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Research Article

Stability of Protein Formulations: Investigation of Surfactant Effects by a Novel EPR Spectroscopic Technique

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Surfactants are known to stabilize proteins and are often employed as additives in protein formulations. We have developed a method to study the interaction of these formulation additives with proteins by using the partitioning behavior of a spin label. In protein-free formulations, 16-doxyl stearic acid partitions into micelles above the critical micelle concentration (CMC) of the surfactant and gives rise to composite electron paramagnetic resonance (EPR) spectra composed of spectra from "free" label and "rotationally hindered" label. We compute the fraction of micelle-associated label by factor analysis and generate a label partition curve. When protein is added to the formulation, surfactant-protein aggregates form at concentrations below the surfactant's CMC. Partitioning of the label into these aggregates causes the EPR spectrum to reflect hindered rotation of the label at lower surfactant concentrations than in the protein-free solutions. A simple model of label partitioning shows that these partitioning shifts can be correlated to the surfactant:protein binding stoichiometry. We have studied the interactions of various non-ionic surfactants like Brij and Tween with recombinant human growth hormone and recombinant human interferon-γ and obtained corresponding binding stoichiometries. These binding stoichiometries match those obtained by other techniques. This technique offers a new method for estimating the protein:surfactant binding stoichiometries.

KEY WORDS: protein stability; formulation; protein–surfactant interaction; label partitioning.

INTRODUCTION

Recombinant proteins are becoming increasingly important as pharmaceutical agents. With more than 150 recombinant proteins in clinical trials and more than a dozen of those having received FDA approval (1), the question of the stability of the protein formulations is of critical importance. Proteins often require various buffers, salts, polymers and surfactants to remain stable and active in solution. To efficiently design stable protein formulations, we need a more comprehensive understanding of the interactions of proteins with the various components of a formulation and their effects on protein stability.

Polymers, polyols and surfactants, including nonionic (2, 3, 4) and anionic surfactants (5–9), have traditionally been used in formulations to stabilize proteins and in the case of blood plasma, to act as anti-viral agents(10). Studies of cationic surfactants have focused on their ability to denature proteins. Protein-surfactant interactions are not well understood, except for sodium dodecyl sulfate (SDS)-protein interactions, which have been studied extensively in the denaturation of proteins and subsequent use in SDS-PAGE electrophoresis. However, the surfactants that are normally used in formulations for their stabilizing properties are nonionic. Nonionic surfactants usually have very low critical micelle concentrations (CMC), making it difficult to study their interactions with proteins (e.g., the CMC of Tween 20 is 0.007% w/w). Although some of the CMC values of the nonionic surfactants used are available in the literature, they are reported in either pure water or weakly buffered systems and change significantly with the addition of various formulation excipients. Protein-surfactant interactions have been studied by various indirect methods such as surface tension (11), viscosity (12), and dye solubilization (13) and by direct measurements such as dialysis (14–18) and ion-selective electrodes (19–22). Traditionally, dialysis has been used to obtain binding stoichiometries. However, in case of surfactants, it is common for the membrane pores to become blocked by the large micellar aggregates (14–18). Recently, surfactant-sensitive electrodes have been used to determine the amount of surfactant bound to proteins by exploiting the electrode's ability to measure the activity of the monomeric surfactant in solution (19–22). However, these electrodes are often unstable, due to the adsorption of the buffer and protein on the electrode surface (18).

Viscosity measurements have been used to show the formation of micelle-like aggregates of surfactants bound to proteins (11, 12). Dye solubilization and steady state fluorescence studies clearly indicate that hydrophobic aggregates form in ovalbumin-SDS systems well below the CMC

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of SDS (13). Also, there are indications that the protein-surfactant aggregates are much smaller than conventional micelles. Oakes (23) has shown based on NMR and EPR data, that micelle-like aggregates are formed in protein-surfactant aggregates. Other NMR studies (24, 25) of sodium trifluorododecylsulfate and sodium *p*-butylphenol butane 1-sulfonate with bovine serum albumin (BSA) give chemical shifts consistent with micelle-like protein-surfactant aggregates.

Nonionic surfactants bind weakly to proteins (26, 27) and, at low concentrations (<1% w/w), do not unfold or denature the protein. Nonionic surfactants like Tween and Pluronic have been studied for their ability to prevent adsorption of proteins to surfaces (4, 28) and also their ability to inhibit aggregation, precipitation and denaturation (2, 3, 29, 30). Mixtures containing Tween 80 or Triton X-100 are extensively used in plasma fractions in an early process stage for viral inactivation (10). Moore *et al.* (31) have reported the use and effectiveness of 9-lauryl ether in enhancing the delivery and bioavailability of methionyl human growth hormone in rats. Nonionic surfactants have also been shown to exhibit hydrophobic protein-surfactant interactions near the surfactant's CMC. BSA has a hydrophobic pocket on the protein surface which can accommodate 2 to 3 molecules of Triton surfactants. The small endothermic enthalpy change associated with BSA-Triton X binding indicates that the interaction is predominantly hydrophobic (32). The milk protein β-lactoglobulin forms a strong 1:1 complex with Tween 20 (33). Nonionic surfactants have also been used for intranasal delivery of proteins (34). The adsorption of insulin on plastic bags is strongly decreased by the addition of Triton X surfactant to the formulation, probably indicating that the surfactant reduces the protein's available hydrophobic surface by binding at the hydrophobic patches on the surface of the protein (4). Tandon *et al.* (30) have shown the effectiveness of various nonionic, ionic and zwitterionic surfactants in increasing the refolding yields of rhodanase denatured with guanidinium chloride. These enhanced refolding yields have been attributed to the reduction in the exposed hydrophobic surface during refolding due to the binding of these surfactants. Cleland and Wang (35) and Cleland and Randolph (36) have recently shown that PEG and polyamino acids enhance the refolding yields of bovine carbonic anhydrase by binding to a hydrophobic folding intermediate.

