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Oxygen-17 and Proton Nuclear Magnetic Relaxation Measurements of Soy Protein Hydration and Protein–Protein Interactions in Solution

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The amount and mobility of "bound" water in neutral aqueous suspensions of a commercial soy protein isolate were quantitated by means of transverse and longitudinal ^{17}O NMR relaxation measurements at 54.2 MHz and 21 °C. Data analysis yielded 33.2 g of "bound" water/100 g of protein and correlation times of 14.2 ns and 32–34 ps for the motions of "bound" water. The hydration of the same protein isolate was investigated by ^{1}H NMR transverse relaxation measurements at 10 MHz and 22 °C. Up to 10% (w/w) protein aqueous dispersions were measured at pH 4.5, 7, 9.1, and 11 in the absence and presence of NaCl. In all cases, a nonlinear protein concentration dependence was observed for relaxation rates. Protein–protein interactions were quantitated by fitting the ^{1}H NMR data by a virial expansion. Transverse ^{1}H NMR relaxation rates showed a linear dependence on protein activity. Data interpretation was based on the effects of the NMR measurements of the ionization of protein groups, the state of protein aggregation, and the binding of salt by the protein.

1. INTRODUCTION

Protein–water interactions have been extensively studied because of their fundamental role in biological systems (Kuntz and Kauzmann, 1974). Recently, soybean protein has become increasingly important as a food ingredient, with its performance in food systems being intimately related to its hydration properties (Kinsella et al., 1985; Chou and Morr, 1979). Water vapor sorption isotherms of various soy protein preparations have been reported (Hagenmaier, 1972; Puri and Bala, 1975; Hermansson, 1977; Hansen, 1978; Chou and Morr, 1979). The amount of water that remains unfrozen in the presence of soy protein has been estimated by using differential scanning calorimetry (DSC) (Muffett and Snyder, 1980) or nuclear magnetic resonance (NMR) (Hansen, 1978; Derbyshire, 1982). Other pertinent work involved measurements of the spontaneous uptake of liquid water by protein powders (Hermansson, 1972; López de Ogara et al.,

1987) or the amount of water retained by the insoluble protein after centrifugation of a protein dispersion (Fleming et al., 1974; Hutton and Campbell, 1981) as well as other empirical approaches to the determination of soy protein hydration (Elgedaily et al., 1982).

Nuclear magnetic resonance relaxation is a noninvasive technique that can provide information about the amount and the mobility of "bound" water (i.e., the fraction of water that is significantly perturbed by the protein) and the extent of various intermolecular interactions (Derbyshire, 1982; Bryant and Halle, 1982; Pessen and Kumosinski, 1985).

The purpose of the present study is to calculate the hydration parameters of soybean protein from ^{17}O NMR relaxation measurements; we also aim to quantitate protein–protein interactions using ^{1}H NMR at various values of pH and ionic strength.

2. THEORY

Among the three different nuclei that can be used in NMR studies of the molecular properties of water (i.e., ^{1}H, ^{2}H, and ^{17}O), oxygen-17 is the one whose NMR relaxation is free from the complications of cross-relaxation and chemical exchange, thus providing the most direct

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means for probing protein hydration (Halle et al., 1981; Piculell and Halle, 1986; Kakalis and Baianu, 1988).

The oxygen-17 nucleus has a spin quantum number $I = {}^5/_2$ and possesses an electric quadrupole moment; the nuclear quadrupole moment (eQ) is coupled to the electric field gradient at the nucleus (eq) that originates from the asymmetric spatial arrangement of electrons around the nucleus. This interaction depends on the orientation of the molecule with respect to the external magnetic field. The quadrupole coupling will fluctuate in time due to the Brownian motion of the molecule, thus providing a highly effective relaxation mechanism and causing a marked broadening of oxygen-17 NMR absorption peaks. The ^{17}O NMR relaxation rates of water bound to macromolecules increase due to the asymmetric electrostatic interactions at the binding site and the longer effective rotational correlation time(s) of bound water. On the other hand, reorientation of the water molecules at the binding site and the fast exchange between "bound" and "free" water both cause a decrease of the ^{17}O NMR relaxation rates.

Analysis of NMR relaxation data is model dependent. The two-state ("bound" and "free") model with fast exchange of water between these two states is a generally accepted point of departure in NMR studies of protein hydration. If there are no additional contributions to relaxation, the observed relaxation rate (R_{obs}) is the weighted average of the bound and free water relaxation rates, R_B and R_F, respectively

$$R_{obs} = P_B R_B + P_F R_F \qquad (1)$$

where P_B and P_F represent the corresponding fractions of bound and free water in the sample.

If C_p is the protein concentration in g of protein/g of water and n_H is the protein hydration in g of bound water/g of protein, then

$$P_B = n_H C_p \qquad (2)$$

and, since $P_F = 1 - P_B$, eq 1 may be rewritten as

$$R_{obs} = (R_B - R_F)P_B + R_F = n_H(R_B - R_F)C_p + R_F \qquad (3)$$

with R_{obs}, R_B, and R_F being either the longitudinal (R_1) or transverse (R_2) relaxation rates. Given a relaxation mechanism, analytical expressions for R_1 and R_2 can be derived from the NMR relaxation theory (Abragam, 1961).

In the two-state (bound and free water), *isotropic* hydration model, the relaxation rates for oxygen-17 (quadrupole relaxation) are

$$R_{1B} = \frac{12\pi^2}{125}K^2\left(1 + \frac{\eta^2}{3}\right)\tau_c\left(\frac{0.2}{1 + \omega^2\tau_c^2} + \frac{0.8}{1 + 4\omega^2\tau_c^2}\right) \qquad (4a)$$

$$R_{2B} = \frac{12\pi^2}{125}K^2\left(1 + \frac{\eta^2}{3}\right)\tau_c\left(0.3 + \frac{0.5}{1 + \omega^2\tau_c^2} + \frac{0.2}{1 + 4\omega^2\tau_c^2}\right) \qquad (4b)$$

where the quadrupole coupling constant for liquid water or ice I_h is $K = e^2qQ/h = 6.67$ MHz and the asymmetry parameter η for the electric field gradient at the nucleus is 0.93 (Halle et al., 1981, and references therein); τ_c is the correlation time for the isotropic motion of bound water and $\omega = 2\pi\nu$ is the angular precession frequency, ν being the NMR frequency used.

