, natural abundance ^{13}C (100.5 MHz) and ^{31}P (161.8 MHz) as well as ^{1}H (400 MHz) NMR measurements were carried out with a GX-400 multinuclear spectrometer (JEOL, Peabody, MA) equipped with a high-resolution, narrow-bore (54 mm), 9.4 T superconducting magnet (Oxford Instruments, U.K.), a DEC-PDP-11/23 dedicated computer with PLEXUS software and a 10 mm tunable (^{31}P to ^{15}N) probe. Generally, 240 mg of lyophilized protein were dissolved in 4 ml of D_2O solutions (6.0%, w/v) that contained 125 mM KCl (casein submicelles) or 80 mM KCl-15 mM $CaCl_2$ (casein micelles) together with 0.02% NaN_3 as an antimicrobial agent and 0.3 mg/ml of sodium 2,2-dimethyl-2-silapentane-5 sulfonate (DSS) as an internal chemical shift standard for ^{13}C- and ^{1}H-NMR. About 4 ml of protein samples in 10 mm high-resolution NMR tubes (Wilmad, Buena, NJ) were used for the NMR measurements.

For the ^{13}C-NMR measurements the pulsewidth was 14 μs (52° nutation angle) and a recycle time of 1.10 s was used. The spectral width was 25 kHz, the acquisition time 0.65 s and a 32K point time-domain array was used for storing the data. In the case of ^{31}P-NMR measurements, a capillary containing 0.12 M aqueous hexamethylphosphoramide (HMPA) solution was placed coaxially in the sample NMR tube. The HMPA peak has a chemical shift of 30.73 ppm vs 85% aqueous phosphoric acid (P.E. Pfeffer, personal communication) and was used as a reference. * The pulsewidth used in ^{31}P-NMR measurements was 21 μs (57° nutation angle), the spectral width 20 kHz, the acquisition time 0.82 s and the recycle time 4.82 s; data were stored in a 32K point time-domain array.

Longitudinal ^{13}C- and ^{31}P-NMR relaxation rates were determined with the inversion recovery method [21] using the 180°-τ-90° pulse sequence. In the case of ^{13}C-NMR measurements, the interpulse delay τ was varied from 5 ms to 2.5 s (six data points) and the pre-acquisition delay was set at 2.8 s. The 90° pulsewidth was 24 μs and 7000 scans were accumulated for each τ value. For the ^{31}P-NMR measurements the 90° pulsewidth was 33 μs and τ was varied from 5 ms to 10 s (11 data points); the pre-acquisition delay was set at 10 s. ^{13}C T_1 values were obtained from a non-linear, three-parameter, least-square fitting of an exponential curve to the experimental points (peak heights) in order to account for slight pulsewidth missettings and/or insufficiently long recycle delays [22]. Both three- and five-parameter fittings were considered for each ^{31}P resonance, using a

* Conversion of pH to pD values was made according to the relation pD = pH + 0.4 [19] where pH is the pH-meter reading for a solution in D_2O with the electrode calibrated in standard H_2O buffers.

* The use of 85% aqueous H_3PO_4 as an external chemical shift standard is undesirable because its broad resonance peak may obscure the neighboring protein peaks. In the experimental setup used, a chemical shift correction due to differences in the volume magnetic susceptibility between the capillary and the sample tube is required: the ^{31}P-NMR chemical shifts in fig. 2 must be increased by 0.06 ppm. The chemical shift values reported in the text have been corrected.

well-tested non-linear regression program run on a Modcomp Classic minicomputer. For comparisons of the goodness of fit between the three- and five-parameter fittings, the *F*-test was used at the 10% level of significance [23].

With the exception of ^{31}P T_1 measurements, proton decoupling was achieved by a bilevel broadband decoupling scheme based on a sequence proposed by Waugh [24]. The decoupler was centered 4.8 ppm downfield from the ^1H resonance of DSS, generating an effective decoupling field of 6.6 kHz. The power output was 9 W during acquisition and 4 W at other times. No r.f. heating of the samples was noticed during the experiments (except as noted in footnote b of table 4).

For the ^1H-NMR measurements, a pulsewidth of 30 μs (77° C nutation angle) and a spectral width of 6 kHz were used; the acquisition time and the recycle time were 1.4 and 3.4 s, respectively. Data were stored in a 16K memory block.

Approx. 30 min were allowed for each sample to reach thermal equilibrium in the magnet before data acquisition. The probe temperature was controlled (± 0.1° C) by means of a thermostated dry nitrogen current. Immediately after each experiment, the sample temperature was also independently checked with a thermocouple thermometer (± 1° C); there were no discrepancies with the probe temperature readings (except as noted in footnote b of table 4).

