Nuclear Magnetic Resonance Study of the Conformation and Dynamics of β-Casein at the Oil/Water Interface in Emulsions

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ABSTRACT A ^{13}C and ^{31}P nuclear magnetic resonance (NMR) study has been carried out on β-casein adsorbed at the interface of a tetradecane/water emulsion. ^{13}C NMR spectra show signals from the carbonyl, carboxyl, aromatic, and C^{α} carbons in β-casein, well resolved from solvent resonances. Only a small fraction of all carbon atoms in β-casein contribute to detectable signals; intensity measurements show that the observable spectrum is derived from about 30 to 40 amino acid residues. ^{31}P NMR spectra show signals from the five phosphoserines on the hydrophilic N-terminal part of the protein. Analysis of T_1 relaxation times of these nuclei, using the model free approach for the spectral density function and the line shape of the α-carbon region, indicates that a large part of the protein is in a random coil conformation with restricted motion and a relatively long internal correlation time. The NMR results show that the conformation and dynamics of the N-terminal part of β-casein are not strongly altered at the oil/water interface, as compared to β-casein in micelle-like aggregates in aqueous solution.

INTRODUCTION

The formation and stabilization of emulsions is an important topic in the food industry. Proteins are known to be suitable emulsifiers. The properties of proteins, however, are greatly influenced by external factors such as pH, temperature, ionic strength, and the presence of other molecules that either occur naturally in foods or are added for technological reasons.

Bovine caseins are disordered, flexible proteins that are often used to stabilize oil-in-water food emulsions. This study concerns one of the main caseins, β-casein, a phosphoprotein consisting of 209 amino acid residues. Its first 50 residues (from the N-terminus) are predominantly hydrophilic and mainly negatively charged at neutral pH, whereas the remaining part is fairly hydrophobic, with little charge (Swaisgood, 1992). Presumably it is the combination of molecular flexibility and amphiphilicity that makes β-casein a good emulsifying agent (Dickinson, 1994). Neutron reflectance measurements have shown that the protein occupies a dense primary layer 2 nm thick at an oil/water interface as well as a low-density secondary layer that extends for 5 to 6 nm into the aqueous phase (Dickinson et al., 1993). It is reasonable to assign the high-density layer to hydrophobic domains that adhere to and cover the oil surface and the low-density layer to N-terminal tails (and probably some loops) that extend into the aqueous phase. Tails from κ-casein form a similar outer layer on casein micelles (Walstra, 1990).

Nuclear magnetic resonance (NMR) spectroscopy may be suitable for studying emulsions because the experimental conditions (temperature, concentration, pH, etc.) during measurement can often be chosen to be similar to those found in industrial processes. ^{1}H NMR studies of casein micelles demonstrate that extended tails are flexible (Griffin and Roberts, 1985; Rollema and Brinkhuis, 1989), and ^{31}P NMR data confirm that phosphoserines (located on the hydrophilic tails) are mobile (Humphrey and Jolley, 1982). No ^{1}H NMR data have been published for casein/oil/water emulsions, probably because oils produce strong interfering signals that obscure much of the spectrum of adsorbed casein.

In this paper we present ^{13}C NMR data on adsorbed β-casein and ^{31}P NMR data on micellar and adsorbed β-casein that provide a quantitative measurement of the time scale and amplitude of protein mobility as well as some information about the secondary structure. ^{13}C and ^{31}P NMR are better suited for this purpose than ^{1}H NMR, because the spectra are better resolved, and one can observe β-casein signals, even in the presence of much stronger oil signals. This is especially true for ^{31}P NMR, because there are no phosphorus atoms in our oil and the spectrum gives site-specific information from the phosphoserines. Furthermore, the interpretation of relaxation times for both nuclei in terms of molecular motion is more straightforward (Wagner, 1993).

