Investigation of Protein-Surfactant Interactions by Analytical Ultracentrifugation and Electron Paramagnetic Resonance: The Use of Recombinant Human Tissue Factor as an Example

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Purpose. The purpose of this work is to utilize electron paramagnetic resonance (EPR) spectroscopy in conjunction with analytical ultracentrifugation (AUC) to investigate the binding of surfactants to proteins with a transmembrance domain. As an example these methods have been used to study the interaction of a nonionic surfactant, C12E8, to recombinant human tissue factor (rhTF) in liquid formulations. The complementary nature of the two techniques aids in data interpretation when there is ambiguity using a single technique. In addition to binding stoichiometries, the possibility of identifying the interacting domains by using two forms of rhTF is explored.

Methods. Two recombinant, truncated forms of human tissue factor were formulated in the absence of phospholipids. Neither of the recombinant proteins, produced in E. coli, contains the cytoplasmic domain. Recombinant human tissue factor 243 (rhTF 243) consists of 243 amino acids and includes the transmembrane sequences. Recombinant human tissue factor 220 (rhTF 220), however, contains only the first 221 amino acids of the human tissue factor, lacking those of the transmembrane region. EPR and AUC were used to investigate the interactions between these two forms of rhTF and polyoxyethylene 8 lauryl ether, C12E8.

Results. Binding of C12E8 to rhTF 243 is detected by both EPR spectroscopy and AUC. Although a unique binding stoichiometry was not determined, EPR spectroscopy greatly narrowed the range of possible solutions suggested by the AUC data. Neither technique revealed an interaction between rhTF 220 and C12E8.

Conclusions. The complementary nature of EPR spectroscopy and AUC make the combination of the two techniques useful in data interpretation when studying the interactions between rhTF and C12E8. By utilizing these techniques in this study, the binding stoichiometry of rhTF 243 to C12E8 ranges from 1.2:1 to 1.3:0.6 based on an aggregation number of 120. This binding is consistent with previously reported activity data that showed an increase in clotting rate when rhTF 243 is in the presence of C12E8 micelles. From the rhTF 220 data, it can further be concluded that the transmembrane domain of rhTF is necessary for interactions with C12E8.

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INTRODUCTION

Nonionic surfactants are already approved for use in several parenteral formulations and are traditionally added to pharmaceutical formulations to reduce protein aggregation during shipping and storage (1–3). If they are present at a concentration that is at or above the critical micelle concentration (CMC), the amphiphilic nature of surfactants enables them to form organized aggregates, or micelles. Their amphiphilic nature also causes nonionic surfactants to interact with hydrophobic surfaces when formulated in an aqueous environment. These surfaces can present themselves in the form of vial-liquid interfaces, air-liquid interfaces and protein-liquid interfaces. While there are a number of physical techniques that have been used to characterize micellar systems, this paper deals with the utilization of two of these techniques, analytical ultracentrifugation (AUC) (4) and electron paramagnetic resonance spectroscopy (EPR) (5,6) to determine the binding stoichiometry of a surfactant-protein complex. The proteins used in this study are two truncated forms of a recombinant human blood coagulation protein, tissue factor (rhTF) (7,8). These recombinant proteins, rhTF243 and rhTF220, expressed in *E. coli*, contain all of the extracellular amino acid residues (1–221) but lack those of the cytoplasmic domain. In addition, rhTF 220, also lacks the transmembrane domain sequence, 222–243.

Previously, analytical ultracentrifugation (AUC) was used to investigate the interactions between polyoxyethylene-8-laurylether (C12E8) and rhTF 243 (4). The buoyant molecular weight (MW_b) of rhTF243 in the presence of C12E8 was determined by sedimentation equilibrium analysis to be 10822 ± 1070. It is not possible to determine MW_b for rhTF243 without any C12E8 because the protein is not soluble without the added surfactant. However, it is possible to estimate the buoyant molecular weight of rhTF243 without surfactant. The expected protein molecular weight based on amino acid composition is 27423. This can be converted to a buoyant molecular weight by multiplying by $(1 - \bar{v}_p\rho)$ where $\bar{v}_p$ is the partial specific volume of rhTF243 and ρ is the solution density. Assuming a density of 1.0 g/cc, the partial specific volume, 0.736 cm^3/g, estimated from the amino acid content (4,9) yields a value of 7240 for the buoyant molecular weight of rhTF243 without any surfactant. This increase in buoyant molecular weight is indicative of a binding interaction between C12E8 and rhTF 243. The purpose of the present study is two-fold. First, we want to confirm the AUC results using an independent technique, electron paramagnetic resonance (EPR) spectroscopy. Second, we want to expand the AUC and EPR studies to also include the rhTF 220-C12E8 system to determine whether or not the transmembrane domain is responsible for the protein-surfactant interactions that lead to the formation of the complexes detected by AUC.