In a fast-exchange, dual-motion, two-state, *anisotropic* model of hydration, bound water molecules are hypothesized to reorient fast around their bonding axes with the protein binding sites while they are tumbling slowly

together with the protein and also to exchange fast (on the NMR time scale) with the free water population.

Then, the following expressions can be used for the ^{17}O NMR relaxation rates (Piculell and Halle, 1986):

$$R_{1B} = \frac{12\pi^2}{125}K^2\left(1 + \frac{\eta^2}{3} - A^2\right)\tau_{Bf} + \frac{12\pi^2}{125}A^2K^2\tau_c\left(\frac{0.2}{1 + \omega^2\tau_c^2} + \frac{0.8}{1 + 4\omega^2\tau_c^2}\right) \qquad (5a)$$

$$R_{2B} = \frac{12\pi^2}{125}K^2\left(1 + \frac{\eta^2}{3} - A^2\right)\tau_{Bf} + \frac{12\pi^2}{125}A^2K^2\tau_c\left(0.3 + \frac{0.5}{1 + \omega^2\tau_c^2} + \frac{0.2}{1 + 4\omega^2\tau_c^2}\right) \qquad (5b)$$

where $\tau_c = \tau_{Bs}$ and τ_{Bf} are respectively the correlation times associated with the slow (nanosecond) and fast (picosecond) motions of bound water. A value of 0.12 is a reasonable estimate for the order parameter A that describes the anisotropic orientation of water molecules in the vicinity of the protein surface (Kakalis and Baianu, 1988, and references therein).

The hydration parameters can be obtained from the slopes of R_1 and R_2 vs C_p plots (Pessen and Kumosinski, 1985). In the case of an anisotropic hydration model, τ_{Bf} must also be calculated: additional ^{17}O NMR relaxation data at a second, sufficiently different NMR frequency (that is, not in the extreme narrowing region) are required for this purpose (Kakalis and Baianu, 1988). Alternatively, a value for τ_{Bf} can be estimated from results on protein model systems (e.g., lysozyme). The ratio of the two intercepts is a function of τ_c alone (eqs 3 and 4), which can thus be calculated. With $\tau_c = \tau_{Bs}$ and τ_{Bf} known, the hydration n_H can be obtained from the slope of either an R_1 or R_2 vs C_p plot.

In the analysis of ^1H NMR relaxation data, it is necessary to consider the effects of cross-relaxation between water molecules and protein methyl groups (Kalk and Berendsen, 1976; Edzes and Samulski, 1978; Koenig et al., 1978) as well as proton chemical exchange between protein ionizable groups and water molecules (Piculell and Halle, 1986; Kakalis and Baianu, 1988). In the presence of cross-relaxation only, R_{obs} in eq 3 may be approximated as

$$R_{obs} = [n_H(R_B - R_F) + R_x]C_p + R_F \qquad (6)$$

where R_x represents the contribution of cross-relaxation (Pessen et al., 1985). A quantitative description of the effect of chemical exchange on the concentration dependence of NMR relaxation rates has not been proposed yet. In the case of proton R_2 measurements, the contribution of R_x to R_{2obs} may be small and can be neglected (see section 4.3).

Equation 3 predicts a linear relationship between R_{2obs} and the macromolecular concentration C_p (Pessen and Kumosinski, 1985)

$$R_{2obs} = R_{2F} + \Delta R n_H C_p \qquad (7)$$

where n_H is the protein hydration and $\Delta R = R_{2B} - R_{2F}$. If n_H is independent of the protein concentration when water is not limiting, deviations from linearity at higher protein concentrations can be attributed to the increasing importance of protein–protein interactions that affect ΔR (Derbyshire, 1982; Pessen and Kumosinski, 1985). These nonidealities can be accounted for if the protein concentration C_p in eq 7 is replaced by the protein activ-

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ity α_p, where

$$\alpha_p = C_p \exp(2B_0 C_p + 2B_{0.5} C_p^{0.5} + 0.667 B_{1.5} C_p^{1.5} + 1.5 B_2 C_p^2 + ...) \quad (8)$$

The virial coefficients B are a measure of the various molecular interactions (Tanford, 1963; Pessen and Kumosinski, 1985). In agreement with general electrolyte solution theory, the sign of the virial coefficients indicates the type of the respective interaction: B is positive for repulsive effects and negative for attractive ones. The B_0 (mL/g) virial coefficient reflects interactions related to the net protein charge Z, the protein excluded volume, and a preferential interaction term (Kumosinski et al., 1987)

$$2B_0 = \frac{Z^2}{4m_s M_p} + \bar{V}_p - \left(\frac{\delta g_s}{\delta g_p}\right)^2 \frac{1}{m_s} \quad (9)$$

where m_s is the salt molarity, M_p is the protein molecular weight, $\bar{V}_p$ (mL/g) is the protein specific volume, and $\delta g_s / \delta g_p$ is the preferential binding term (g of preferentially bound salt/g of protein). The B_2 coefficient represents interactions due to fluctuating multipoles. For isoionic protein solutions (i.e., in the absence of any added ions), the $B_{0.5}$ and $B_{1.5}$ terms must be included in eq 8 in order to account for attractive effects due to charge fluctuations (Kirkwood and Shumaker, 1952; Timasheff et al., 1957; Pessen and Kumosinski, 1985). This correction for nonidealities has been experimentally verified by ^1H NMR of β-lactoglobulin solutions (Kumosinski and Pessen, 1982), ^2H NMR of bovine casein micelles and submicelles in solution (Kumosinski et al., 1987), and ^1H NMR of corn zein samples (Myers-Betts and Baianu, 1989).

3. MATERIALS AND METHODS

3.1. Materials. The soybean protein isolate used (Soybean Protein Grade II, no. 38388) was obtained from U.S. Biochemical Co. (Cleveland, OH) and had a reported protein content of 91.5% ($N \times 6.25$, dry basis). All reagents used were analytical grade or "ACS certified".

Samples were prepared by adding a measured volume of solvent to a preweighed amount of powder. The samples were well mixed by stirring over a period of 3 h in order to reach maximum solubility (Shen, 1976a) and hydration (Hermansson, 1972; Hansen, 1978). Occasionally, samples were left overnight in the cold room; this did not affect NMR relaxation rates, provided enough time was allowed for the samples to reach room temperature prior to the measurements. In all cases, solubility was below 100%; the more concentrated samples were very viscous slurries. Protein concentration (% w/w) was calculated from (g of powder)/(g of powder + g of solvent) $\times$ 100. The density of H_2O solvents was taken as 1.0 g/mL, and that of D_2O as 1.1 g/mL.