2.3. HPLC of casein samples

Degradation of casein(s) by microorganisms or endogenous milk proteases can result in small, mobile protein fragments that may dominate their high-resolution NMR spectra. It is therefore important to ensure that caseins remain intact during the several hours of NMR data acquisition. For that purpose, an HPLC system was employed consisting of a Varian 5000 liquid chromatograph (Varian Associates, Palo Alto, CA), a 6-way Rheodyne (Cotati, CA) sample injector with a 20 μl sample loop, a 250 × 4.6 mm C4 HPLC column (Hi-Pore RP-304, Bio-Rad, Richmond, CA) and a Varian CDS 401 data system. Casein samples were diluted with 0.1% trifluoroacetic acid (TFA) to a final protein concentration of 10 μg/μl; aliquots of 20 μl were injected onto the column and the chromatogram was developed with a linear gradient of acetonitrile (ACN), from 30% ACN/0.1% TFA to 50% ACN/0.1% TFA, over 31 min at a flow rate of 1.5 ml/min. The rate of ACN addition was then changed to bring ACN to 75% over 9 min and the column was regenerated by a reverse gradient to the starting solvent. The eluant absorbance was continuously monitored at 280 nm with a Varian UV-50 variable-wavelength detector.

The HPLC results were in agreement with previous data [25]; κ-, α_{s2}-, α_{s1}-, β- and γ-caseins (in this order of increasing retention time) were clearly resolved. Caseins were found to be virtually intact in all samples at the end of the NMR measurements.

2.4. Electron microscopy

Samples for transmission electron microscopy (TEM) were prepared by dispensing 20 μl of micelle solution on a Formvar-coated specimen grid and, at 1-min intervals, washing with five drops of distilled water, 2% uranyl acetate, blotting with Whatman no. 1 filter paper and air drying. Samples were observed using a Zeiss 10B TEM instrument (Carl Zeiss, Thornwood, NY) at 60 kV and various instrumental magnifications. The micrographs showed an average micelle diameter of 1000 Å; larger particles (diameter approx. 3000 Å) were also present. These sizes are well within the micelle size distribution in skim milk (see section 1). The general micelle morphology was similar to that previously reported [26].

3. Results and discussion

3.1. ^{13}C-NMR spectral assignments

Tentative assignments of the ^{13}C-NMR resonances of the casein micelles and submicelles (fig. 1) were made by comparing the observed chemical shifts with those of other diamagnetic proteins and peptides [10,14,15], after taking into account the sample's amino acid composition [2]. The pro-

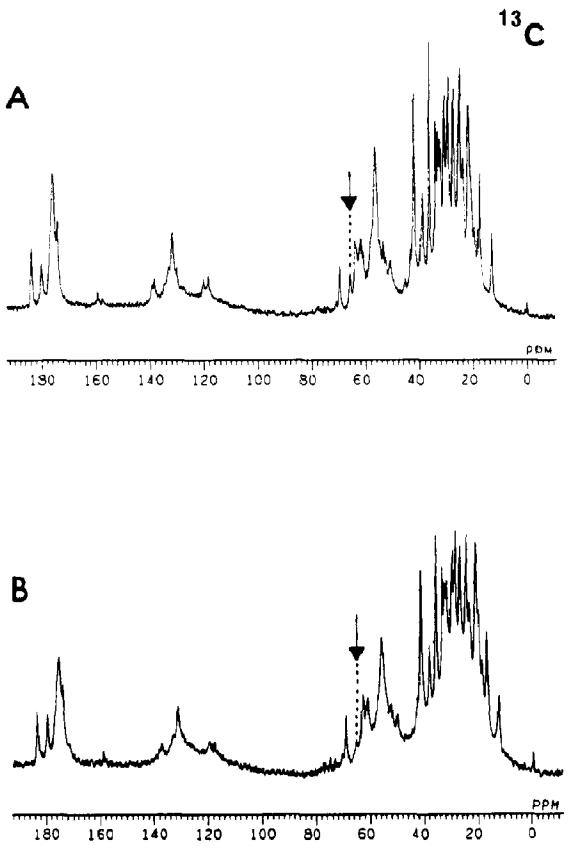

Fig. 1. Natural abundance $^{13}C\{^1H\}$ NMR spectra (100.5 MHz) of whole casein submicelles (A) and micelles (B) at 26°C. The protein concentration was 6.0%. Experimental details are given in section 2. Each spectrum is the result of 60000 scans; the applied exponential line broadening is 10 Hz. The arrows indicate the peak assigned to the βCH_2 of phosphoserines (table 1) which appears broadened in the presence of calcium (B). The lower signal-to-noise ratio of the micelle spectrum (also evident in figs 2 and 3) is mostly likely due to some casein sedimenting on the NMR tube walls during sample spinning; the decreased resolution in B vs A is probably the result of the different magnetic susceptibility of the micelle particles from the surrounding solution and the concomitant decrease in the field homogeneity.

posed assignments are given in table 1; recognizing that overlapping resonances could confound the assignments, these must be viewed with care and represent only preliminary values. In addition, differences in the chemical shift of some groups between reported values and those measured for caseins are likely to reflect differences in the protein structure. Residues of a certain type may well occur in different chemical environments; this would cause a chemical shift nonequivalence for their ^{13}C-NMR resonances. The non-symmetric lineshape of certain individually assigned resonances (e.g., Ile δCH_3) is a result of this nonequivalence. Nevertheless, many of the observed resonances do coincide with well-defined positions found in other proteins.