MATERIALS AND METHODS

Chemicals

rhTF 243 and rhTF 220 were produced at Genentech, Inc. Unlike the naturally occurring protein, neither of the recombinant proteins, which are expressed in *E. coli*, are glycosylated.

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rhTF 243 is a 243 amino acid peptide that includes the extracellular and transmembrane domains of the protein, while rhTF 220 only contains the extracellular domain. Both proteins were purified from *E. coli* extracts via immunoaffinity chromatography and formulated into PBS (10 mM sodium phosphate, isotonic NaCl, pH 7.3) containing C12E8 surfactant. Immediately prior to conducting experiments that required initial surfactant concentrations lower than that of the formulation, rhTF was either exhaustively dialyzed against surfactant-free PBS buffer or chromatographed on an a surfactant removal column, Extracti-Gel D™ (Pierce Chemical Corp.) (10,11). C12E8 purchased from Sigma Chemical Co. was dissolved in PBS buffer and added to the samples for subsequent analysis. C12E8 concentrations were assessed using a chromogenic assay (12).

Methods to Characterize C12E8-rhTF Interactions

Analytical Ultracentrifugation

Methods for the sedimentation equilibrium and velocity experiments have been reported previously (4) and are only summarized here. Data were collected using Model E and XLA analytical ultracentrifuges equipped with UV absorption optical systems. NONLIN, a non-linear least squares fitting program, and Kaleidagraph™ software were used to fit the equilibrium data to a single ideal species model. In order to convert the measured buoyant molecular weights into molecular weights, the partial specific volume of a rhTF243-C12E8 mixed micelle had to be determined. The process used as described by Shire (4) was an iterative computation that assumed a particular stoichiometric model. The partial specific volume of neat surfactant determined by high precision densitometry was used in the computations to estimate the partial specific volume of the rhTF243-C12E8 complex which was then used to obtain the complex molecular weight. The process of this computation and potential ambiguities are reviewed further in the discussion. Sedimentation equilibrium was also used to determine the molecular weight of rhTF220 as a function of C12E8 concentration. Three samples at a rhTF220 loading concentration of 0.1, 0.2, and 0.4 mg/mL at 0, 0.01 and 0.04% C12E8 were analyzed by sedimentation equilibrium at 20000 and 25000 rpm at 10°C. The buoyant molecular weights were converted to weight average molecular weight by using a partial specific volume of 0.727 cm^3/g that was computed from the theoretical amino acid composition (9). Densities of the solution were determined with a Metler-Parr DMA 35 density meter.

EPR Spectroscopy Probe Partitioning

Another method used to determine the surfactant binding stoichiometry to rhTF is electron paramagnetic resonance (EPR) spectroscopy probe partitioning. This method has been described in detail by Bam *et al.* (5,6). It takes advantage of the competitive micellization that occurs in systems where protein binds surfactant. Protein surfactant binding shifts the onset of (mixed) micelle formation to lower surfactant concentrations. Consequently, probe molecules partition into micellar environments at lower surfactant concentrations if such mixed micelles form. The surfactant:protein binding stoichiometry corresponds to the molar ratio of surfactant to protein that yields the maximum shift in the fraction of the probe partitioned into a (mixed) micellar environment as a function of surfactant concentration (5).