The initial sample pH was 7.0; it was adjusted (± 0.01 pH unit) by slowly adding 2 or 6 N HCl or NaOH with a microsyringe while continuously stirring. The temperature of every sample was measured (± 0.1 °C) with a digital thermometer before and after each NMR measurement.

3.2. Measurements. Proton NMR transverse relaxation rate measurements were made on resonance with a PC-10 NMR Process Analyzer (IBM Instruments, Danbury, CT) at 10 MHz using the Carr–Purcell–Meiboom–Gill (CPMG) pulse sequence (Farrar and Becker, 1971). The decay of the transverse magnetization/spin–echo maximum amplitude was monitored with a dual-beam storage oscilloscope (Tektronix Model 5113).

In all experiments two or three independent series of measurements (all in triplicate) were made at room temperature. The error in T_2 values was generally <5%. Transverse relaxation rates were corrected for magnetic field inhomogeneity, assuming a T_2 value of 3 s for liquid water at 22 °C; 0.1 or 0.5 M NaCl does not significantly affect the relaxation rates of water.

Natural-abundance oxygen-17 NMR measurements were car-

ried out at 54.19 MHz with a GX-400 multinuclear spectrometer (JEOL Inc., Peabody, MA) equipped with a high-resolution, narrow-bore (54 mm) 9.4-T superconducting magnet (Oxford Instruments Inc., U.K.), a DEC PDP 11/23 dedicated computer with PLEXUS software, and a 10-mm tunable (^{31}P to ^{15}N) probe. About 4 mL of well-dispersed and thoroughly mixed protein in D_2O (not pH adjusted; pD = 7.4) was analyzed in 10-mm high-resolution NMR tubes (Wilmad, Buena, NJ) at 21 $\pm$ 1 °C. The ^{17}O NMR 90° pulse width for D_2O was 27.5 μs, and 100 scans were sufficient for a signal-to-noise ratio of 150:1 (with 5-Hz exponential line broadening applied). The spectral width was 25 kHz, the acquisition time was 0.33 s, and a 16K point time-domain array was used for storing the data with adequate resolution.

Transverse ^{17}O NMR relaxation rates of duplicate samples were obtained from line-width measurements at half-height, $\Delta\nu_{1/2}$, of the absorption signals according to $R_2 = \pi\Delta\nu_{1/2}$. The exchange broadening that contributes significantly to the ^{17}O $\Delta\nu_{1/2}$ of H_2O solutions is small for D_2O (Halle et al., 1981). The combined correction for field inhomogeneity and for exchange broadening was determined by comparing the $\Delta\nu_{1/2} = (\pi T_2)^{-1}$ measured with a single pulse for D_2O with the value calculated from T_1 measurements (T_1 = 5 ms), considering that $T_1 = T_2$ for pure D_2O.

Longitudinal ^{17}O NMR relaxation rates were measured in duplicate with the inversion–recovery method (Farrar and Becker, 1971) using the 180°–τ–90° pulse sequence; τ was varied from 100 μs to 30 ms (8 data points) and the preacquisition delay was set at 25 ms. The 90° pulse width was determined separately for each protein concentration and was found to vary from 27.5 (for D_2O without protein) to 29 μs (5% protein in D_2O). T_1 values were obtained from a nonlinear, three-parameter, least-squares fit of an exponential curve to the experimental points (Figure 1). The error in the T_1 measurements is estimated to be <5%.

3.3. Data Analysis. The τ_c and n_H were calculated from the ^{17}O NMR relaxation data as detailed under Theory (section 2). The equation for τ_c was solved numerically (solution range 0.1–100 ns) with a Fortran program based on Newton's method.

Data points of the observed proton relaxation rate vs protein concentration were fitted by eqs 7 and 8 by using a nonlinear regression program in Fortran. The program is based on a Gauss–Newton algorithm and was run on a Modcomp Classic minicomputer.

For comparisons of the goodness of fit, the F test was used with the following F value (Motulsky and Ransnas, 1987)

$$F = \frac{(SSR_1 - SSR_2)/(df_1 - df_2)}{SSR_2/df_2} \quad (10)$$

where SSR refers to the sum of squares of the residuals and df to the number of degrees of freedom (number of data points minus number of fitting parameters). Subscript 1 refers to the simpler model, the one with the fewer parameters. F distribution tables were consulted for ($df_1 - df_2$) and df_2 degrees of freedom (Beyer, 1984).

4. RESULTS AND DISCUSSION

4.1. Evidence for Fast Exchange. In the case of aqueous soybean protein dispersions the decay of the proton spin–echo maximum amplitudes in the CPMG pulse sequence follows a single exponential (Figure 2) and the line shape of the ^{17}O NMR absorption peak is approximately Lorentzian (Figure 3), in agreement with a fast-exchange model for protein hydration (Derbyshire, 1982).

4.2. Calculation of the Hydration Parameters. Oxygen-17 NMR relaxation rates were found to increase linearly with soy protein concentration up to 4% (w/w) (Figure 4); deviations at 5% (w/w) (and higher) protein concentration are attributed to protein–protein interactions (Pessen and Kumosinski, 1985).