3.2. ^{31}P-NMR spectral assignments

Approx. 40% of the serines of whole casein (6.3 mol%) are selectively phosphorylated [2]. None of the peaks in the ^{31}P-NMR casein spectra (fig. 2) are due to nonprotein milk components [17,28,29]. The spread of the ^{31}P-NMR chemical shifts and their difference from those of free phosphoserine (4.0 ppm [17]) and α_{s1}-casein in 8 M urea (4.3 ppm [30]) are most likely related to differences in the microenvironments of casein phosphoserines when complexed in whole casein. The overlapping ^{31}P-NMR resonances (approx. 3.89, 3.70 and 3.57 ppm) of the micelle sample (fig. 2B) are partly resolved into three main asymmetric peaks (4.21, 3.81 and 3.54 ppm) in the submicelle sample (fig. 2A). The NMR spectral patterns and the chemical shift values observed in this study by re-forming casein micelles through Ca^{2+} addition are somewhat comparable to those reported by Belton et al. [28] for native milk (micelles) and EDTA disaggregated colloid (submicelles); if one takes into account the difference in pH/p*D* between our samples and their samples as well as the dependence of the SerP chemical shifts on pH/p*D* [28] the values are nearly equal.

3.3. The binding of calcium

Phosphorylated serine residues are generally considered to be involved in the binding of calcium by caseins [1,4]. Such binding may be directly probed by ^{31}P-NMR [31,32]. The upfield shift (approx. 0.4 ppm) of ^{31}P-NMR resonances due to deprotonated phosphoserines of salivary proteins A and C in the presence of calcium, combined with the effect of their dephosphorylation upon calcium binding, suggested that phosphoserines

Table 1

^{13}C-NMR chemical shifts [a] of whole casein

Assignment	Chemical shift (±0.02 ppm)	
	Submicelle	Micelle
Ile δCH_3	12.84	12.78
Ile γ_2CH_3, Ala βCH_3	17.11	17.05
Ala βCH_3, Ile γ_2CH_3	17.41	17.39
Val γ_2CH_3	19.13–19.44	19.17–19.37
Thr γCH_3, Val γ_1CH_3	20.35	20.32
Val γ_1CH_3, Thr γCH_3	20.54	20.53
Leu δ_2CH_3	21.07	21.03
Leu δ_1CH_3	21.48	21.43
Lys γCH_2, *cis* Pro γCH_2	23.42	23.44
trans Pro γCH_2, Arg γCH_2, Leu γCH	24.76	24.72
Ile γ_1CH_2	26.98	26.92
Lys δCH_2	27.20	27.12
Glx βCH_2	28.89	28.87
Arg βCH_2, His βCH_2 *	29.34	29.29
trans Pro βCH_2, Val βCH	30.21	30.16
cis Pro βCH_2	31.98	31.95
Not assigned *	32.38	32.29
Lys βCH_2	32.74	32.67
Gln γCH_2	33.64	33.62
Glu γCH_2	36.19	36.14
Asx βCH_2	38.58	38.63
Ile βCH_2	38.86	38.92
Phe, Tyr βCH_2	39.24	39.18
Leu βCH_2	41.12	41.07
Lys ϵCH_2	41.80	41.80
Arg δCH_2	43.16	43.09
Gly αCH	44.35	44.35
Pro δCH_2	50.21–50.91	50.50
Backbone αCH	52–60	
cis Pro αCH	60.94	60.99
trans Pro αCH	61.57	61.62
Ser βCH_2OH *	62.90–63.46	63.16–63.56
Ser βCH_2OPi *	65.80 [b]	65.60 [b]
Thr $\beta CHOH$	69.64	69.57
Tyr ϵCH	118.15	118.04 [c]
Tyr ϵCH, His δ_2CH	–	119.06 [c]
His δ_2CH *	119.93	121.55
Tyr γC, Phe ζCH	129.80	129.77
Phe ϵCH, Tyr δCH	131.37	131.43
Phe δCH, Tyr δCH	131.80	131.74
His ϵ_1CH	133.09	132.85–133.27
His γC, Phe γC	138.06	137.62 [c]
Phe γC, His γC	138.92	138.79 [c]
Tyr ζC	157.03–157.70	– *
Arg ζC *	159.57	159.45
Backbone CO	174.32–175.03	174.32–174.85
Backbone CO, Asn γCO	175.94–176.67	175.83–176.66
Asp γCO	179.90	179.99
Gln δCO	180.24	180.21
Glu δCO	183.79	183.77