A hydrophobic, non-reactive nitroxide, 16-doxyl stearic acid, served as the spin probe for this study. In order to determine the binding stoichiometry for each rhTF: C12E8 system, two sets of data were analyzed, one with and one without the protein. For each pair of sample sets, the surfactant concentration was allowed to vary from zero to well above the surfactant's CMC. Spectra containing 5–10 averaged scans centered at a magnetic field of 3470 G were collected at a constant frequency of 9.75 GHz. The scan width was 60 G. Spectra were analyzed using a factor analysis program written by Heller based on Malinowski's factor analysis theory (13,14). There were two non-zero eigenvectors, indicating that there are two observable populations of probe molecules. By multiplying each of these non zero eigenvectors by the eigenvalues of the Z matrix, two basis spectra were obtained. These basis spectra were assigned to populations within and outside micellar environments on the basis of their associated nitrogen hyperfine coupling constants and rotational diffusivities. Double integration of the basis spectrum resulting from micelle-associated probes, multiplied by the appropriate eigenvectors and divided by the double integral of the experimental spectrum yielded the fraction of the total probe found in the micelle associated fraction.

RESULTS

EPR Probe Partitioning: Binding Stoichiometries

Figure 1 shows the probe partitioning curves for 16-doxyl stearic acid in the presence and absence of rhTF 243, which contains the transmembrane domain, as a function of C12E8 concentration. In the presence of the protein, less surfactant is required to form a micelle-like environment. As stated above, protein-surfactant mixed micelles form at lower surfactant concentrations than pure surfactant micelles (5,6). The surfactant-protein binding stoichiometry coincides with the maximum in a plot of the difference between the two curves as a function of the overall C12E8:protein molar ratio (Fig. 2). C12E8 exhibits an ill-defined binding stoichiometry to rhTF of 50 to 100 moles of surfactant per protein molecule. If we assume an aggregation number of 120 molecules of C12E8 per micelle,

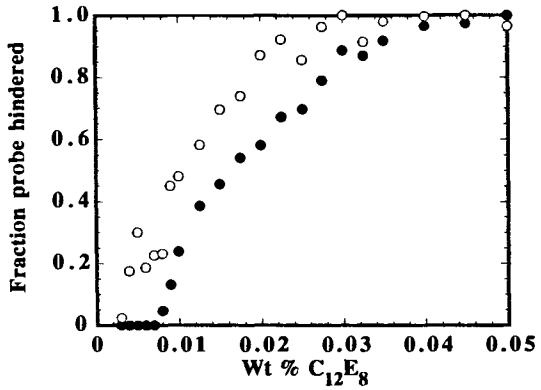

Fig. 1. EPR determination of probe partitioning of 16-doxyl stearic acid in the presence (○) and absence (●) of rhTF243 as a function of C12E8 concentration. The solid line is the result of a 3rd order polynomial fit to the data.

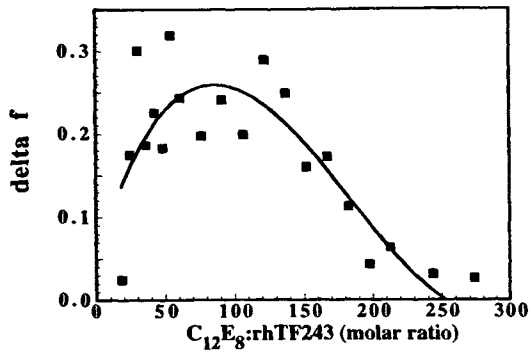

Fig. 2. Binding stoichiometry of C12E8-rhTF243 complexes as determined by EPR difference titration.

as in the previous study, the results correspond to approximately 0.4 to 0.8 micelles per complex. Unlike rhTF 243, the presence of rhTF 220 does not significantly affect the probe partitioning. The partitioning plots for 16-doxyl stearic acid in the presence and absence of rhTF 220 essentially overlap as seen in Fig. 3. Thus, no binding of C12E8 to rhTF 220 is detectable by EPR.

Analytical Ultracentrifugation-Binding Stoichiometry of rhTF 220

The sedimentation equilibrium data for rhTF220 in the presence and absence of C12E8 can be analyzed as a single ideal sedimenting species. A typical sedimentation equilibrium experiment is shown in Fig. 4 for rhTF220 in the presence of 0.04% C12E8. The solid line is the result of fitting the data to a single ideal sedimenting species and the insert shows the residuals to the fit. The buoyant molecular weights of rhTF 220 in the presence and absence of C12E8 are presented in Table I with standard errors from determinations at two rotor speeds. The buoyant molecular weight and weight average molecular weights are essentially independent of the C12E8 concentration. This suggests that C12E8 micelles do not interact with rhTF 220 and confirms the determination of binding by EPR. Furthermore, the molecular weight from 6 analyses (two rotor speeds and three samples) is 25900 ± 1500 (standard error) which is in excellent agreement with the theoretical monomer molecular weight of 24800.