Use of the fast-exchange, two-state, dual-motion, anisotropic model (eqs 5) requires first an estimate of τ_{Bf}, the
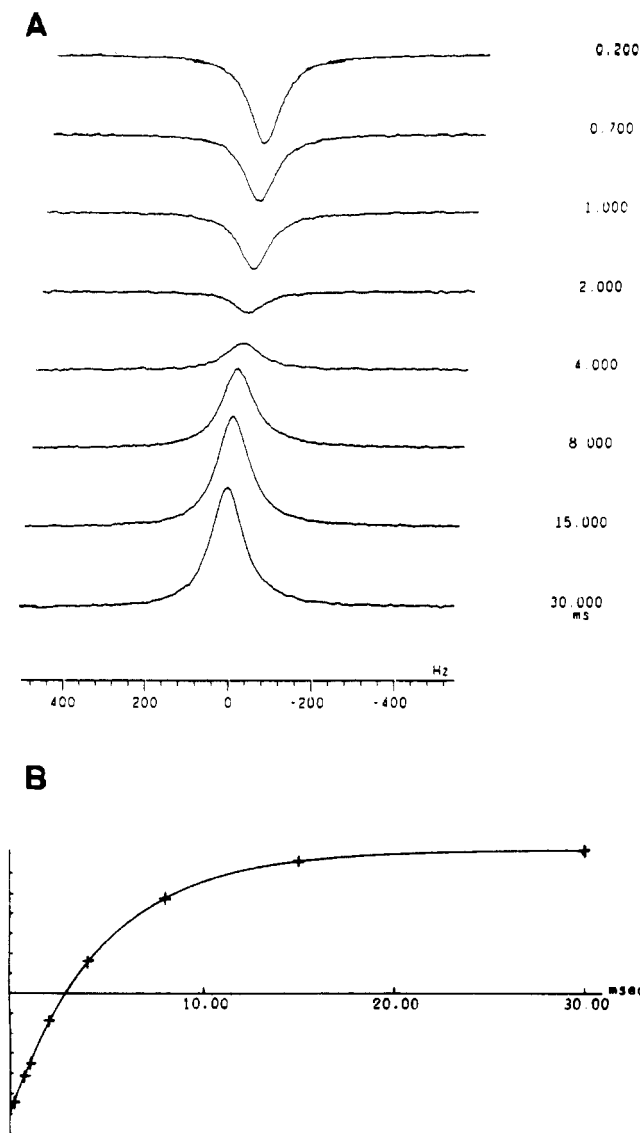
A

B

Figure 1. Oxygen-17 NMR peak intensities of D_2O as a function of interpulse delay τ from a T_1 determination by inversion-recovery. (A) A set of spectra for a 2% (w/w) dispersion of soy protein isolate in D_2O at 21 °C and neutral pH (pD = 7.4). (B) A three-parameter exponential fit of the experimentally determined peak heights H with the expression $H = A + B \exp(-\tau/T_1)$ that is obtained by means of the PLEXUS software.
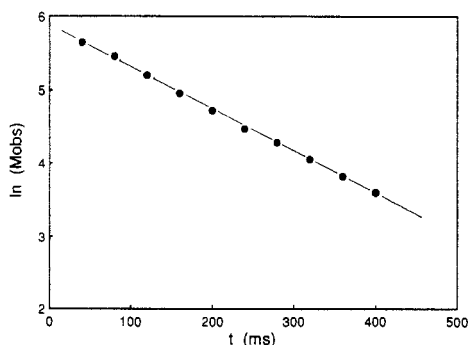

Figure 2. Logarithm of the observed ^1H NMR transverse magnetization ($M_{y,obs}$, spin–echo maxima in the CPMG pulse sequence) vs time for an 8% (w/w) soy protein isolate dispersion in H_2O at pH 7 and 22 °C. The good linear fit ($r = -0.9996$) implies that the decay of the transverse magnetization is a single exponential.

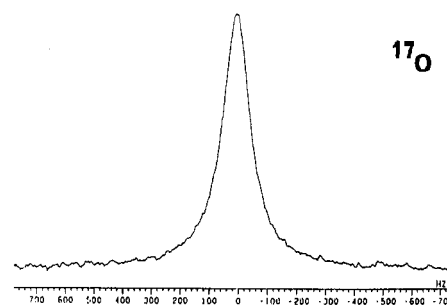fast correlation time of bound water. In the case of lysozyme solutions, $\tau_{Bf} = 29$ ps in the absence of added

Figure 3. Oxygen-17 NMR spectrum (54.19 MHz) of a 5% (w/w) soy protein isolate dispersion in D_2O at neutral pH and 21 °C. The ratio of the line widths at 50% and 0.55% of the peak heights is 1.0:14.0 (compared to 1.0:13.5 for a true Lorentzian), in agreement with the expected nearly exponential decay (Halle and Wennerström, 1981). In the case of lysozyme hydration studies by ^{17}O NMR, the correction due to the deviation of the decay from a single exponential was estimated to be small, comparable to the error of the measurements (Kakalis and Baianu, 1988).
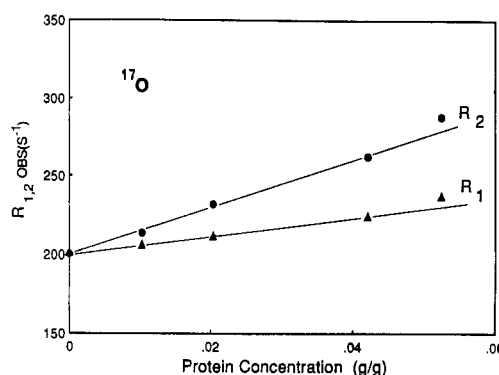

Figure 4. Dependence of the oxygen-17 longitudinal R_1 (▲) and transverse R_2 (●) NMR relaxation rate (s^{-1}) on protein concentration C_p (g of protein/g of water) for soybean protein dispersions in D_2O at 21 ± 1 °C and neutral pH. The points represent experimental values. The straight lines are $R_1 = 588.54C_p + 199.99$ ($r = 0.9999$) and $R_2 = 1501.39C_p + 199.62$ ($r = 0.9986$). The good linear fits in the protein concentration range from 0.00 to 0.04 g/g of water imply that in this range the protein activity does not differ greatly from the protein concentration.

Table I. Hydration Estimates of Commercial Soybean Protein Isolates

sample	hydration, g of water/g of protein
soy protein, grade II (U.S. Biochemical Co.)[a]	0.33[c]
ACP-950 (Anderson Clayton)[b]	0.36[d]
EdiPro N (Ralston Purina)[a]	0.32[e]

[a] Neutral pH. [b] Sample pH not reported. [c] From ^{17}O NMR at 21 °C, according to a fast-exchange, two-state, *anisotropic* model (present study). [d] From ^1H NMR, unfrozen water at -50 °C (Hansen, 1978). [e] From differential scanning calorimetry, unfrozen water at -30 °C (Muffett and Snyder, 1980).

salt (Kakalis and Baianu, 1988). This value refers to 27 °C and for that reason represents a lower limit for the present study. We assumed a τ_{Bf} value in the range of 32–34 ps, also consistent with the calculated correlation times of bound D_2O for powdered lysozyme at 20 °C (Lioutas et al., 1986). The calculated slow correlation time τ_{Bs} or τ_c then is 14.2 ns, and the hydration amounts to 33.2 g of bound water/100 g of protein.