are part of the protein calcium-binding sites [31] for these proteins. A similar upfield shift (0.25–0.32 ppm) of phosphoserine ^{31}P-NMR resonances of ovalbumin in the presence of magnesium has also been attributed to metal binding by phosphoserine residues [32]. The generally upfield shifts (up to 0.3 ppm) of ^{31}P-NMR casein resonances upon the addition of calcium (fig. 2, section 3.2) are consistent with, though better resolved than, previous ^{31}P-NMR data on bovine milk [28]. They are also consistent with the reported effect of calcium on the ^{31}P-NMR chemical shifts of purified β-casein [33]. When considered in conjunction with the effect of α_{s1}-casein dephosphorylation on calcium binding [34] as well as the upfield shift (0.20 ppm) and the marked broadening (table 1 and fig. 1) of the βCH_2 ^{13}C-NMR resonance of casein phosphoserines in the presence of calcium, these observations strongly suggest that casein phosphate groups are involved in the binding of calcium. This is in contrast to proteins such as parvalbumin [35] where calcium binding is associated with highly specific side chain/backbone binding sites.

Micelle formation is accompanied by several ^{13}C-NMR chemical shift differences between micelles and submicelles (table 1). There is one new peak at 119.06 ppm (Tyr ϵCH and/or His δ_2CH) in the micelle ^{13}C-NMR spectrum. The calcium effect on the chemical shift of Arg ζC (table 1) may be related to the inhibition of casein coagulation by rennin when casein arginines are chemically modified [36]. Other notable changes are the increased intensities of the two micelle resonances at 29.29 (Arg and/or His βCH_2) and

[a] vs internal DSS. At neutral pD, DSS also has peaks at 17.68, 21.80 and 57.07 ppm which, in our spectra (fig. 2), are buried under the protein peaks.

[b] Phosphoserine. The βCH_2 chemical shift of free SerP at pD 7.6 and 24°C is 65.29 ppm. As in Ser [27], an additional downfield shift may result from the amino acid's incorporation into a polypeptide chain.

[c] Broad/weak signal; approximate/uncertain assignment.

* Asterisks denote the most significant chemical shift differences between micelles and submicelles.

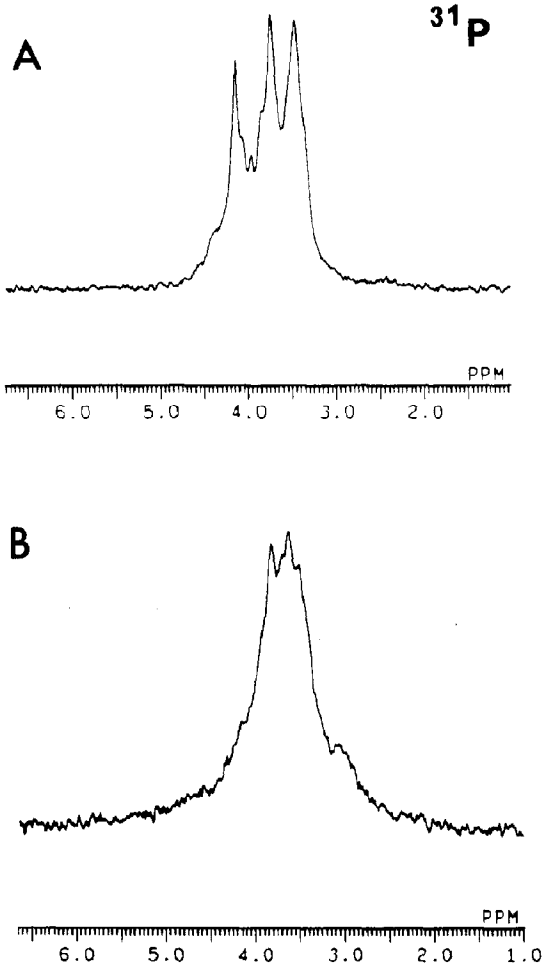

Fig. 2. Natural abundance $^{31}P\{^1H\}$ NMR spectra (161.8 MHz) of whole casein submicelles (A) and micelles (B) at 26°C. The protein concentration was 6.0%, the ionic strength 125 mM and the p*D* 7.6 (submicelles) or 7.4 (micelles). The number of scans was 600 and the applied exponential line broadening 2 Hz. Other experimental details are described in section 2. The general upfield shifts in B vs A are most likely due to the added calcium rather than the small difference in p*D* (0.2 units) between micelles and submicelles. The ^{31}P chemical shifts of whole casein are pH-independent above pH 7 [28].

32.29 ppm (not assigned). Raman and infrared spectroscopic studies have indicated calcium-induced conformational changes in whole casein that may be related to the above mentioned spectral differences [3,37].