With the rising evidence of the stabilizing nature of surfactant-protein interactions in liquid formulations, a method is needed to determine the surfactant-protein binding stoichiometries and their effect on formulations. The following describes a technique for evaluating these binding stoichiometries without chemical modification or immobilization of the protein.

MATERIALS AND METHODS

Tween 20, Tween 40, Tween 80, Brij 52 and Brij 92 were purchased from Sigma Chemical Co. and were used without further purification. The spin label, 16-doxyl stearic acid was also purchased from Sigma. Recombinant human growth hormone (rhGH) and recombinant human interferon-γ (rhIFN-γ) were provided by Genentech Inc. Deionized water from a MilliQ water purification system (Millipore Corp., Bedford, MA) was used to prepare all buffers and samples.

rhGH was lyophilized from a volatile ammonium carbonate buffer to yield a salt-free protein powder. The protein formulations were made by dissolving lyophilized rhGH into the formulation buffer. rhGH was used at a concentration of 5 mg/ml and rhIFN-γ was used at a concentration of 0.5 mg/ml. The rhGH formulation buffer consisted of 45 mg/ml mannitol, 0.25% phenol in 10 mM sodium citrate buffer, pH 6.0. The rhIFN-γ formulation buffer consisted of 40 mg/ml mannitol in 5 mM sodium succinate buffer, pH 5.0.

The buffers were prepared in deionized water and then filtered through a 0.22 μm filter to remove particulates. A solution of 16-doxyl stearic acid was prepared by dissolving 0.5 mg of the spin label in 10 μL of isopropanol, then diluting with 990 μL of the formulation buffer. A stock solution was then prepared by diluting 100 μL of the above solution with 900 μL of the formulation buffer. Any undissolved label was removed by first centrifuging for 10 min. and then filtering through 0.22 μm Millipore syringe filters. 10 μL of this stock solution was the mixed with varying amounts of surfactant stock solution and the volume was brought to 100 μL by the addition of buffer, giving a maximum spin label concentration of 20 μM. EPR spectra were taken at every 10 μL addition of the surfactant stock solution, until the resulting spectrum did not change.

After equilibrating the sample for 15 min., the X-band EPR spectra of each sample was measured by using a Bruker ESP 300 spectrometer at a field modulation of 100 kHz. The spectrometer settings were maintained at a modulation amplitude of 1.0 Gauss, a scan time of 20 sec, a scan width of 80 Gauss divided into 1024 intervals, and a frequency of 9.75 GHz. Microwave power was set at 10 mW. Each spectrum was obtained by signal averaging over 10 to 20 scans. Digitized spectra were transferred to a HP series 735 workstation for further analysis. Spectra were analyzed using a spectral fitting program (37) based on the EPR spectral simulation code of Schneider and Freed (38).

To measure the nitrogen hyperfine splitting parameter values (a_n) of 16-doxyl stearic acid in solvents of different polarities, 50 μg of the spin label was dissolved in 200 μL of solvents of varying dielectric constant and their EPR spectra were recorded and analyzed as described above (39).

At surfactant concentrations below the CMC, the EPR spectrum is dominated by the signal from the rapidly rotating spin label. As the concentration of the surfactant is increased, the spectrum increasingly indicates the presence of a micelle-associated label and this label is rotationally hindered. Above the CMC of the surfactant, the label begins to partition into the micelles, resulting in a mixed spectrum composed of the spectrum of the freely rotating label and the micelle-associated label. At concentrations well in excess of the CMC, essentially all the label partitions into the micelles and the spectrum reflects only micelle-associated label.

Since the spectra were composed of overlapping spectra which is indicative of multiple spin label environments, they were deconvoluted using factor analysis (40). A data matrix (D) was created with each spectrum as a column composed of the 1024 data points. Therefore, the data matrix was always a 1024 × *n* matrix with *n* being the number of spectra,

with each spectrum taken at a different surfactant concentration.

$$[D] = \begin{bmatrix} spect.1 & \cdots & spect.n \\ \cdot & & \cdot \\ \cdot & & \cdot \\ \cdot & & \cdot \end{bmatrix}^{n} 1024$$

Then, a covariance matrix (Z) was created:

$$[Z] = [D]^T[D]$$

This matrix Z was then diagonalized and the eigenvalues and the eigenvectors were found using computer analysis programs based on the methods reported by Press *et al.* (41). The number of significantly non-zero eigenvalues was indicative of the number of populations in the spectra. Among all the groups of spectra that were analyzed, there were always two significantly non-zero eigenvalues. The eigenvectors corresponding to these largest eigenvalues were then used to generate two basis spectra, which may be combined linearly to form the mixed spectra. The basis spectra obtained from factor analysis were plotted with the spectra of label without any surfactant and under surfactant concentrations greatly exceeding the reported CMC of the surfactant. The two basis spectra were found to match almost exactly with the experimental spectra obtained using label without any surfactant and label with surfactant at concentrations well above the CMC of the surfactant. These spectra were defined as the free label (Figure 2) and rotationally hindered label (Figure 3), respectively. The integral of an absorption spectrum was used to determine the concentration of spin label in the sample. The spectrum was recorded in the derivative mode, hence the double integral of the EPR spectrum yielded the concentration of the spin label in the sample. The eigenvectors along with the double integral of each basis spectrum provided the molar ratio of the two populations that produced the mixed spectra. The fraction of the total amount of label that is micelle-associated was then calculated and plotted against the surfactant concentration, generating the label partition curves.