EXPERIMENTAL

Initial emulsions containing 20% *n*-tetradecane oil and 9 mg/ml bovine β-casein in imidazole buffer (50 mM, pH 6.5, 0.01% NaN_3, and 20 mM

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EDTA for the ^{31}P samples) were prepared by homogenizing the mixture for more than 20 passes at 100 Pa using a laboratory homogenizer (Delta Instruments). *n*-Tetradecane oil (99.5%) was obtained from Merck and used without further purification. The emulsion was diluted three times with buffer and centrifuged at 7000 × *g* for 45 min. The cream was removed and resuspended in buffer so that the oil fraction was as in the initial emulsion. This was repeated twice. In the final step the cream was resuspended in D_2O to an oil fraction of 0.4. D_2O served as an NMR lock solvent. The volume-surface average droplet diameter of the washed emulsion as determined with laser diffraction (Coulter Laser LS130) was about 0.5 μm (see Fig. 1). This implies a surface area of about 5 m^2/ml emulsion. With a saturated surface load of at least 2 mg/m^2 per milliliter of emulsion, 10 mg of β-casein is adsorbed at the oil/water interface. To improve the NMR linewidths, solutions were dialyzed (Spectrapor; molecular weight cutoff, 12–14,000) against imidazole buffer to remove paramagnetic ions. The concentration of unadsorbed β-casein in the water phase after the last wash was calculated to be less than 0.05% of the total amount in the emulsion. Proteins readily adsorb to the oil/water interface; therefore a saturated surface load is obtained at extremely low protein concentrations (about 2.5 mg/l) (Graham and Phillips, 1979; Walstra and De Roos, 1993). Protein desorption from the interface is very unlikely, because protein adsorption is practically irreversible. The freshly prepared emulsions were used immediately for NMR experiments. The free β-casein samples were prepared by dissolving 150 mg β-casein in 3 ml of imidazole buffer (50 mM imidazole, 20 mM EDTA, pH 6.5). This solution was dialyzed according to the same procedure as the emulsion samples. A volume of 2 ml D_2O imidazole buffer was added to give a final concentration of 3% (w/v). At this concentration, most of the β-casein exists predominantly in the form of micelle-like aggregates (Andrews et al., 1979). NMR measurements were made on Bruker AMX-300 and AMX-500 spectrometers at 295 K using two-level broad-band ^1H decoupling. For ^{13}C NMR, an external dioxane chemical shift reference was used. Signal-to-noise ratios were poor; for example, the spectrum shown in Fig. 2 was accumulated in 58 h.

THEORY

There are two main relaxation mechanisms for ^{13}C and ^{31}P spins. When ^1H-^{13}C dipolar interactions dominate, the spin-lattice relaxation time, T_1, for ^{13}C nuclei is given by (Abragam, 1961; McCain, 1987)

$$\frac{1}{T_1} = \left(\frac{\mu_0}{4\pi}\right)^2 \frac{1}{20} \gamma_H^2 \gamma_C^2 \hbar^2 \sum_I r_{CI}^{-6} \times [J(\omega_I - \omega_C) + 3J(\omega_C) + 6J(\omega_I + \omega_C)], \quad (1)$$

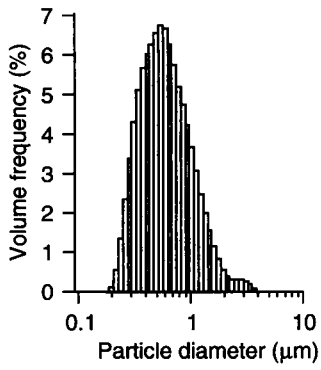

FIGURE 1 Volume frequency (%) of diameters of oil droplets versus diameter immediately after emulsification.

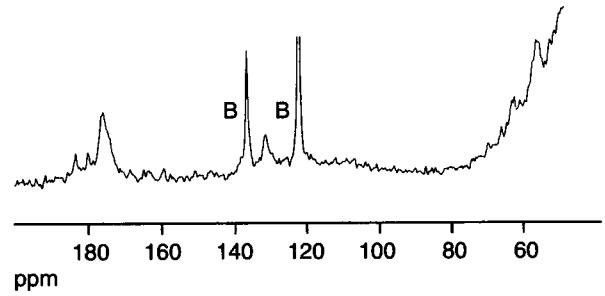

FIGURE 2 ^{13}C NMR spectrum of a β-casein/tetradecane emulsion suspended in buffered D_2O (50 mM imidazole, pH 6.5) at 295 K. The trace rises to the right as it approaches the very strong tetradecane spectrum (off scale). Peaks marked B are from the imidazole buffer, and the remainder are from β-casein, including signals at 183.4, 180.0, 176.0, 131, 63, and 56.3 ppm.

where $\hbar$ is Planck's constant divided by 2π, r_{CH} is the ^1H-^{13}C internuclear distance, γ_H and γ_C are the gyromagnetic ratios, and ω_H and ω_C are the spin precessional frequencies of ^1H and ^{13}C nuclei, respectively. The equation for ^1H-^{31}P relaxation can be obtained by replacing C by P everywhere in Eq. 1.