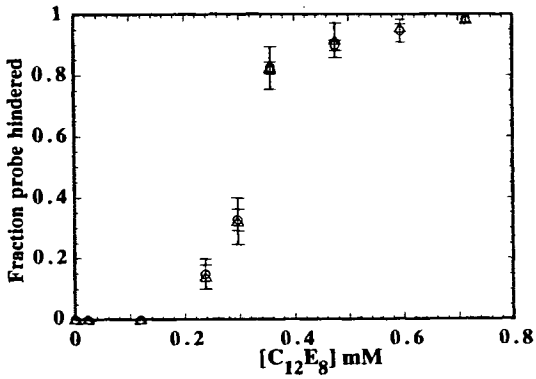

Fig. 3. EPR determination of probe partitioning of 16-doxyl stearic acid in the presence (○) and absence (△) of rhTF220 as a function of C12E8 concentration.

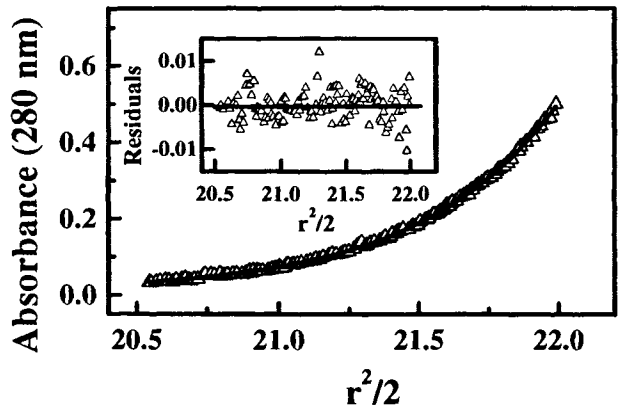

Fig. 4. Sedimentation equilibrium of rhTF220 in the presence of 0.04% C12E8 at 25000 rpm (Δ) and 10°C. The solid line through the data is the resulting non-linear least squares fit to a single ideal sedimenting species model. The inset shows the residuals to the fit.

DISCUSSION

The molecular weight (MW_c) of a complex comprised of N_p proteins and N_s surfactants can be expressed as:

$$MW_c = MW_p * N_p + MW_s * N_s, \quad (1)$$

where the subscripts c, p, and s are complex, protein, and surfactant, respectively. Using the AUC data, it is also possible to calculate MW_c by the following:

$$MW_c = MW_b/(1 - \rho\bar{v}_c), \quad (2)$$

where MW_b is the buoyant molecular weight of the complex, ρ is the solution density, and $\bar{v}_c$ is the partial specific volume of complex (4) which can be determined from the partial specific volume of protein and surfactant (4, 15):

$$\bar{v}_c = (\bar{v}_p + \delta_s\bar{v}_s)/(1 + \delta_s), \quad (3)$$

δ_s is the amount of surfactant bound to protein on a weight to weight basis and can be expressed as:

$$\delta_s = (MW_s * N_s)/(MW_p * N_p). \quad (4)$$

The above four equations were used to determine the binding stoichiometry of C12E8 by AUC, using an iterative technique, assuming 120 C12E8 monomers in a micelle, and only one micelle per complex (4). Thus, assuming that fractional micelles, complexes containing less than a value of 120 C12E8 molecules, were not allowed, a binding stoichiometry of 1.2 rhTF 243 molecules per C12E8 micelle was determined from the AUC data (4). The resulting average molecular weight of

Table I. Molecular Weights Determined by Sedimentation Equilibrium of rhTF220[a]

wt. % C12E8	Molecular weight
0	26200 ± 1300
0.01	25500 ± 1800
0.04	25900 ± 1300

[a] Each value is the result of the average ± SE of determinations on one sample at two rotor speeds.