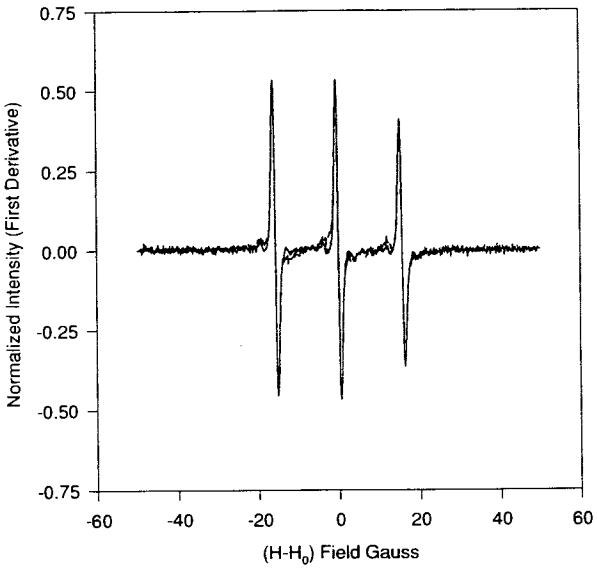

Figure 2. Comparison of free label spectra obtained from pure label without any surfactant (solid line) in rhGH formulation buffer and basis spectrum obtained from factor analysis (dashed line).

REFRACTIVE INDEX STUDIES

The EPR results were compared to binding measurements that were made utilizing a recently developed system for studying ligand-protein interactions. The system, Bia-

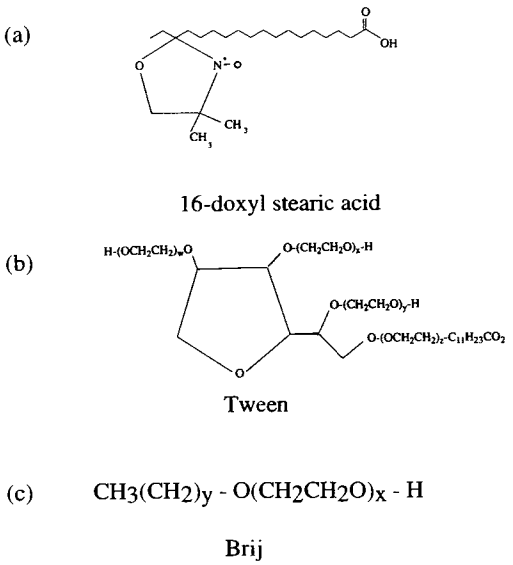

Figure 1. Chemical structure of the various surfactants and spin label used in the analysis. 16-doxyl stearic acid, Tween (w+x+y+z=20). Tween 20- $R=C_{11}H_{23}CO_2$ (laurate); Tween 40- $R=C_{16}H_{31}CO_2$ (palmate); Tween 80- $R=C_{17}H_{33}CO_2$ (oleate).

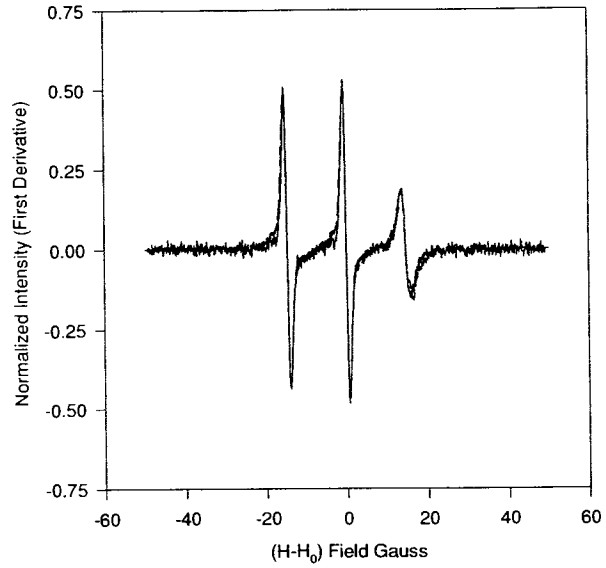

Figure 3. Comparison of micelle-associated spectra obtained from label with 2mM Tween 20 much above the CMC (solid line) in rhGH formulation buffer and basis spectrum obtained from factor analysis (dashed line).

core® (Pharmacia, Piscattaway, NJ), was designed to measure the changes in refractive index upon ligand binding to immobilized protein. The protein, rhGH, was immobilized on a Biacore® Sensorchip which consisted of a gold plate with activated linker arms and a layer of dextran. Wild type rhGH was immobilized on the plate by amine coupling at the amino terminus and a $^{137}Q \rightarrow C$ mutant of rhGH was immobilized by thiol coupling to the free thiol (^{137} C) as shown in Figure 4. The measurements were then performed by flowing running buffer, 10 mM Hepes, 120 mM NaCl, pH 7.4, across the plate. Samples containing surfactants in the same buffer were then injected (30 μL). The concentrations of surfactants used was 0.1 to 10 mg/ml for Tween and 0.00139 to 0.0125 mg/ml for Brij. The injection was followed by a continuous flow of running buffer and the change in refractive index (R) was measured. The refractive index increased to at least two fold above background (samples run in cell without protein) in each case. The stoichiometry of binding, n, was then calculated from the changes in the refractive index upon ligand binding to the immobilized protein.

$$n = \frac{R_s}{R_p}\frac{MW_p}{MW_s} \quad (1)$$

The refractive index change from the protein immobilization, Rp, is first measured to quantitate the amount of protein on the surface (R_P = 1461 for wild type; 1092 for mutant) and then the refractive index change upon addition of surfactant to the immobilized protein, Rs, is measured. These refractive index measurements along with the molecular weights of the protein (MW_p = 22,127 Da) and surfactants (MW_s) were then used to calculate the stoichiometry. To assure maximum binding, the surfactant solutions were tested at different concentrations until saturation was achieved.