When spin-lattice relaxation is caused by chemical shift anisotropy, T_1 is given by (Abragam, 1961; McCain, 1987)

$$\frac{1}{T_1} = \frac{1}{15} \gamma_P^2 B_0^2 \Delta\sigma^2 J(\omega_P), \quad (2)$$

where B_0 is the static field strength and $\Delta\sigma$ is the difference between the extreme values of the diagonalized chemical shift anisotropy tensor. The chemical shift anisotropy relaxation rate depends on the magnetic field strength. Therefore it is possible to determine the type of mechanism by measuring relaxation rates at different field strengths.

The T_1 values provide information about molecular motion, but not directly; the data must be interpreted. Relaxation theory involves many independent variables, but the experiment often provides only a few data points; therefore it is usually necessary to find a way to simplify the analysis. If internal motion within the β-casein is quite rapid compared to rotational motion of the emulsion particle, the large difference in time scale will allow a separation of the two effects. Typically, to further simplify the problem, we assume that there is only one time scale for internal motion.

Relaxation times are related to molecular motions through the spectral density function $J(\omega)$. For the analysis of our data, the model-free function (Lipari and Szabo, 1982a,b) is used, given by

$$J(\omega) = \frac{2}{5}\left(\frac{S^2\tau_M}{1 + (\omega\tau_M)^2} + \frac{(1 - S^2)\tau}{1 + (\omega\tau)^2}\right), \quad (3)$$

where

$$\frac{1}{\tau} = \frac{1}{\tau_M} + \frac{1}{\tau_e}, \quad (4)$$

and

$$S = \frac{1}{2}\langle 3\cos^2\theta - 1\rangle. \quad (5)$$

The parameter τ_M is the correlation time for overall rotational motion and τ_e is the effective correlation time for internal motion. The angular brackets indicate an ensemble average, and θ is the angle between the principal axis of the chemical shift tensor and the surface normal of the oil droplet, if relaxation is determined by chemical shift anisotropy. If relaxation is determined by dipolar couplings, θ is the angle between the ^{13}C-H internuclear vectors and the surface normal. S is the generalized order parameter, which is a measure of the spatial restriction of the motion. S^2 is a number between 0 and 1. If the tail of the protein is completely rigid, $S = 1$ and the only motion is that of the oil droplet on which the protein is adsorbed. If protein motion is totally unrestricted, $S = 0$, although the converse need not be true. In effect, S provides information about the amplitude of internal motions. For more detailed information, the order parameter should be interpreted in terms of a specific model.

For the emulsions used in our work, where the average particle diameter is about 0.5 μm, τ_M is about 10^{-2} s according to the Stokes-Einstein equation. This is several orders of magnitude larger than τ_e (which is on the order of 10^{-9} s); to a good approximation, Eq. 3 reduces to

$$J(\omega) = \frac{2}{5}(1 - S^2)\frac{\tau_e}{1 + (\omega\tau_e)^2}. \quad (6)$$

RESULTS AND DISCUSSION

^{13}C NMR spectra

Fig. 2 shows part of the ^{13}C NMR spectrum of β-casein adsorbed on a tetradecane-water interface. The trace rises toward the right as it approaches a tetradecane peak; NMR signals from tetradecane are much stronger than those from β-casein, because the sample contains approximately 10^4 molecules of tetradecane per β-casein molecule. A control experiment with pure tetradecane showed no signals above 50 ppm, whereas another control experiment using 2% β-casein in imidazole/D_20 buffer (where the β-casein exists mostly as micelle-like aggregates) gave NMR spectra that exhibit peaks in the same positions as in our emulsion spectra. Therefore we confidently assign all the peaks that we observe above 50 ppm to β-casein, with the exception of two narrow peaks at 122 and 136 ppm from the imidazole buffer. β-Casein peaks below 50 ppm are obscured by the intense tetradecane spectrum.