Table II. Parameters Computed from AUC and EPR[a]

N_s/N_p	δ_s	$\bar{v}_c$	MW_c	N_s	N_p
50	0.98	0.849	74074	68.1	1.36
100	1.96	0.887	100421	124	1.24

[a] Solved using equations (1) to (4) and values of $\bar{v}_p$ = 0.736, MW_p = 27423, MW_s = 538.8, $\bar{v}_s$ = 0.964, ρ = 1.00579 and MW_b = 10822 as previously described (4).

the rhTF 243-C12E8 complex, 99300 is 3.6 times the protein molecular weight and 1.5 times the average molecular weight of a C12E8 micelle, 67000 (16). The computed molecular weight computed from the buoyant molecular weight is dependent on the partial specific volume of the complex (equation (2)) which in turn is dependent on δ_s (equation (4)) which yields the stoichiometry of the complex. Thus, there is not a closed form to the solution of these equations (i.e., 5 unknowns, N_p, N_s, MW_c, $\bar{v}_c$, and δ_s with 4 equations), and an iterative technique as outlined by Shire (4) must be used. However, it is possible that a protein surfactant mixed micelle could have a surfactant content with an aggregation number which differs from that of a pure surfactant micelle, and may not be well defined. The determination of N_s/N_p by EPR difference titration (Fig. 2) suggests a range of 50 to 100. This range further defines the limits of the AUC determinations. Thus setting N_s/N_p to 50 or 100 yields values of N_p, N_s, MW_c, $\bar{v}_c$, and δ_s by solving equations (1) through (4). The results for this computation using $\bar{v}_p$ = 0.736, MW_p = 27423, MW_s = 538.8, $\bar{v}_s$ = 0.964, ρ = 1.00579 and MW_b = 10822 (4) are shown in Table II. So although initially the possibility of the range of solutions existing was not considered, the previously reported result of a complex with a ratio of 1.2 moles of rhTF 243 per C12E8 micelle is supported by the EPR data. Interactions between rhTF 243 and C12E8 are detected using both techniques. Although the EPR yields an ill-defined binding stoichiometry, it is useful in narrowing the range of the possible solutions suggested by the AUC data. In particular, the EPR data strongly suggests that the majority of complexes do not contain more than one micelle since the EPR technique results in a ratio of N_s/N_p that is not greater than expected for one micelle of surfactant. The observed binding interaction between rhTF 243 and C12E8 is consistent with previously published rhTF 243 activity assays linking the presence of surfactant micelles and the improved ability of rhTF 243 to clot blood (4). From the previous study, the time required for rhTF 243 to clot blood is minimum at a surfactant concentration that coincides with the CMC of C12E8. Neither AUC or EPR detects interaction between rhTF 220 and C12E8. Because rhTF 220 lacks the transmembrane region, it appears that the transmembrane domain of tissue factor is necessary for the interaction with the surfactant micelle. We suspect the surfactant micelles provide an environment similar to that found in a lipid membrane; thus, in the absence of the interacting domain, no binding should occur.

CONCLUSIONS

AUC and EPR probe partitioning give complementary results when used to investigate rhTF-C12E8 interactions. Binding can be detected using either technique. However, the AUC technique does not always allow distinction between the possible physical characteristics of these interactions. EPR probe partitioning clarifies the ambiguity of the type of complex formed by decreasing the range of possible solutions.

Using both techniques to investigate the interactions between two truncated forms of rhTF and C12E8, the nature of the C12E8-rhTF 243 complex is elucidated. The data suggests that approximately 1.2 to 1.3 molecules of rhTF 243 form a complex with 1 to 0.6 C12E8 micelles (based on an aggregation number of 120). Neither the AUC nor the EPR data are precise enough to rule out the possibility that some micelles contain more than one tissue factor molecule. Nonetheless, although the buoyant molecular weights from 2 AUC determinations have an ~10% error, these data are consistent with the majority of the micelles containing one tissue factor molecule. Furthermore an average value of 1.2 TF molecules per micelle, assuming a surfactant aggregation number of 120, and no more than one micelle in the complex, suggests that no more than 25% of the species contain two Tissue Factor molecules. If complexes are present with more than two TF molecules per micelle then the amount of micelles with more than one TF molecule will decrease because of the greater contribution of those complexes to the weight average molecular weight. Only rhTF 243, and not rhTF 220, interacts with C12E8, indicating the transmembrane domain of rhTF is necessary for interactions with the surfactant molecules.

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