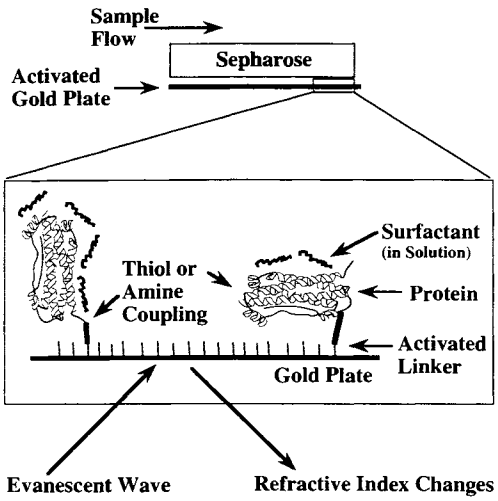

Figure 4. Diagram of refractive index method used to assess rhGH-surfactant interactions. rhGH was immobilized on the gold plate through either amine coupling or thiol coupling chemistry. For the amino coupling of wild type rhGH, the most reactive group is the terminal amine group and the orientation of the protein for this coupling is depicted by the protein molecule on the left side of the inset. The $^{137}Q \rightarrow C$ rhGH mutant was linked to the plate via the free thiol. This linkage would result in a protein orientation as shown by the protein molecule on the right side of the inset. A sample containing the surfactant is passed across the immobilized rhGH on the gold plate. Binding of the surfactant to the protein results in a change in refractive index and this change is measured by applying an evanescent wave of light to the opposite side of the gold plate. (rhGH structure: DeVos, A. M., Ultsch, M., and Kosiakoff, A. A. (1992) *Science* **255**, 306–312; Ribbon diagram program: Kraulis, P. J. (1991), *J. Appl. Cryst.* **24**, 946–950).

RESULTS AND DISCUSSION

To ascertain whether the spin label, 16-doxyl stearic acid interacts with the protein, a solution of the spin label at various concentrations was prepared in buffer and the EPR spectra were recorded. rhGH or rhIFN-γ was added to this solution and after equilibrating the EPR spectrum of the label was recorded again. There was no change in the spectrum upon addition of protein as shown in Figure 5. If the spin label bound to the protein, then the label would be more rotationally hindered than the freely rotating spin label in solution. Since there was no change in the rotational diffusivity of the spin label as indicated by virtually identical EPR spectra, the spin label used did not bind to the surface of the protein.

When protein was added to the formulation, the label became rotationally hindered at lower surfactant concentrations (Figures 6, 7 and 8). Figure 6 shows a plot of the fraction of spin label that is rotationally hindered vs. the concentration of surfactant Tween 40. It should be emphasized that the pure surfactant curves for Tween 40 show a different behavior in Figures 6 and 8 because they are in different formulations for rhGH and rhIFN-γ respectively, which differ significantly in their excipients. The error bars are noted on the pure surfactant curve; the error is largest on the steepest portions of the curve and smallest on the horizontal portion. The fraction of hindered label curve shifts to lower

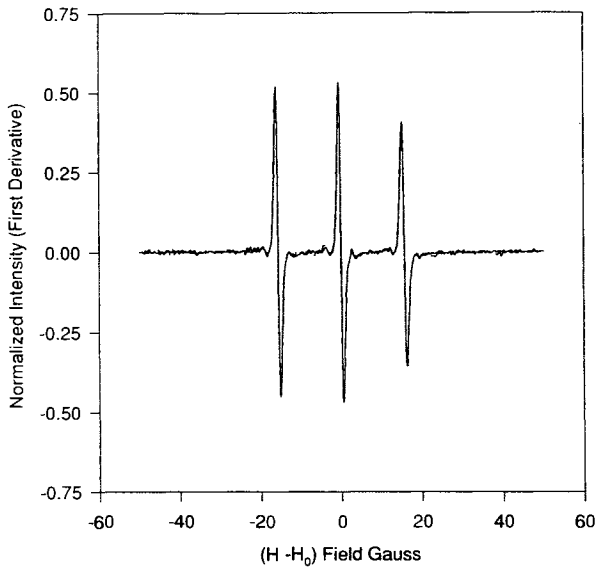

Figure 5. Comparison of the spectra obtained from the spin label 16-doxyl stearic acid in absence of any surfactant. 16-doxyl stearic acid in 10 mM sodium citrate buffer, pH 6.0 with 5 mg/ml rhGH (dashed line). 16 doxyl stearic acid in 10 mM sodium citrate buffer, pH 6.0 without rhGH (solid line).

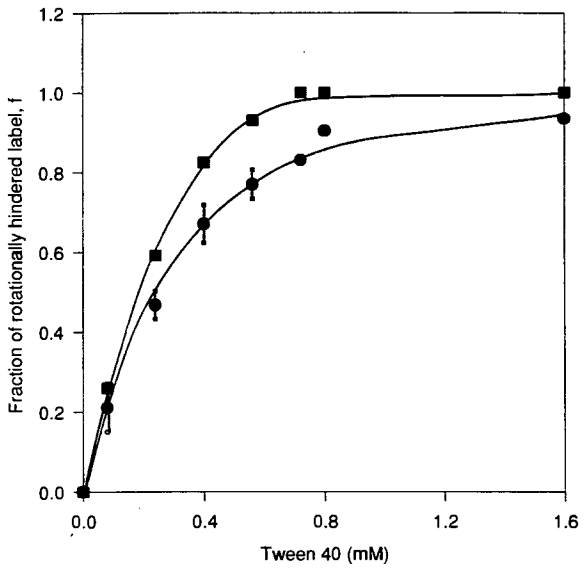

Figure 6. Shift in the label partition curve of Tween 40 in formulation buffer upon addition of 5 mg/ml rhGH:Tween 40 in formulation without rhGH (circles). Tween 40 in formulation with 5 mg/ml rhGH (squares). Error bars are indicated on the protein-free label partition curve. The lines through the data points have no theoretical significance.