If the protein were completely rigid and attached to particles as large as those in our emulsion, its peaks would be undetectable; they would be both extremely broad and easily saturable. Therefore, we do not expect to see signals from those portions of the protein that are relatively rigid. The fact that protein signals are observed demonstrates that at least part of the β-casein is internally mobile, and the fact that the spectrum resembles that of β-casein in solution suggests that the same parts of the protein are mobile in both emulsions and micelle-like aggregates.

It is not possible to assign peaks in the ^{13}C NMR spectrum of β-casein to individual carbon atoms; we lack the information to make such an assignment, and even if we could identify them, individual peak intensities would be barely above the noise level. Instead, peaks are observed that are the superimposed signals of many carbons that have nearly identical chemical shifts. For example, in a typical protein the α-carbon atoms of 15 different amino acids provide signals that cluster in the narrow range from 53 to 58 ppm, and four other amino acids contribute signals near 63 ppm, whereas other carbon resonances seldom appear in this range (Wüthrich, 1976). Therefore, the broad peak that is observed (using a large, 50-Hz line-broadening parameter to blend the overlapping lines) centered on 56.3 ppm and the smaller peak near 63 ppm (too weak to be accurately measured) can be assigned to unresolved α-carbon peaks.

Similarly, minor peaks at 183.4, 180.0, and the major peak at 176.0 ppm (with a weak shoulder at a somewhat lower chemical shift) are derived from carbonyls in peptide bonds and from carboxylates on side chains. The peak near 131 ppm is from aromatic carbons (Wüthrich, 1976). Even though our NMR signals cannot be assigned to individual carbon atoms, they do provide structural information. The chemical shift of an α-carbon varies with the secondary structure of the protein; when amino acids are incorporated into an α-helix, their α-carbon and their carbonyl chemical shifts increase by more than 0.7 ppm and 0.5 ppm, respectively, as compared to the random structure, whereas in a β-strand, the shifts are lower by the same amounts (Wishart and Sykes, 1994). Using tabulated chemical shift data (Wishart and Sykes, 1994) and the amino-acid sequence for the first 50 N-terminal residues on β-casein (Swaisgood, 1992) (assumed to be the mobile region that is the source of the observed signals), the average chemical shift of α-carbon atoms in the configuration is calculated to be 56.56 ppm (excluding amino acids such as proline that have α-carbon shifts outside the 53 to 58 ppm range) and 63.40 for the remaining α-carbons (excluding glycine at 45 ppm); for carbonyl carbons in the random configuration the average shift (including all amino acids) is 175.96 ppm. These calculated shifts are not highly dependent on amino acid composition, so if mobile regions other than the N-terminal tail were included, the calculated shifts would be nearly the same. The major peaks that are observed (at 56.3 and 176.0 ppm) are closest to those calculated for the random configuration (56.56 and 175.96); therefore our results suggest that a large portion of the protein has no well-defined secondary structure. In turn, these results are consistent with large-amplitude internal motion; strong secondary structure tends to restrict internal mobility.

Spin-lattice relaxation times, T_1, were measured using the method of progressive saturation (McCain, 1987). Results were obtained at the following chemical shift values: 183.4

ppm, 2.8 s; 180.0 ppm, 3.8 s; 176.0 ppm, 2.16 s (the shoulder on this peak had about the same relaxation time); and 56.3 ppm, 1.95 s (Fig. 3). Peaks at 131 and 63 ppm had similar T_1 values but were too weak for accurate measurement. Our measured relaxation times are averages of many individual values; each carbon atom in the molecule has a unique T_1. The results in Fig. 3 appear to fit a single-exponential recovery curve quite well; however, it is the nature of these functions that an average of a wide distribution of exponentials is indistinguishable from a single exponential within our range of experimental error. Therefore, we cannot determine from our data whether relaxation times are all about the same or whether individual carbon atoms have T_1 values that vary from the average.

Because α-carbon atoms and carbonyl carbon atoms occupy adjacent positions on the protein main chain, it is assumed that they experience virtually the same internal motion; then, by using the two experimental relaxation times, two parameters can be determined that describe the motion, e.g., time scale and amplitude. Although the system is much more complex than we have assumed, the results cannot support a more detailed analysis.