The latter hydration value (33.2%) is in good agreement with reported estimates of the hydration of commercial soy protein isolates (Table I); it is also consistent with the hydration of other soy protein prepara-

tions (Hansen, 1978) and soy protein fractions such as 7S globulins (28% hydration; Hansen, 1978) and glycinin (36% hydration; Badley et al., 1975).

The evaluation of the soy protein hydration parameters can also be accomplished by recognizing that due to the large size of the soy proteins and the employed high NMR frequency, the ^{17}O NMR measurements are beyond the low-frequency dispersion curve that reports on the protein molecules' rotation, at a point where R_{1B} and R_{2B} are frequency independent. R_{1B} contains information about the fast-motion component only, whereas R_{2B}, through the zero-frequency spectral density term, carries information about the slow component as well (Halle et al., 1981). Then eqs 5 become

$$R_{1B} = \frac{12\pi^2}{125}K^2\left(1 + \frac{\eta^2}{3} - A^2\right)\tau_{Bf} \qquad (11a)$$

$$R_{2B} = \frac{12\pi^2}{125}K^2\left(1 + \frac{\eta^2}{3} - A^2\right)\tau_{Bf} + \frac{3.6\pi^2}{125}A^2K^2\tau_c \qquad (11b)$$

R_{1B} and R_{2B} can be obtained from the slopes of the concentration dependences of R_{1obs} and R_{2obs} (Figure 4), as noted above, assuming that soy proteins bind 0.32 g of water/g of protein (Kuntz and Kauzmann, 1974; Table I). From $R_{1B} = 1639$ s^{-1} and eq 11a we obtain $\tau_{Bf} = 31$ ps; then from $R_{2B} - R_{1B} = 3053$ s^{-1} and eqs 11 we get $\tau_c = \tau_{Bs} = 16.8$ ns.

The exact nature of the motion(s) that correspond to the calculated correlation times is not clear. The slow motion (τ_{Bs}, nanosecond time scale) may be associated with the protein tumbling in solution, the translational diffusion of water molecules along the protein surface, and the exchange of water molecules between the bound and free states (Bryant and Halle, 1982; Piculell and Halle, 1986). The fast motion (τ_{Bf}, picosecond time scale) may be related to the reorientation of water molecules around hydrogen bonds (Bryant and Halle, 1982) and/or the libration of hydrated amino acid side chains (Bryant and Halle, 1982; Pessen and Kumosinski, 1985). The calculated correlation times indicate that the mobility of bound D_2O is not much lower than that of free D_2O (4.7 ps at 20 °C): bound water is essentially "fluid".

The hydration parameters of soy protein according to an isotropic model (eqs 4) are 1.52 g of bound water/100 g of protein and a bound water correlation time of 3.3 ns. Whether a protein surface induces a preferential orientation to bound water molecules or not is a subject of debate. Here we simply note that the analysis of NMR data according to an anisotropic model yields hydration values for β-lactoglobulin (Kumosinski and Pessen, 1982), bovine casein (Kumosinski et al., 1987), lysozyme (Kakalis and Baianu, 1988), and soy protein (present study) that are in agreement with their hydration measurement by other methods and in line with general estimates of protein hydration (Kuntz and Kauzmann, 1974).

4.3. Extent of Cross-Relaxation. Cross-relaxation between H_2O and protein protons dominates ^1H longitudinal NMR measurements of protein hydration; its contribution to T_2 measurements is less important, particularly at lower NMR frequencies (Kalk and Berendsen, 1976; Edzes and Samulski, 1978; Sykes et al., 1978; Myers-Betts and Baianu, unpublished results). At 10 MHz, the ^1H transverse NMR relaxation rate of an 8% (w/w) soy protein dispersion in 10% H_2O–90% D_2O was found to be only 8% lower than the R_{2obs} of the same sample in 100% H_2O. This value (8%) is a measure of the relative contribution of intermolecular processes to the observed transverse ^1H NMR relaxation and essentially repre-
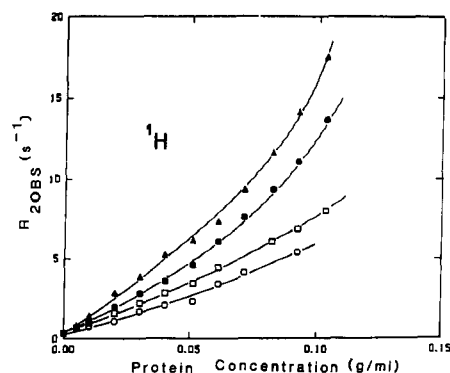

Figure 5. Concentration dependence of 10-MHz ^1H NMR transverse relaxation rates for aqueous soy protein dispersions with no added salt at 22 ± 2 °C and pH 4.5 (O), 7.0 (□), 9.1 (●), and 11.0 (▲). T_2 values were obtained with the CPMG pulse sequence.

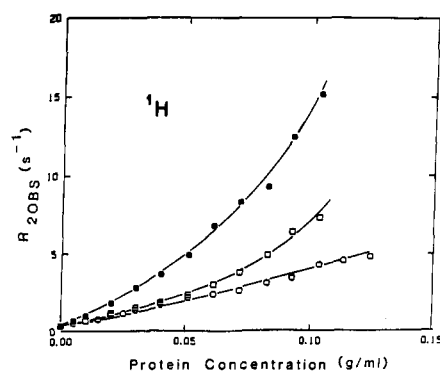**Figure 6.** Concentration dependence of 10-MHz ^1H NMR transverse relaxation rates for soy protein dispersions in 0.1 M NaCl at 22 ± 2 °C and pH 4.4 (O), 7.0 (□), and 9.0 (●). T_2 values were obtained with the CPMG pulse sequence.

Table II. Calculated Hydration Products $\Delta Rn_H{}^a$ for Aqueous Soy Protein Dispersions at 22 ± 2 °C from Nonlinear Regression of ^1H NMR Relaxation Rates on Protein Concentration[b]

	pH			
	4.5 ± 0.2	7.0 ± 0.1	9.1 ± 0.3	11.0 ± 0.2
no added salt	36.3 ± 6.0	54.1 ± 2.3	57.8 ± 2.7	140.3 ± 17.2
0.1 M NaCl	31.4 ± 2.5	23.3 ± 3.2	58.3 ± 6.2	
0.5 M NaCl		28.6 ± 2.5		

[a] In mL g^{-1} s^{-1}. The protein concentration was in g of protein/mL of solvent. There are no numerical differences with eqs 3 and 7 (C_p in g of protein/g of water) since the H_2O density is 1 g/mL. [b] According to eqs 7 and 8.

sents an upper limit for the extent of cross-relaxation.