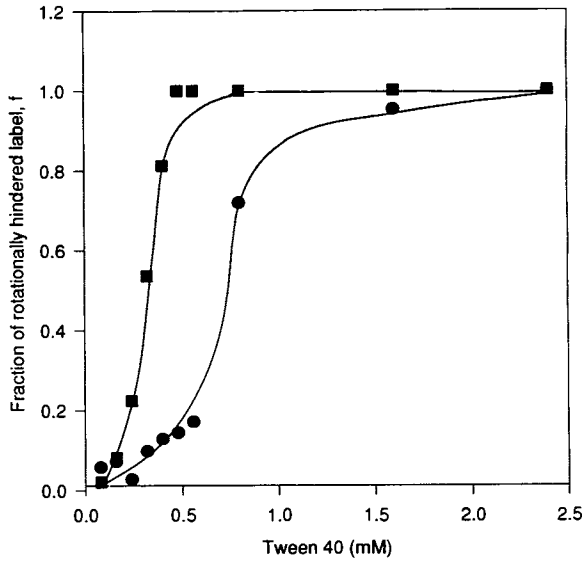

Figure 8. Shift in the label partition curve of Tween 40 in formulation buffer upon addition of 0.5 mg/ml rhIFN-γ:Tween 40 in formulation without rhIFN-γ (circles). Tween 40 in formulation with 0.5 mg/ml rhIFN-γ (squares). The lines through the data points have no theoretical significance.

Tween concentrations when 5 mg/ml rhGH is present in the formulation. Similar curves for rhGH-Tween 20 and rhIFN-γ-Tween 40 are plotted in Figures 7 and 8 respectively. This effect is understandable if the surfactant-protein binding is stronger than surfactant-surfactant binding. Surfactant-protein aggregates will then form at a concentration lower than the surfactant's CMC, and the label can partition into these aggregates. Therefore, in the presence of protein, a mixed spectrum was obtained at lower surfactant concentrations than those required in the protein-free formulation and this change resulted in a shift of the label partition curves to lower surfactant concentrations.

To obtain the binding stoichiometry of the surfactant to the protein, the fraction of the label that is rotationally hindered in the formulation containing protein was subtracted from the same fraction for the formulation without protein. The difference of the fractions was then plotted against the molar ratio of surfactant to protein (Figures 9, 10 and 11).

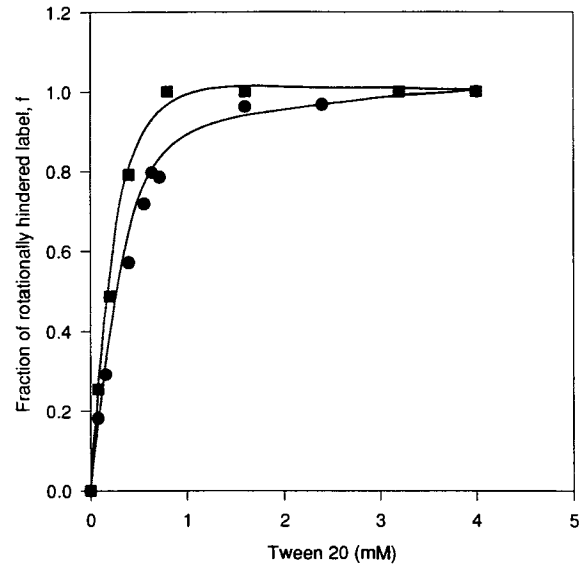

Figure 7. Shift in the label partition curve of Tween 20 in formulation buffer upon addition of 5 mg/ml rhGH:Tween 20 in formulation without rhGH (circles). Tween 20 in formulation with 5 mg/ml rhGH (squares). The lines through the data points have no theoretical significance.

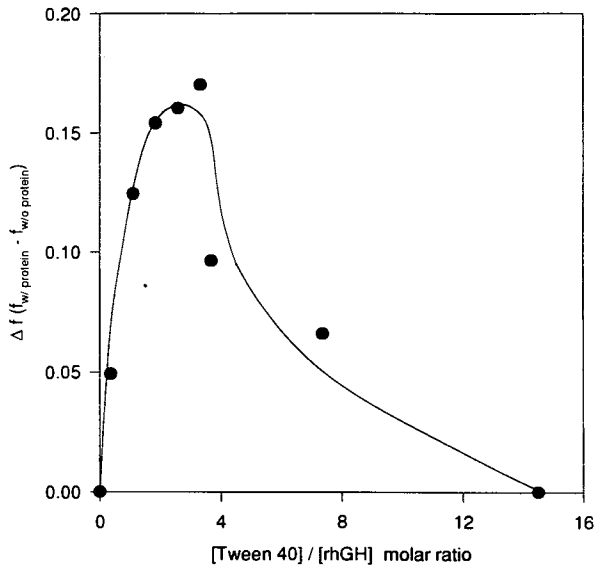

Figure 9. Difference in the fraction of micelle-associated label plotted as a function of the molar ratio of Tween 40:rhGH allows the determination of the binding stoichiometry. Δf is obtained by subtracting the fraction of rotationally hindered label in absence of rhGH from the fraction in the presence of 5 mg/ml rhGH. Data calculated from the results in Figure 6.