Applying the relaxation Eqs. 1, 2, and 6; assuming that α-carbon relaxation is dominated by the dipole-dipole mechanism from one ^{1}H at 0.112 nm (McCain and Markley, 1986) and that carbonyl carbons are relaxed by chemical shift anisotropy (with $\Delta\sigma$ = 193 ppm, a value taken from the carbonyl group in acetone; Pines et al., 1972); and using S = 0, the curves shown in Fig. 4 were calculated. These curves represent theoretical lower limits on T_1 at the field strength of our NMR spectrometer. T_1 values that lie above the curves imply $S > 0$, which may be calculated from

$$S^2 = 1 - \frac{T_1(\text{at } S = 0)}{T_1(\text{at } S > 0)}. \quad (7)$$

Experimental α-carbon and carbonyl T_1 values are indicated in Fig. 4 as horizontal lines above the curves. The lines span

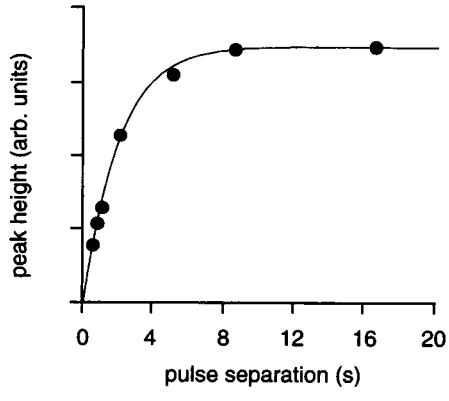

FIGURE 3 Results from a T_1 measurement at 56.3 ppm. Spectra were obtained using equal numbers of 90° transmitter pulses at different repetition rates. The curve represents a least-squares fit of measured peak heights to an exponential recovery function. It gave a relaxation time of 1.95 s.

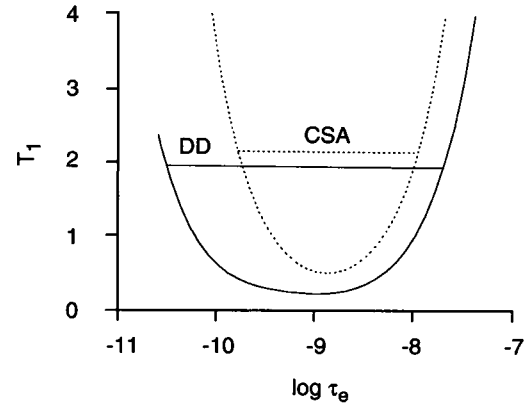

FIGURE 4 Theoretical T_1 relaxation times versus the effective correlation time for internal motion, τ_e, compared with experimental T_1 values. The solid curve represents dipole-dipole (DD) relaxation times of α-carbon atoms, and the dashed curve gives the relaxation times of carbonyl carbon atoms by the chemical shift anisotropy (CSA) mechanism (calculated using $S = 0$). The solid and dashed horizontal lines are experimental T_1 values measured at 56.3 and 176.0 ppm, respectively; the extension of the horizontal lines inside the theoretical curves shows the range of correlation times that are consistent with the experimental data if $S > 0$.

the range over which the theory is consistent with experimental data. Using Eq. 7, these data can be transformed into Fig. 5, a plot of all possible order parameters and correlation times that are consistent with experiment. Assuming that internal motions are the same at both types of carbon, we expect to identify their common values of S and τ_e as a point on Fig. 5 where the two curves cross. There is no crossing point, but the two curves do approach each other closely at $\tau_e \approx 1$ ns and $S \approx 0.9$. Considering potential errors (experimental errors in the measurement of T_1, the range of T_1 values that must make up the single values that we measure, and simplifying assumptions that may be responsible for errors in our calculations), we believe that the curves do