4.4. Protein Concentration Dependence of the ^1H NMR R_2 Measurements. Proton NMR transverse relaxation rates increased linearly with soy protein concentration up to approximately 5% (w/w) soy protein (Figures 5 and 6). The marked deviations from linearity at higher protein concentrations are due to protein–protein interactions (Pessen and Kumosinski, 1985). The results obtained from the nonlinear regression analysis of the data according to eqs 7 and 8 are presented in Tables II–IV. In the investigated protein concentration range (0–10% (w/w)), the use of virial coefficients other than B_0 was not necessary in order to make a statistically significant contribution to the goodness of fit (F test, 10% level of significance). The only exception is the data at pH 11, where additional use of B_2 resulted in a significant improvement of the fit (F test, 1% level of significance).

The virial coefficients of a given protein sample can be determined by means of various types of measure-

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Table III. Calculated Virial Coefficients B_0 (mL/g)[a] from Nonlinear Regression Analysis of ^{1}H NMR Relaxation Rates at 10 MHz on Soy Protein Concentration at 22 ± 2 °C According to Eqs 7 and 8

	pH			
	4.5 ± 0.2	7.0 ± 0.1	9.1 ± 0.3	11.0 ± 0.2
no added salt	2.3 ± 0.9	1.5 ± 0.2[b]	3.8 ± 0.2	−4.1 ± 1.4[c]
0.1 M NaCl	0.6 ± 0.3	5.2 ± 0.7	4.3 ± 0.5	
0.5 M NaCl		4.4 ± 0.4		

[a] See footnote a of Table II. [b] No pH adjustment; contains the least amount of salt ions. [c] At pH 11 a B_2 value of 64.6 ± 11.4 (mL/g)^2 is also required for improved fitting.

Table IV. Calculated[a] RMS Values for the Fitting of the Soy Protein NMR Relaxation Data by a Virial Expansion of Eq 7 Using Eq 8

	pH			
	4.5	7.0	9.1	11.0
no added salt	0.192	0.081	0.135	0.164
0.1 M NaCl	0.111	0.178	0.330	
0.5 M NaCl		0.136		

[a] RMS = $\{[\sum(R_{2obs} - R_{2calc})^2]/(\text{no. of data points} - \text{no. of fitting parameters})\}^{1/2}$; R_{2calc} values obtained from eqs 7 and 8, using ΔRn_H and B values from Tables II and III.
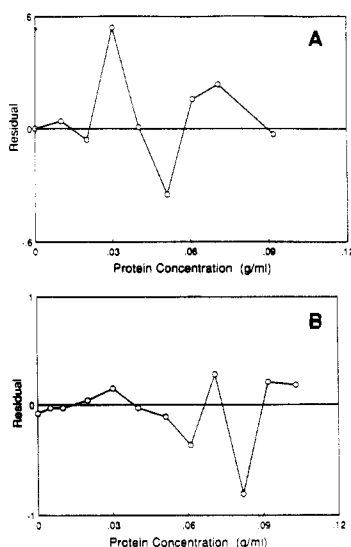

Figure 7. Representative residual plots for soy protein isolate in (A) H_2O, pH 4.5, and in (B) 0.1 M NaCl, pH 9.1: ($R_{2obs} - R_{2calc}$) vs protein concentration C_p. The RMS values are 0.192 (A) and 0.330 (B). The random distribution of positive and negative residuals indicates the absence of systematic error and suggests that the equation used is appropriate for the data.

ments (e.g., light scattering, osmotic pressure, and so on) that yield similar results (Tanford, 1963). Thus, the β-lactoglobulin virial coefficients from ^{1}H NMR relaxation are in agreement with those obtained from light scattering (Kumosinski and Pessen, 1982). However, the lysozyme virial coefficients from ^{1}H, ^{2}H, and ^{17}O transverse NMR relaxation measurements are in disagreement (Myers-Betts and Baianu, unpublished results), presumably because different nuclei monitor different processes. In the event of such a possibility for soy proteins, the virial coefficients obtained from ^{1}H NMR relaxation may be viewed with some caution. However, this does not diminish the importance of our measurements since the focus of the present study is the variation of the virial coefficients with pH and ionic strength rather than their absolute values.

4.5. Effect of pH. Either ΔR or n_H may be responsible for the observed effect of pH on the soy protein
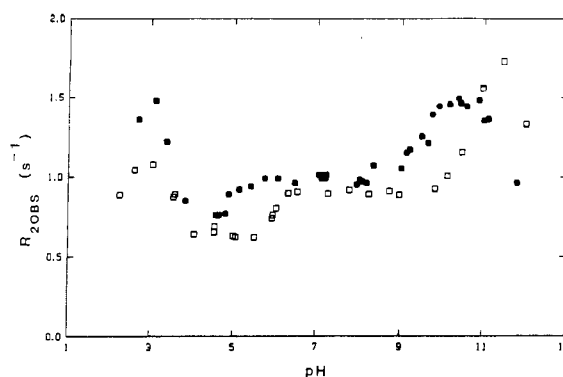

Figure 8. pH dependence of 10-MHz ^{1}H NMR transverse relaxation rates for 1% (w/w) soy protein dispersions in H_2O (●) or with 0.1 M NaCl added (□) at 22 ± 2 °C. T_2 values were obtained with the CPMG pulse sequence.

hydration product ΔRn_H (Table II). The soy protein hydration n_H is minimal around the isoelectric point (pH 4.5) where the net protein charge is zero (Hermansson, 1972; Hagenmaier, 1972; Chou and Morr, 1979; Hutton and Campbell, 1981). This is expected since protein charged groups are the primary sites of protein–water interactions (Kuntz and Kauzmann, 1974). Interestingly, the pH dependence of soy hydration is similar to that of its solubility (Shen, 1976b). The increase of ΔRn_H with pH above the isoelectric point (Table II) may then be attributed to the increase of n_H with pH as a result of protein side-chain ionization (notably carboxyl groups). The accompanying increase in protein solubility and the concomitant replacement of protein–protein interactions by protein–water interactions may also be responsible for the augmented hydration.