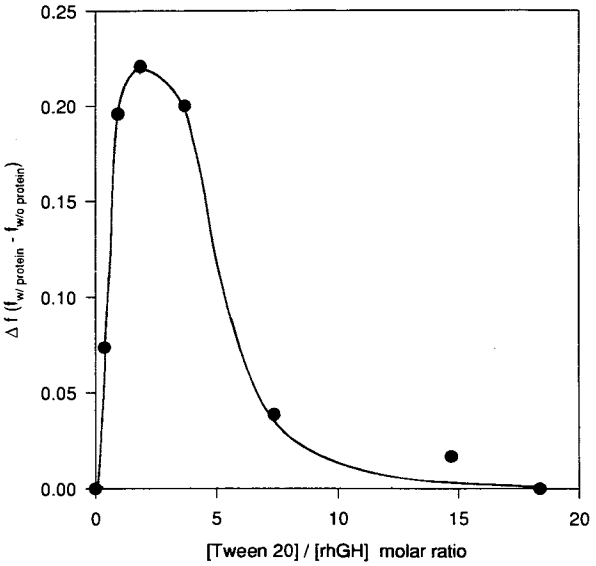

Figure 10. Determination of Tween 20 binding stoichiometry. Δf is obtained by subtracting the fraction of rotationally hindered label in absence of rhGH from the fraction in the presence of 5 mg/ml rhGH. Data calculated from Figure 7.

Assuming a simple equilibrium partitioning of the label between the micelles and the bulk solution, the difference between the fractions will pass through a maximum at a surfactant:protein ratio which results in saturation of the surface of the protein with the surfactant molecules.

At equilibrium, we may write a set of equations defining the formation of aggregates from surfactant monomers and the partitioning of label between the aggregates and free solution:

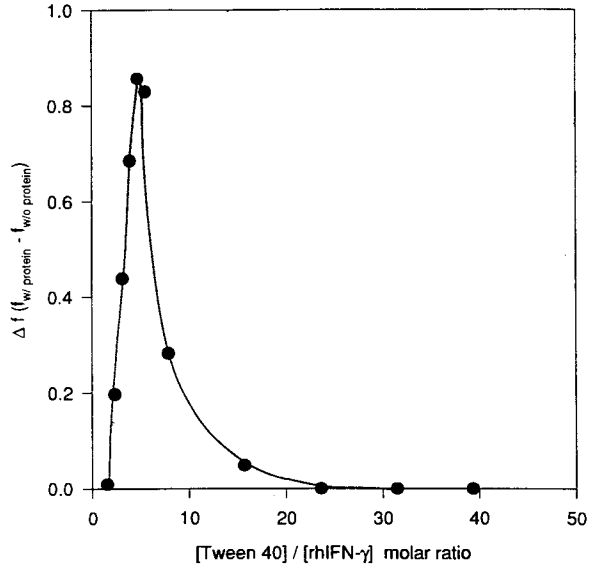

Figure 11. Difference in the fraction of micelle-associated determines the Tween 40:rhIFN-γ binding stoichiometry. Δf is obtained by subtracting the fraction of rotationally hindered label in absence of rhIFN-γ from the fraction in presence of 0.5 mg/ml rhIFN-γ. Data calculated from results in Figure 8.

$$nS_1 \xleftrightarrow{K_{mic}} S_n \quad \text{Micelle} \tag{1}$$

$$L_f + S_n \xleftrightarrow{K_{aggm}} L_{aggm} \quad \text{Label partition into micelle} \tag{2}$$

$$mS_1 + P \xleftrightarrow{K_p} PS_m \quad \text{Surfactant bound to protein} \tag{3}$$

$$L_f + PS_m \xleftrightarrow{K_{aggp}} L_{aggp} \quad \text{Label partition into protein-surfactant aggregate} \tag{4}$$

Where S_1 is the surfactant monomer, S_n is the surfactant micelle, L_f is the free label, L_{aggp} is the (surfactant-protein aggregate)-associated label, L_{aggm} is the micelle-associated label, P is the protein, PS_m is the surfactant-protein aggregate and K_{mic}, K_{aggm}, K_p, K_{aggp} are the respective equilibrium constants.

An analysis of the nitrogen hyperfine splitting parameter of 16-doxyl stearic acid shows that the polarity of the local environment around the label that has partitioned into the micelles is not distinguishable from that of a label in surfactant-protein aggregates. Therefore we assume that:

$$K_{aggm} \sim K_{aggp} \tag{5}$$

We also assume that:

$$K_p \gg K_{mic} \tag{6}$$

Since we cannot distinguish between the micellar aggregates and the protein-surfactant aggregates using EPR spectroscopy, we define [Aggregate] as the total concentration of micelles and the protein-surfactant aggregates in solution and $[L_{agg}]$ as the total concentration of aggregate associated label in solution with an equilibrium constant, K.

$$L_f + \text{Aggregate} \xleftrightarrow{K} L_{agg} \tag{7}$$

Defining the fraction of total label that is associated with aggregates as f, and assuming that there is strong label partitioning into these aggregates, whether micellar or protein surfactant aggregates,

$$f \equiv L_{agg}/(L_{agg} + L_f) \tag{8}$$

$$\Delta[\text{Aggregate}] \propto \Delta f \tag{9}$$

where [Aggregate] is the total concentration of aggregates (micelle and surfactant-protein aggregates) in solution, Δ [Aggregate] is the difference between aggregate concentrations with and without protein at a given surfactant concentration, f is the fraction of total label associated with aggregates (fraction of rotationally hindered label) and Δf is the difference between the fraction of label associated with aggregates for solutions with and without protein at a given surfactant concentration. Strong label partitioning into surfactant aggregates can be assumed because the label, 16-doxyl stearic acid, is highly hydrophobic and should partition into a more hydrophobic environment of a micellar core or protein-surfactant aggregates. Strong label partitioning is also indicated by the fact that the spectra of freely rotating

labels were undetectable at surfactant concentrations significantly above the CMC even though EPR is more sensitive to the rapid motion of such free labels in solution.