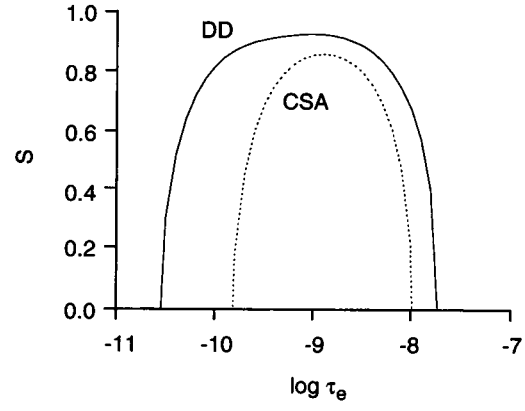

FIGURE 5 Values of the order parameter, S, and of the effective correlation time for internal motion, τ_e, that are consistent with experimental data, assuming dipole-dipole (DD) relaxation of α-carbon atoms and relaxation of carbonyl carbon atoms by the chemical shift anisotropy (CSA) mechanism. If α-carbon and carbonyl carbon atoms were to experience internal motion at the same amplitude and time scale, the two curves should cross.

meet to a reasonable approximation somewhere in the broad range between about $S = 0.7$ and $S = 0.9$ and with τ_e between about 3×10^{-10} and 6×10^{-9}.

Peak intensities can be used to estimate the number of NMR-detectable carbon atoms in β-casein. The imidazole buffer provides a convenient reference intensity. Taking into account the volume fraction of the aqueous phase (0.6), and its buffer concentration (50 mM), as well as the concentration and molecular mass of β-casein (10 mg/ml, 24 kDa), the 122 ppm peak of imidazole (from two equivalent carbon atoms) should have an intensity that is 144 times as great as the signal from one carbon atom in every casein molecule. Accurate intensity measurements require low repetition rates (see Fig. 3); the data discussed below were obtained from six different T_1 experiments with pulse separations of 5.0 s or greater. The number of detectable carbons was calculated as 144 times the ratio of the casein peak integral to the 122 ppm peak integral.

The combined integrals from peaks at 56 and 63 ppm (the α-carbons) are equivalent to 26 ± 10 carbon atoms. The intensity of the 131 ppm peak (aromatic carbons) indicates 11 ± 6 atoms, and combined integrals from peaks at 176, 180, and 183 ppm (carbonyl carbons) indicate a total of 45 ± 20 atoms. Error limits are twice the standard deviation. Systematic errors are possible in the α-carbon data because it was necessary to estimate a suitable baseline for integration; the 56 and 63 ppm peaks are affected by nearby tetradecane signals (see Fig. 2).

The amino acid sequence of β-casein includes a highly charged N-terminal region that is about 50 residues long; the remainder is mostly hydrophobic. Using the published sequence (Swaisgood, 1992) and assuming that only the first 50 residues are NMR detectable, we calculate that the signal should be derived from 49 α-carbons (the glycine peak is out of range), six aromatic carbons (five from one phenylalanine and one from a histidine), and 63 carbonyl carbons (some residues—glutamic acid, for example—have more than one carbonyl). Experimental data (except for aromatic carbons) suggest a somewhat shorter detectable region (perhaps 30 or 40 residues), but considering the wide range of experimental error and the possibility of substantial systematic error, our data are consistent with a mobile tail that approaches 50 residues in length. Certainly, we do not detect signals from every residue in β-casein.

^{31}P NMR spectra

Fig. 6 displays the ^{31}P NMR spectra of β-casein in solution and of β-casein emulsions obtained at two magnetic field strengths. All other conditions were kept constant. The solution spectra both show four peaks in an intensity ratio of about 1:1:2:1 (from low to high field), accounting for all five phosphoserine residues in the β-casein molecule. This is in agreement with the β-casein NMR spectra reported by Humphrey and Jolley (1982); they assigned peak 4 (Fig. 6, *A* and *C*) to phosphoserine residue 35, which is the phosphoserine furthest from the N-terminus.

The spectrum of β-casein in solution at 121.48 MHz (Fig. 6 *C*) has a very broad-base region. This may indicate that there are two populations of free β-casein present in the sample. At the concentrations used in this experiment, β-ca-