3.4. The configuration of Pro residues

Although peptide bonds in proteins show an overwhelming preference for the *trans* configuration, both *cis* and *trans* isomers have been observed for peptide bonds preceding proline [38]. The *cis-trans* isomerization of the X-Pro peptide bond is slow on the NMR time scale and well-separated signals for the β- and γCH_2 groups of Pro *cis* and *trans* isomers can be detected by ^{13}C-NMR [39–41]. In a series of model peptides the β-carbon of Pro was shown to have a chemical shift of 30.5 ± 0.6 ppm (*trans*) or 32.2 ± 0.4 ppm (*cis*); the corresponding γ-carbon resonances are at 25.1 $\pm$ 0.5 and 23.4 ± 0.3 ppm, respectively [39]. Since the ^{13}C-NMR relaxation rates in the two isomeric species are similar, the β- and γCH_2 intensities of the *cis*- and *trans*-proline can provide a reliable estimate of the relative amounts of the two isomers [40,41]. Proline is the single most abundant amino acid (approx. 12 mol %) of whole casein [2] and it is likely to play an important role in determining the proteins' structure *. The limited resolution in the ^{13}C-NMR spectra of whole casein (table 1) does not allow a rigorous quantitative determination of the *trans/cis* ratio. The relative intensities of the peaks related to γ- and βCH_2 groups of *trans*- and *cis*-Pro (fig. 3) are consistent with the prevalence of the *trans* isomer (> 85%); the *cis* isomer may also be present (< 15%), in agreement with molecular modelling on individual caseins [43] and ^{13}C-NMR data on other peptides and proteins [39–42].

3.5. Molecular dynamics of casein

The correlation time τ_c that characterizes the overall isotropic tumbling of casein submicelles and micelles can be estimated from the equation

$$\tau_c = \frac{4\pi r_s^3 \eta}{3kT}$$

* In addition to X-Pro, there is considerable X-Pro-Pro and Pro-X-Pro, particularly in β-casein [2]. In model tripeptides, there is a decrease in the stability of X-Pro-Pro in the order *trans-trans*, *cis-cis*, *trans-cis* and *cis-trans* [42]. To our knowledge, there have been no reports on the effect of Pro-X-Pro on protein structure.

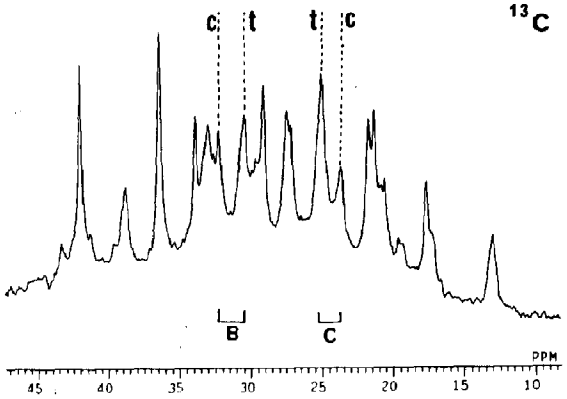

Fig. 3. The upfield region of the ^{13}C-NMR spectrum of a 6.0% whole casein submicelle solution after 19 h of signal accumulation at 26°C. The applied exponential broadening is 10 Hz; other experimental conditions are given in section 2. The positions of the resonances associated with βCH_2 (B), and γCH_2 (C) groups of the Pro ring are indicated, as well as the effect of the X-Pro peptide bond conformation: c, *cis*; t, *trans* configuration (table 1).

where r_s is the particle's Stokes radius, η the viscosity of the medium, k Boltzmann's constant (1.3806×10^{-16} erg K^{-1}) and T the absolute temperature. If r_s is as low as 40 Å for casein submicelles, then for $\eta = 0.87$ CP (water at 26°C), τ_c equals 56 ns; in the case of casein micelles ($r_s = 500$ Å) τ_c is 110 μs. The mere observation of high-resolution ^{13}C-NMR spectra (fig. 2) for so slowly tumbling particles strongly suggests the occurrence of considerable fast local motion(s) within both the casein submicelles and micelles.

The linewidths at half-height, $\Delta\nu_{1/2}$, of well-resolved and individually assigned Lorentzian absorption peaks (after correcting for the digital as well as an estimated 2 Hz inhomogeneity broadening) can provide a rough estimate of the motional time scale of the corresponding chemical groups. In the case of ^{13}C-NMR, at the magnetic field employed (9.4 T), the relaxation of protonated carbons is dominated by the ^{13}C-^{1}H dipole-dipole interactions (DD), whereas the chemical shift anisotropy (CSA) is the major relaxation pathway for the non-protonated carbons [10,14, 15]. In the event of isotropic motion characterized by a single correlation time τ_c, under conditions of complete proton decoupling, $\Delta\nu_{1/2}$ is given by the equations:

$$\Delta\nu_{1/2}^{DD} = \frac{n\hbar^2\gamma_C^2\gamma_H^2}{20\pi r_{CH}^6}$$

where

$$f(\tau_c) = 4\tau_c + \frac{\tau_c}{1+(\omega_C-\omega_H)^2\tau_c^2} + \frac{3\tau_c}{1+\omega_C^2\tau_c^2} + \frac{6\tau_c}{1+\omega_H^2\tau_c^2} + \frac{6\tau_c}{1+(\omega_C+\omega_H)^2\tau_c^2}$$

and

$$\Delta\nu_{1/2}^{CSA} = \frac{\gamma_C^2 H_0^2(\Delta\sigma)^2}{45\pi} \cdot \left(4\tau_c + \frac{3\tau_c}{1+\gamma_C^2 H_0^2\tau_c^2}\right)$$

The gyromagnetic ratios γ_C and γ_H are 6728 and 26753 rad s^{-1} G^{-1}, $\omega_C = 6.317 \times 10^8$ rad s^{-1}, $\omega_H = 2.512 \times 10^9$ rad s^{-1} and H_0 is 94000 G. The C–H bond length is 1.09×10^{-8} cm, $\hbar = 1.055 \times 10^{-27}$ erg s and the chemical shift anisotropy $\Delta\sigma$ for carbonyl and aromatic carbons is 2×10^{-4}; n is the number of protons directly attached to the carbons. At high fields, chemical shift anisotropy is the dominant relaxation mechanism in ^{31}P-NMR of phosphoproteins [16,17]. In this case, $\Delta\sigma = 1.1 \times 10^{-4}$ and $\gamma_P = 10840$ rad s^{-1} replaces γ_C in eqs 2 and 3. Although the assumption of isotropic motion for protein side chains is generally an oversimplification, it may be a reasonably accurate description for immobile groups within spherical proteins and protein assemblies (such as submicelles and micelles) and can serve as a point of departure for a discussion of mobilities as detected as detected by NMR.

Calculated and experimentally measured linewidths are listed in table 2 (these values represent an upper limit because of possible contributions from slower baseline resonances). Their comparison, however, clearly suggests fast segmental motion for Lys side chains within the casein submicelles. The decrease in mobility upon micelle formation is much less than what is expected from the increased protein size where, in the absence of local motion, peaks would be too broad to be detected. In the case of ^{31}P-NMR, the limited

Table 2

Calculated and observed ^{13}C- and ^{31}P-NMR linewidths of certain casein submicelle and micelle resonances at 26 °C

Resonance		Submicelle	Micelle
Methylene carbon	calc.	153 Hz [a]	301.1 kHz [b]
	obs. [c,d]	25 Hz	60 Hz
Non-protonated carbon	calc.	25 Hz [a]	49.8 kHz [b]
	obs. [e]	26 Hz	60 Hz [f]
Phosphorus	calc.	20 Hz [a,g]	39.1 kHz [b,g]
	obs. [h]	–	–

[a] For isotropic rotation with $\tau_c = 56$ ns.
[b] For isotropic rotation with $\tau_c = 110$ μs.
[c] The Lys ϵCH_2 (table 1).
[d] The reported linewidths may represent an upper limit, since they were measured with respect to the apparent spectral baseline; possible broad humps (due to slow protein motions) in the envelope of aliphatic resonances were not taken into account.
[e] The Glu δCO (table 1).
[f] Asymmetric lineshape (fig. 4).
[g] Considering CSA relaxation only; there may be an additional 10–15% contribution to the linewidths from relaxation through dipolar interactions [14].
[h] The low resolution does not allow linewidth measurements.

resolution, although the best achieved to date (fig. 2), does not allow accurate measurement of linewidths. However, ^{31}P-NMR linewidths in micelles are much narrower than the calculated 39.1 kHz, indicating fast local motion of phosphate groups within casein micelles, in agreement with studies on individual caseins [44–46].

^{13}C-NMR relaxation measurements can provide information concerning the mobility of chemical groups within protein molecules on the timescale of 10^{-7} to 10^{-12} s [10,14]. The (average) rotational correlation time for the backbone carbon atoms of a native globular protein is a reliable estimate of the correlation time for the overall tumbling of the entire protein molecule [47]; higher mobilities are generally observed for side chain groups that are further away from the protein backbone [10,14,15,47]. Some results of ^{13}C-NMR

Table 3

Longitudinal ^{13}C-NMR relaxation times of certain submicelle and micelle resonances at 37 °C

Resonance	Submicelles		Micelles	
	chemical shift (ppm)	T_1 (ms) [a]	chemical shift (ppm)	T_1 (ms) [a]
α CH	56.33	388	56.21	395
Lys ϵCH_2	41.80	589	41.80	471
Glu γCH_2	36.19	313	36.14	276
Phe δ,εCH	131.60	385	131.60	476
Thr βCHOH	69.64	475	69.57	388
Ser βCH_2OH	62.90	323	63.16	319
SerP βCH_2OPi	65.80	267 [b]	65.60	223 [b]

[a] The error in the ^{13}C T_1 measurements is of the order of 10%.
[b] Due to the low signal-to-noise ratio, the error in these particular measurements is of the order of 25% (submicelles) or 40% (micelles).