At concentrations below the CMC of the surfactant, we do not observe any mixed spectra in the absence of protein because there are no aggregates into which the spin labels can partition. However, when we add protein to the solution at the same surfactant concentration, the surfactants bind to the protein surface. At a given surfactant concentration, the difference between the concentration of aggregates in solutions with and without protein indicates the amount of surfactant-protein aggregates. As we add more surfactant to the solution, the surface of the protein molecules becomes progressively saturated with the surfactant molecules, until it reaches the binding stoichiometry. The amount of label that can partition into aggregates is directly proportional to the amount of aggregates in the solution. Therefore at surfactant levels below the binding stoichiometry the difference in the label partitioning curves (Δf) increases and at surfactant levels above the binding stoichiometry the difference in the label partition curves decreases. When this difference is plotted against the molar ratio of surfactant to the protein, the maximum in this curve occurs at the approximate binding stoichiometry. It is possible that these protein-surfactant aggregates may lead to micelle formation by acting as nuclei for micelles or the protein may substitute for surfactant in the micelle thereby reducing the CMC. On the other hand, the micelle formation may be completely independent of the protein-surfactant binding. In any case, from the maximum in the curve, we can obtain protein-surfactant binding stoichiometries. An implicit assumption in this analysis is that the binding stoichiometries obtained will approximate the true values when the surfactant-protein binding is much stronger than the surfactant-surfactant binding, i.e. $K_p \gg K_{mic}$. This assumption is reasonable because K_{mic}, which is the strength of the interaction between surfactant molecules, is small in case of nonionic surfactants. Nagarajan (42) has recently shown that, in the case of SDS/polyethylene oxide (PEO) system, $K_p > K_{mic}$, the polymer-surfactant binding is stronger than the surfactant-surfactant binding. A similar scenario is thus reasonable for surfactant-protein interactions. As these two binding strengths come closer together, the surfactant molecules will not preferentially bind to the protein and the maximum in the label partition shift curves will no longer represent the true surfactant-protein binding stoichiometry.

Binding stoichiometries calculated for various Tween and Brij surfactants with rhGH are given in Table 1. The small difference in relative hydrophobicities of the Tweens tested here cannot account for the difference in binding stoichiometries. The differences in hydrophobicity are the result of differences in the chain of the Tween molecules which all have a sorbitan hydrophilic group. For Tween 20, which has a twelve carbon alkyl chain, the binding stoichiometry was in the range of 2.5 to 3.5 (Tween 20/rhGH). Tween 40, which consists of greater than 90% palmitic acid (sixteen carbon alkyl chain), bound to rhGH in a range of 2.5 to 3 Tween 40 molecules per rhGH. Finally, Tween 80, with an eighteen carbon alkyl chain, either does not bind to rhGH or binds at very low stoichiometries. Tween 80 did not significantly shift the label partition curves. This small effect could be attributed to the fact that, among the different Tweens, the CMC of Tween 80 is the lowest. Therefore, Tween 80 could exhibit stronger surfactant-surfactant interactions than surfactant-protein interactions, leading to preferential formation of surfactant-surfactant aggregates over surfactant-protein aggregates. Thus, the addition of protein has no significant effect on the partitioning of the spin label and the curve does not shift. On the other hand, there could be fewer Tween 80 sites per rhGH so that the shift in the label partition curves is small.

Table 1. Binding stoichiometries obtained by the EPR method of CMC-shift compared with those obtained by refractive index studies

	Surfactant:rhGH binding stoichiometry		
		(Refractive Index)	
Surfactant	(EPR)	rhGH	137Q → C
Tween 20	2.5-3.5	8.7-9.2	6.7
Tween 40	2.5-3	Not studied	Not studied
Tween 80	No shift*	2.2-3.8	3.3
Brij 52	Not studied#	1.6-3.8	1.3
Brij 92	3.5-4	3.2-5.2	2.0

* No shift in the label partition curves was observed with Tween 80.
# Brij 52 was not studied because of solubility problems.

The rhGH-surfactant binding stoichiometries measured by EPR were also assessed by the refractive index method (Biacore®). For the wild type rhGH, the surfactant binding stoichiometries are significantly greater for Tween 20, but are approximately the same for Brij 92. The higher values for the Tween 20 - rhGH interactions may result from micelle formation at the rhGH surface providing a higher refractive index change than that observed for stoichiometric binding. When the mutant rhGH (^{137}Q →C) was immobilized, the binding stoichiometries decreased for all the surfactants. The differences in observed stoichiometry for the wild type and mutant rhGH immobilized on the gold plate were probably due to burying of the exposed hydrophobic surfaces. Thus, the lower stoichiometry for the mutant rhGH may have resulted from removal of potential binding sites. A small increase in the binding stoichiometry was observed with a decrease in alkyl chain length for both Tween 80 to Tween 20. Overall, these results indicated that the rhGH may bind to the hydrophobic alkyl chains of the surfactants. Previous studies have demonstrated the presence of fatty acid binding sites on the surface of rhGH (43, 44).

If the surfactant-protein interactions are hydrophobic, then a more hydrophobic protein than rhGH would be expected to bind more surfactant. To assess this hypothesis, a very hydrophobic protein, rhIFN-γ, was studied with Tween 40. The hydrophobicity index for rhGH and rhIFN-γ according to two different hydrophobicity scales, the Von Heijne hydrophobicity scale (45) and the bulk hydrophobic character developed by Manavalan and Ponnuswamy (46), shows that rhIFN-γ is more hydrophobic than rhGH. Based on the hydrophobic index of Von Heijne respective hydrophobicities for rhGH and rhIFN-γ are 301 and 271 (Von Heijne index), and 410 and 379 (Bulk hydrophobicity), with the more positive indicating more hydrophilic. The shift in the