Proteins of the soy isolate undergo ionic strength and pH-induced association–dissociation reactions (Kinsella et al., 1985) which are manifested in the viscosity of soy protein dispersions (Lee and Rha, 1979; Shen, 1981). An increase in the sample viscosity will result in slower tumbling of the protein molecules in solution and longer correlation times τ_c for the bound water molecules, thus leading to an increase of R_{2B} (Derbyshire, 1982) and consequently R_{2obs}. For the same reason, protein dissociation at extreme pH is expected to cause a decrease in R_{2B} due to a reduction in the sample viscosity (Lee and Rha, 1979).

The dependence of R_{2obs} on pH for soy proteins in H_2O (Figure 8) may thus be rationalized as the combined effect of pH on the protein ionized groups, the protein solubility, and the protein aggregation state and denaturation, which, in turn, affect the sample viscosity. Below the isoelectric point (pH 4.5), increased protein solubility and higher net protein charge may both enhance the soy protein hydration, n_H, apparently despite the protonation of carboxyl groups; the increasing viscosity below pH 4 might be responsible for longer bound water correlation times τ_c and higher R_{2B} values. All three factors favor an increase in R_{2obs} with decreasing pH in this pH range. Generally, the same reasoning might apply to the pH ranges 4.5–6 and 9–10. The leveling off of R_{2obs} in the pH range 6–9 is most likely the result of opposing trends whose effects on R_{2obs} balance each other: the viscosity minimum at pH 6 (Lee and Rha, 1979), the deprotonation of His residues above pH 7, and the increasing protein solubility with pH. The maximum in R_{2obs} at pH 10.5 (Figure 8) is probably related to a viscosity variation at this pH range due to the onset of soy protein dis-

sociation and to changes in the ionization of Lys and Tyr residues that affect n_H.

4.6. Effect of Ionic Strength. Salt appears to decrease the hydration of soy protein isolates at neutral pH (Elgedaily et al., 1982; López de Ogara et al., 1987). The effect of NaCl on NMR measurements of soy protein hydration may be related to the effect of salt on protein solubility and charge (subsection 4.5). The solubility of soy protein is affected by NaCl concentration in a pH-dependent manner (Shen, 1976b, 1981; van Megen, 1974). Salt generally decreases the viscosity of soy protein dispersions. Additionally, (hydrated) ions bind to protein charged groups, thus increasing the protein charge and the amount of bound water (Kuntz and Kauzmann, 1974; Kumosinski, 1988).

The pH variation of $\Delta R n_H$ in the presence of 0.1 M NaCl shows a minimum at pH 7 (Table II). This may not be related to the solubility of soy protein: its pH profile in 0.1 M NaCl is not fundamentally different from that in H_2O, and the soy protein is still least soluble around pH 4.5 (van Megen, 1974; Shen, 1976b). The number of protein charged groups and consequently ion binding to them are not expected to be at a minimum around pH 7. A possible explanation is the viscosity minimum around pH 6 (Lee and Rha, 1979) and the viscosity-reducing effect of salt for soy protein aqueous dispersions (Hermansson, 1975): the combined effect would be faster tumbling for protein molecules, and thus decreasing τ_c for bound water molecules and reducing R_{2B} values.

The variation of $\Delta R n_H$ with ionic strength at pH 7 (Table II) is similar to that of the solubility for a denatured soy protein isolate (Shen, 1981): the addition of salt causes an initial decrease, and then a plateau is reached. Salt should decrease ΔR (see above) whereas ion binding to the soy proteins (Kumosinski, 1988) should increase n_H. Salt-induced changes in the aggregation state of the proteins (Kinsella et al., 1985) may also affect n_H and ΔR. All three factors are probably involved in the observed variation of the hydration product $\Delta R n_H$ with the ionic strength.

The generally lower R_{2obs} values obtained in the presence of NaCl (Figure 8) are most likely due to the viscosity-decreasing effect of salt (Hermansson, 1975). The overall trends of R_{2obs} vs pH in the absence or presence of salt are similar (Figure 8). The observed displacement of the R_{2obs} maximum at alkaline pH toward higher pH values in the presence of salt may be related to the salt-mediated suppression of electrostatic repulsions and the consequent stabilization of soy protein quaternary structure (Kinsella et al., 1985). The reasons for the widening of the R_{2obs} toward higher pH (Figure 8) are not clear.

4.7. Virial Coefficients and Protein Activity. The sign of the calculated virial coefficients (Table III) indicates that protein–protein repulsions dominate in all cases. The absence of nonzero $B_n/2$ terms is most likely due to the charge-screening effect from the ions of the present salt and the base/acid added for the pH adjustment. By itself, the soy protein isolate used has a reported ash content of 3.8% (w/w) and an estimated 50 mM sodium content for a 10% (w/w) protein dispersion in H_2O without any pH adjustment (pH 7.0); this amount must be sufficient to suppress the effect of charge fluctuations at low protein concentrations (Pessen and Kumosinski, 1985; Myers-Betts and Baianu, unpublished results).

In the following discussion it is assumed that the net protein charge and the ion binding to the protein are the dominant contributions to B_0 whereas the effect of the
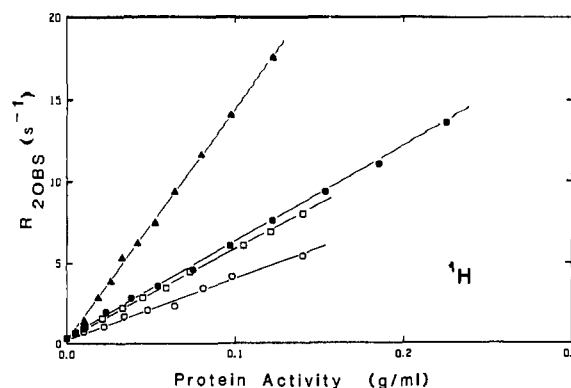

Figure 9. Dependence of the ^1H NMR transverse relaxation rates on protein activity for soy protein dispersions in H_2O at 22 ± 2 °C and pH 4.5 (O), 7.0 (□), 9.1 (●), and 11.0 (▲). Protein activities were calculated by using eq 6 and the corresponding B values from Table III. The correlation coefficients are 0.9952, 0.9996, 0.9996, and 0.9979 for data at pH 4.5, 7.0, 9.1, and 11.0, respectively.