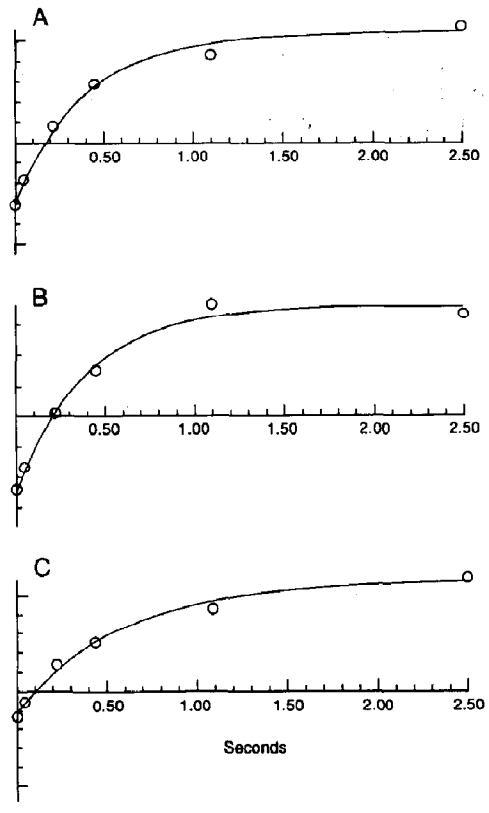

Fig. 4. Exponential decay for αCH backbone resonances of submicelles (A) and micelles (B). Peak height in arbitrary units is plotted vs time in s. T_1 values were calculated from these curves as described in section 2.2. The decay curve for a typical distal side chain carbon (ϵCH_2 of lysine) is shown (C) for submicelles only; the micelles' data were identical and are not shown.

longitudinal relaxation measurements for whole casein are given in table 3. It is most interesting that the T_1 values of the submicelle and micelle αCH envelopes are essentially identical. For both submicelles and micelles the αCH resonances exhibit well-defined single-exponential decay (fig. 4A and B). This of course is reflected in the nearly identical T_1 values calculated from these curves and given in table 3. Since the αCH carbon is an integral part of the protein backbone, the similarity in the decay curves suggests that the dynamic states of the submicelle protein chains are not affected by incorporation into casein micelles. The corresponding correlation time according to an isotropic rotation model (no internal motion) is approx. 8 ns [48]. Consideration of a more involved model with a 10 ps internal libration at a mean angle of 20° in addition to the overall isotropic rotational tumbling [49] results in a reduction of τ_c by a factor of 2. These values (8 or 4 ns) are comparable to those of small monomeric globular proteins, well below the 131 ns expected for the overall tumbling of the submicelles of 94 Å-radius. It appears that there is considerable

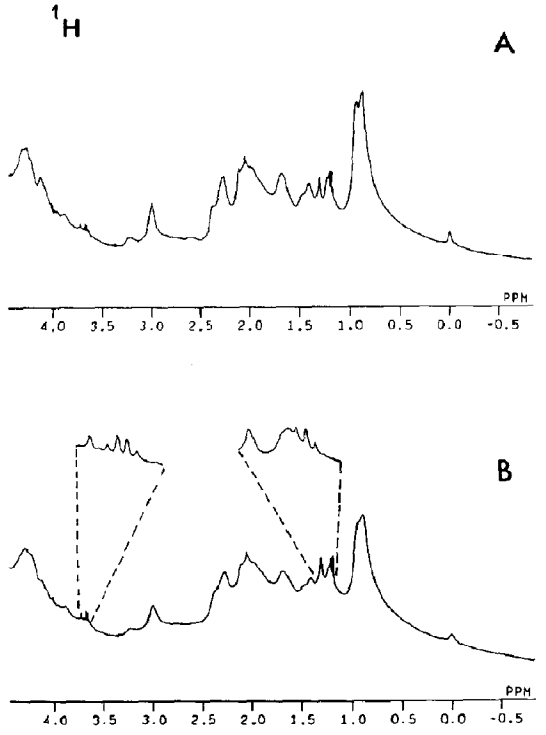

Fig. 5. The upfield region of the ^{1}H-NMR spectra (400 MHz) of 6.0% whole casein submicelles (A) and micelles (B) at 37°C. Each spectrum is the result of 500 scans; the applied exponential broadening is 0.5 Hz. Other experimental details are given in section 2. The expanded regions provide a clearer view of the fine details at 1.15 and 3.70 ppm, which are identical for submicelles and micelles. These sharp resonances are most likely due to non-protein components such as residual lactose (3.7 ppm) and/or the carbohydrate moiety of κ-casein [13,50,51]. An alternative assignment for the 1.15–1.25 ppm peaks is Ile γ_2CH_3 and/or Thr γCH_3 [52].