label partition curve is more dramatic in case of rhIFN-γ, as can be seen in Figure 8, probably a reflection of its higher hydrophobicity than rhGH. As shown in Figure 11, the titration of rhIFN-γ with Tween 40 went through a maximum at approximately a 6 to 1 Tween 40 to rhIFN-γ ratio. The transition from a single component to a mixed spectrum occurred at Tween 40 concentrations above 0.5 mM and this transition was very sharp as shown in Figure 8. The sharp transition in case of rhIFN-γ compared to rhGH may have resulted from a strong association between the surfactant and rhIFN-γ or the ability of Tween 40 to form a micelle with less surfactant in the presence of rhIFN-γ. The combined rhIFN-γ and rhGH results indicated that the amount of surfactant required for binding to the surface can be correlated to the hydrophobicity of both the protein surface and the surfactant. In case of rhIFN-γ, six molecules of Tween 40 are required to saturate the surface of the protein. This is understandable since rhIFN-γ is a much more hydrophobic protein than rhGH and accordingly, we would expect that more residual hydrophobic surface would require more of the same surfactant to saturate it.

The effect of the spin label concentration in the shifts in the partition curves was studied by varying the label concentration over the range of 20 μM to 200 μM. Identical results were obtained at each label concentration. The variation in the values generated by these experiments was small as shown on the Tween 40 label partition curve with rhGH (Figure 6). The label partition curves for the Tweens and Brij did not have distinct inflection points, indicating that the CMC in the presence of protein occurs over a wide concentration range. Nonionic surfactants generally have micelles that are less structured and more polydisperse in aggregation number, and may account for the gradual change in the micelle formation. This loose structure of the micelles was further probed by examining the nitrogen hyperfine splitting parameter (a_n) values of the spin labels associated with the micelles.

The nitrogen hyperfine splitting parameter (a_n) reflects the polarity of the local environment surrounding the spin label since it is related to the dielectric constant (40). The values of a_n can be obtained from the fitting programs as discussed in the Materials and Methods section. An analysis of the a_n values indicates that the rotationally hindered label is in a more non-polar environment than the freely rotating label. The free label yields an a_n value close to the value obtained in pure water, and in buffer. The a_n value of the rotationally hindered label spectrum reveals that the local environment has a polarity lower than water but considerably higher than a liquid hydrocarbon. This results leads us to believe that the rotationally hindered label is either partitioned inside the micelles or is associated with a cluster of surfactant molecules which has made the environment less polar. To ascertain that the reduced value is not the result of the interaction of the spin label with surfactant monomers in the absence of micelles, we measured the a_n values in the formulation buffer below the CMC of Tween 20 and found that the value is identical to the a_n value in the absence of any surfactant ($a_n = 15.74$ G). Also, the a_n value in carbon tetrachloride (14.24 G) is not affected by Tween addition, even at 0.8 mM Tween 20 ($a_n = 14.24$ G). The a_n value in formulation buffer with 50μM Tween 20 (below the CMC) is 15.7 G, whereas the a_n value in formulation buffer with 4mM Tween 20 (which is significantly above the CMC) is 14.8 G. These results, combined with the fact that the a_n value shows a marked decrease in formulation buffers with surfactant concentrations above the CMC, confirm that the rotationally hindered label has a lower a_n value because it is associated with a micelle-like cluster of surfactant molecules. Further, there is no difference between the a_n value obtained for a label associated with protein-surfactant aggregates and a label associated with micelles. These results suggest that the polarity of the local environment of a label associated with the protein-surfactant aggregate is similar to that of a nonionic micelle. Therefore, we can conclude that the driving force for the partitioning of a label into surfactant micelles is approximately the same as that for partitioning into surfactant-protein aggregates. This conclusion also supports the assumption that we made earlier that the equilibrium constants for label partitioning into surfactant micelles and surfactant-protein aggregates are approximately equal, i.e. $K_{aggm} \sim K_{aggp}$. It should be noted that in both the micelle and the surfactant-protein aggregate, the label is in an environment that is only moderately non-polar as indicated by the a_n values, perhaps indicating significant penetration of water into the micellar core, this is to be expected for nonionic micelles, since they are less structured than their ionic counterparts.

CONCLUSIONS

The above results indicate that the protein-surfactant interactions can be probed by exploiting the shift in the label partition curves. This method can be applied to any protein-surfactant system, and requires no chemical modification of immobilization of the protein. Label partition curves were generated with and without protein in different formulations. The shift in the label partition curve on addition of protein can be used to obtain the surfactant:protein binding stoichiometry. We have studied the interactions of various nonionic surfactants like Brij and Tween, and report their binding stoichiometries with rhGH and rhIFN-γ. The stoichiometries obtained match those obtained by other techniques. This technique offers an easy way to determine the protein-surfactant binding stoichiometry for use in protein formulations in which the knowledge so far is mostly empirical, primarily due to the lack of a simple methodology for studying these interactions. This new technique can thus be of help in determining the effect of surfactants on protein stability. If the binding stoichiometries can be correlated to the hydrophobicity, molecular weight of the surfactant and the hydrophobic surface area exposed on the protein, then formulation scientists will be able to better predict the amount and type of surfactant required to stabilize a given protein formulation. Similar studies using the fluorescent probe ANS are under way to study the effect of various excipients in a formulation on the CMC of the surfactant and how this relates to the stability of the formulation.

ABBREVIATIONS

EPR	Electron paramagnetic resonance spectroscopy
NMR	Nuclear magnetic resonance spectroscopy
SDS	Sodium dodecyl sulfate

BSA	Bovine serum albumin
PAGE	Polyacrylamide gel electrophoresis
CMC	Critical micelle concentration
rhGH	Recombinant human growth hormone
rhIFN-γ	Recombinant human gamma interferon
MW	Molecular weight
PEG	Polyethylene Glycol
ANS	8-Anilino-1-napththalenesulfonic Acid

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