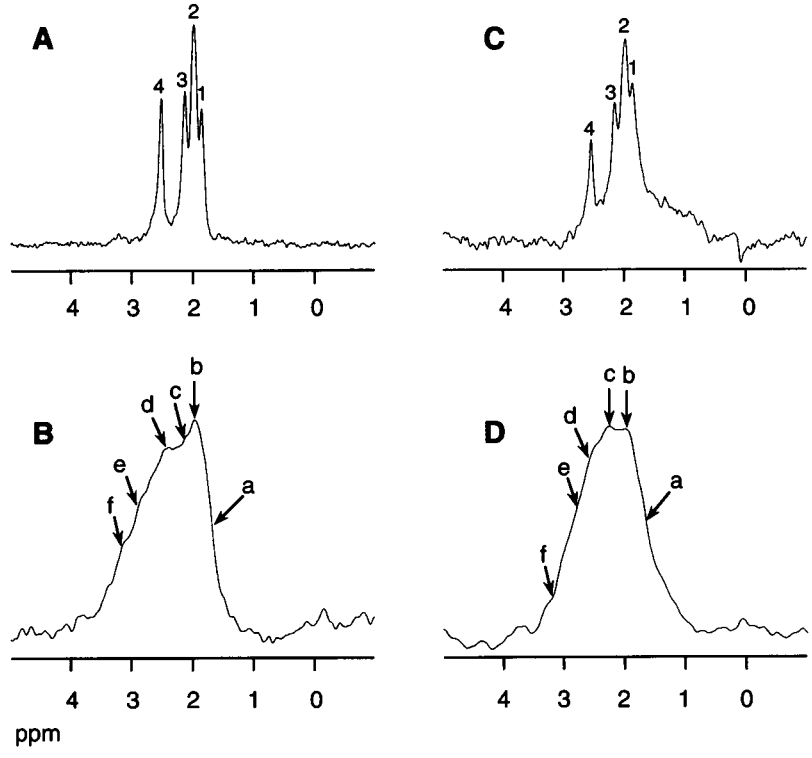

FIGURE 6 ^{31}P NMR spectra of β-casein at pH 6.5 in solution and of the β-casein stabilized oil/water emulsion at two different magnetic fields. (*A*) β-Casein in solution at 202.46 MHz; (*B*) β-casein-stabilized oil/water emulsion at 202.46 MHz; (*C*) β-casein in solution at 121.48 MHz; (*D*) β-casein stabilized oil/water emulsion at 121.48 MHz. The labels 1–4 and a–f refer to the positions used in the determination of the relaxation data in Table 1.

TABLE 1 Longitudinal relaxation times of β-casein in solution and the β-casein-stabilized oil/water emulsion at two different magnetic fields

Longitudinal relaxation time T_1 (s) of β-casein in solution				
ν_P (MHz)	Peak 1	Peak 2	Peak 3	Peak 4
202.46	1.40 ± 0.03	1.44 ± 0.02	1.57 ± 0.06	1.58 ± 0.08
121.48	1.94 ± 0.08	2.00 ± 0.05	2.24 ± 0.10	2.51 ± 0.10

Longitudinal relaxation time T_1 (s) of adsorbed β-casein						
	Position a	Position b	Position c	Position d	Position e	Position f
202.46	1.37 ± 0.08	1.75 ± 0.14	1.70 ± 0.10	1.60 ± 0.09	1.74 ± 0.19	1.80 ± 0.19
121.48	2.01 ± 0.15	2.07 ± 0.09	2.20 ± 0.18	2.28 ± 0.15	2.16 ± 0.19	2.05 ± 0.34

The different peaks and positions are given in Fig. 6. The parameter ν_P is the phosphorus NMR frequency.

sein exists predominantly in the form of micelle-like aggregates (Andrews et al., 1979). As was suggested in the same article (Andrews et al., 1979) and confirmed by Humphrey and Jolley (1982), the highly charged N-terminal region is located at the surface of the β-casein "micelle," where there is extensive penetration of water. This β-casein tail, which contains all five phosphoserine groups, has a certain degree of freedom, and therefore four peaks are resolved in the spectrum. However, a population of β-casein molecules that have their N-terminal regions inside the "micelle" would have less freedom of motion and therefore broader peaks. Adding EDTA to our emulsion reduced the peak widths by about 25%; ^{31}P NMR spectra are very sensitive to traces of paramagnetic metal ions, and the addition of EDTA removes these ions.

The two emulsion spectra do not show resolved peaks. They were analyzed using a 20-Hz line broadening. The reason for broad peaks in this case is slow overall motion of the oil particle on which β-casein is adsorbed. This limits the rate at which chemical shift anisotropy is averaged over all angles.