protein excluded volume is less significant (eq 9). In the absence of added salt the B_0 is low at the isoelectric point (pH 4.5), where electrostatic repulsions and protein solubility are at a minimum, and also at pH 7.0, where salt ion content is low and ion binding to the protein is less significant. As expected, B values increase at pH 9.0 and 11.0 due to the higher protein charges and the larger extent of electrostatic repulsions. The observed increase in B_0 with increasing salt concentration at neutral pH is most likely due to the progressively higher binding of chloride ions to the proteins: the increasingly negative protein charge results in stronger electrostatic repulsions. This is in agreement with the light scattering data that show increasingly positive B_0 values for BSA in solution with increasing NaCl concentration (Timasheff et al., 1957). The effect is not significant at pH 9.0 probably because of the intrinsic negative protein charges that do not favor anion binding to the proteins. In the absence of considerable ion binding at the protein's isoelectric point (pH 4.5), the decrease in B_0 with increasing salt concentration at pH 4.5 may be due to the general salt-induced suppression of electrostatic interactions (m_s in the denominator of the first term in eq 9).

Protein activities α_p can be calculated from the obtained virial coefficients (Table III) and the protein concentration C_p (eq 8). The plot of R_{2obs} vs α_p is found to be linear in all cases (Figures 9 and 10), as predicted by Pessen and Kumosinski (1985).

5. CONCLUDING REMARKS

We have illustrated the usefulness of solute concentration dependent ^{17}O NMR relaxation measurements for the study of the hydration of complex biomaterials such as soy proteins (present study) and myofibrillar proteins (Lioutas et al., 1988). A soy protein isolate is a complex and dynamic system of interacting proteins; this does not allow rigorous data interpretation at the molecular level. Work on purified soy protein fractions is required for that purpose. Furthermore, the soy protein that we used is one of the several commercially available isolates whose hydration properties may be affected by the method and the technical conditions of production. However, the changes in hydration parameters and molecular interactions of agriculturally important materials as a function of pH, ionic strength, temperature, composition of protein blends, etc. are significant since they may lead to a better understanding of their behavior during process-
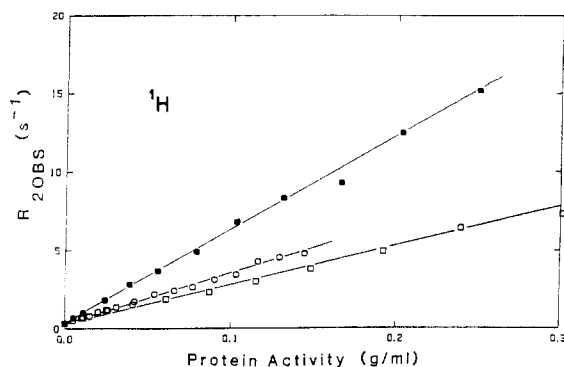
Figure 10. Dependence of the ^1H NMR transverse relaxation rates on protein activity for soy protein dispersions in 0.1 M NaCl at 22 ± 2 °C and pH 4.4 (O), 7.0 (□), and 9.0 (●). Protein activities were calculated by using eq 6 and the corresponding B_0 values from Table III. The correlation coefficients are 0.9974, 0.9976, and 0.9981 for data at pH 4.4, 7.0, and 9.0, respectively.

ing. A quantitative analysis of the pH dependence of the NMR relaxation rates (Figure 8) will be presented in a subsequent report.

ACKNOWLEDGMENT

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Effects of Controlled Sulfitolysis of Bovine Serum Albumin on Droplet Size and Surface Area of Emulsions

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Bovine serum albumin (BSA) was modified by oxidative sulfitolysis to cleave 10 and 17 disulfide bonds. The average droplet diameter and surface area of oil in water emulsions stabilized by native and modified BSA were determined with a computerized imaging system at three oil volume fractions (ϕ), namely, 0.22, 0.42, and 0.62. At ϕ = 0.22, no difference in the emulsifying properties of the proteins was observed. At ϕ = 0.42 and 0.62, the emulsions stabilized by modified BSA had smaller average diameters and greater surface areas than emulsions stabilized by native BSA. The emulsifying activity of BSA was enhanced by the increased flexibility and by exposure of hydrophobic segments of BSA resulting from the cleavage of disulfide bonds.

Proteins stabilize emulsions by mechanisms that involve reduction of interfacial tension, formation of an interfacial film around the droplets, and repulsion between droplets (Graham and Phillips, 1979; Dickinson and Stainsby, 1982). Different proteins possess a range of emulsifying properties reflecting their dynamic structure and the presence of hydrophobic and hydrophilic regions (Kinsella, 1982; Nakai, 1983). These properties, which vary with different proteins, make some proteins useful as emulsifying agents in formulated and fabricated food systems (Kinsella, 1984). The mechanisms accounting for the emulsifying activity of proteins have been the subject of many studies (Graham and Phillips, 1979; Nakai et al., 1980; Waniska et al., 1981; Shimizu et al., 1986; Kato et al., 1986; Das and Kinsella, 1989), but an understanding of the exact role of proteins in the formation and stabilization of emulsions has not been fully elucidated.

Emulsions are formed by the input of energy to increase the interfacial area and by including a surfactant to stabilize the dispersed droplets. As surfactants, proteins migrate to and adsorb at the interface, orient polar groups to the aqueous phase and nonpolar groups to the apolar phase, and lower the interfacial tension (Davis and Rideal, 1963; Graham and Phillips, 1979). The solubility, hydrophobic/hydrophilic balance, size, and net charge of a protein determine how rapidly it reaches the interface and thereby partially determine the initial emulsion characteristics. Once adsorbed to the interface, the protein may partially unfold, spread, and interact to form a continuous cohesive film (Graham and Phillips, 1979; Kinsella, 1984; Leman and Kinsella, 1989). The degree of unfolding at the interface is determined by the surface area present relative to the amount of protein available and the flexibility of the protein. At high oil volume fraction (ϕ), the protein unfolds as much as possible to cover maximum surface area. If the amount of protein present is relatively large, unfolding will not be as complete (Phillips, 1981). The capacity of a protein to cover maximum interfacial area greatly reflects (among other factors) its conformational flexibility; e.g., the flex-