Table 4

Longitudinal ^{31}P-NMR relaxation times of certain casein submicelle and micelle resonances [a,b]

Resonance	Submicelles		Micelles	
	Chemical shift (ppm)	T_1 (ms) [c]	Chemical shift (ppm)	T_1 (ms) [c]
Phosphorus	4.21	929 (87%)	3.70	602 (67%)
		5616 (13%)		656 (33%)
	3.81	398		
	3.54	223 (88%)		
		1573 (12%)		

[a] Data points (peak heights) corresponding to antiphase spectral patterns (and possibly erroneous peak intensities) were not taken into account. The contribution of each relaxation component (whenever more than one was considered) is given in parentheses.

[b] These particular measurements were conducted under continuous 9 W proton decoupling which caused substantial r.f. heating. The samples' temperature rose to 36°C, 10°C above the controlled (26°C) probe temperature.

[c] The error in the ^{31}P T_1 measurements is approx. 5%.

mobility of the protein backbone(s) within the casein submicelle.

^{13}C-NMR T_1 resonances of protein side chains also exhibit exponential decay (the decay curve for ϵCH_2 lysine of submicelles is shown in fig. 4C). The T_1 values calculated from these curves appear to be similar, within experimental error, for submicelles and micelles (table 3). However, in this case, the isotropic rotation model is an unrealistic approximation, and an estimation of the respective correlation times requires a more appropriate model. Nevertheless, the results do suggest similar

mobilities for protein side chains within these two structures.

The most downfield major ^{31}P resonance (4.21 ppm) of submicelles (fig. 2A) appears to relax considerably more slowly than the other two (table 4). Upon calcium binding and micelle formation, the longitudinal relaxation times of all phosphoserines become comparable (approx. 600 ms). However, the characterization of the corresponding change(s) in the dynamic state of the SerP side chains requires more involved motion models than the simple isotropic rotation. In the case of macromolecules such as phosphoproteins, the fitting of multiple relaxation data (e.g., T_1 measurements at different magnetic fields) to theory can rarely be accomplished by assuming a single correlation time [17,46].

3.6. ^{1}H-NMR of casein

Our ^{1}H-NMR spectra (fig. 5) are fairly similar to those previously reported [11–13] for native casein micelles. There is little or no difference in the casein ^{1}H-NMR spectra as the submicelles are incorporated into micelles after the addition of calcium, in agreement with fig. 4 of ref. 13 where EDTA was added to dissociate the micellar complexes. There have been attempts to assess the dynamic state of casein by ^{1}H-NMR [11–13]. However, the extensive spectral overlap does not permit any estimates of mobility from linewidths. It appears that the broad-featured casein ^{1}H-NMR spectra are the result of limited spectral resolution rather than restricted molecular mobility and thus they are quite compatible with fast motion, as detected by ^{13}C- and ^{31}P-NMR.

4. Concluding remarks

^{1}H-NMR data have suggested the existence of some fast motion within casein micelles [11–13]. In one study, the mobile protein regions were exclusively identified with the glycomacropeptide segment of κ-casein, thus supporting a 'hairy' micelle model where flexible glycomacropeptide chains extend into solution from the surface of rigid protein cores [11]. It is unclear how such a rigid structure will at the same time be loosely packed in order to explain the ready penetration of lactose, salt and proteolytic enzymes into the micelle [1]. Also, from the viewpoint of signal intensity it is unlikely that the evidence of mobility in our ^{13}C-NMR spectra of submicelles and micelles is solely due to the glycomacropeptide which comprises less than 4% mol of whole casein and less than 0.3% protein in our samples. Other ^{1}H-NMR results have indicated that only part of the observed mobility in micelles is due to κ-casein [12]. Indeed, ^{13}C-NMR resonances due to Arg and Phe (which do not occur in glycomacropeptide) can be equally resolved in both submicellar and micellar casein.

Multinuclear NMR spectroscopy is a noninvasive technique that can provide a wealth of information, even about large protein assemblies such as casein micelles. Thus, the monitoring of the binding of calcium or other metal to (phospho)-proteins by NMR may provide useful insights into a wide range of related biological problems, such as the regulation of calcium in the oral cavity by salivary proteins [26] and the mineralization of the bone [53]. Also, caseins, because of their well-studied association-dissociation reactions [4], provide a good model for correlating ^{31}P-NMR relaxation data with the state of protein aggregation [46]. The quantitative assessment of the casein dynamics in particular can be used to evaluate critically proposed structural models and to study the effect of experimental conditions such as pH, temperature and ionic strength on its structure.

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