Longitudinal relaxation times, T_1, were measured using the inversion recovery pulse sequence (McCain, 1987). The values for β-casein in solution and adsorbed β-casein, calculated with a least-squares fit to the peak heights, are given in Table 1. The relatively long relaxation times, typical of smaller mobile molecules, are consistent with some motional freedom and a lack of secondary structure in the N-terminal region of β-casein. The values for β-casein in solution are in agreement with those reported by Humphrey and Jolley (1982), and the T_1 values increase from peak 1 to 4.

The experimental ^{31}P NMR spectrum (Fig. 6) is partially but not completely resolved. Therefore, we cannot measure the relaxation times of individual peaks unambiguously. We can, however, measure the apparent T_1 at several positions across the spectrum (positions *a* to *f* in Fig. 6) to look for evidence of different relaxation times. Relaxation times for adsorbed β-casein do not show significant differences for positions *a* to *f*. Either all relaxation times are similar or each position is a weighted average of different phosphoserines, or both. It can be seen, however, that the relaxation times are in the same range as those of β-casein in solution, suggesting similar motional freedom in the phosphoserine groups of the two preparations.

The T_1 values of Table 1 show longer relaxation times at lower field for both the dissolved and adsorbed protein. This indicates that the chemical shift anisotropy relaxation mechanism plays a dominant role. In our analysis of the ^{31}P NMR results, we assume that only the chemical shift anisotropy relaxation mechanism is active.

The generalized order parameter S and a rotational correlation time τ_e were determined with a least-squares fit to the T_1 values at two different magnetic fields using Eqs. 2 and 6 and the chemical shift anisotropy of cyclic thioxophosphonate ($\Delta\sigma$ = 200 ppm) (Gorenstein, 1984) in Eq. 2 as an approximation for an orthophosphate group.

TABLE 2 Order parameter *S* and the internal rotational correlation time τ_e determined from the relaxation times of Table 1

β-Casein in solution				
	Peak 1	Peak 2	Peak 3	Peak 4
S	0.86	0.87	0.88	0.89
τ_e (ns)	1.49	1.49	1.40	1.12

Adsorbed β-casein						
	Position a	Position b	Position c	Position d	Position e	Position f
S	0.87	0.85	0.88	0.89	0.87	0.83
τ_e (ns)	1.32	2.32	1.77	1.40	1.98	2.70

Longitudinal relaxation times of β-casein in solution were fitted using the same model-free spectral density as that of a β-casein stabilized oil/water emulsion. The following assumptions were made; they are similar to those used in the analysis of ^{13}C relaxation data: 1) the highly charged N-terminal region of β-casein is located near the surface of the oil particle, 2) tails move as if on the surface of a cone, 3) the "micelles" are so large that their rotational correlation time is much larger than that of the internal rotational correlation time of the N-terminal tail of β-casein. The results of the fit are shown in Table 2.

The order parameters show a more restricted motion for peak 4, phosphoserine-35, which is in agreement with the assumption that it is located closest to the micellar surface. The behavior of the individual phosphoserine groups in the tail is difficult to disentangle, because there is a likely overlap of signals from these groups, which means the calculated order parameters and rotational correlation times are not unique.

For further research, we suggest an investigation of the response of β-casein tails to changes in pH, temperature, calcium ion activity, or the presence of other surface-active molecules.

CONCLUSION

The NMR results give the following picture of a β-casein/oil/water emulsion. We see mobile regions with little secondary structure that are fairly stiff ($S \approx 0.9$) and in which the motions are rather slow ($\tau_e \approx 1$ ns). The time scale is approximately correct for motions that involve entire protein tails; for comparison, the correlation time for rotation of a particle 2 nm in diameter in water at room temperature is a few nanoseconds. Undoubtedly, there are also faster internal motions that occur throughout the protein, but the T_1 data are not sensitive to correlation times faster than about 10^{-11} s.

Our results appear to be consistent with a "hairy sphere" model, in which N-terminal tails extend from β-casein molecules on the surface. Their relative stiffness may be caused by a combination of crowding and mutual electrostatic repulsion. Assuming that protein tails are free to move within a cone that has its apex at their point of attachment to the emulsion particle, an order parameter of 0.9 is consistent with tail motion through an angle of up to 15° from a vector normal to the particle surface.

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