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EFFECT OF ANIONIC SURFACTANTS, NONIONIC SURFACTANTS AND NEUTRAL SALTS ON THE CONFORMATION OF SPIN-LABELED ERYTHROCYTE MEMBRANE PROTEINS

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SUMMARY

Erythrocyte membranes have been solubilized with anionic surfactants (sodium dodecyl sulfate and sodium deoxycholate), neutral salts (guanidine·HCl and KSCN), and a nonionic surfactant (Triton X-100). Conformational changes are observed by means of covalent attachment of *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)-maleimide to sulfhydryl groups of the membrane proteins.

Conformational changes are produced by the anionic surfactants and neutral salts and are correlated with solubilization. Sodium dodecyl sulfate produces a unique effect; the conformational change lags the solubilization, and the threshold of the conformational change coincides with the critical micelle concentration. At concentrations giving partial solubilization, the conformation of the unsolubilized protein in the membrane is essentially identical with the conformation of the solubilized proteins. Superimposed spectra for a given agent have isoslopic points and suggest that solubilization with a particular agent may be a two-state process. Sodium dodecyl sulfate shows a third state at high concentrations.

Conformational changes are not detected in protein solubilized by the nonionic surfactant.

INTRODUCTION

The erythrocyte membrane is widely studied both for its own sake and as a model of membrane structure in general. Although some 20–40% of the protein of the erythrocyte membrane can be solubilized by manipulation of ionic strength at neutral or near-neutral pH, the remainder requires a solubilizing agent. Solubilization of essentially the entire erythrocyte membrane has been accomplished by use of sodium dodecyl sulfate[1–3], sodium deoxycholate[4] and Triton X-100[5]; partial solubilization has been observed with urea[1,7], and neutral salts such as guanidine·HCl[6] and KSCN[8]. Solubilization has also been accomplished by a variety of organic solvents and acids[9,10], which will not be discussed here.

Dodecyl sulfate and guanidine are generally denaturing as far as enzymatic activity of membrane proteins is concerned[6,11,12] and deoxycholate probably

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inhibits activity[13] while Triton X-100 preserves activity[5]. KSCN is apparently inhibiting to enzymatic activity[8] but its effects are reversible. In all cases, the effect of the perturbing agent depends on its concentration.

A large number of physical and chemical techniques have been used in the study of the interaction of these agents with the red cell membrane. The techniques used have varied from study to study, so that direct comparison among agents is difficult. A recent study using the spin label technique[1] has compared the effects of a number of these agents.

Previous work has focused on the structure of the protein at the particular concentration which solubilizes the protein or membrane. In this paper the effect of anionic and nonionic surfactants and neutral salts on solubilization and structure of membrane proteins of human erythrocyte membranes is studied as a function of concentration. The spin label technique[14,15] is used to monitor the conformational changes of the protein during solubilization. We have found that different solubilizing agents differ markedly in their effect on protein structure and in the relation between conformational changes and solubilization.

MATERIALS AND METHODS

Erythrocyte ghosts were prepared by the method of Dodge *et al.*[16] from outdated blood donated by the Spokane Regional Blood Bank. After hemoglobin was removed, the ghosts were suspended in 50 mM NaCl–5 mM phosphate buffer, pH 7.5, to maintain membrane stability. Spin label (approx. 1 mg/80 mg protein) was dissolved in approx. 0.05 ml of acetonitrile, diluted with buffered saline, and added to the ghost suspension. After incubation overnight at 4 °C the suspension was washed with buffered saline until spin label was no longer present in the supernatant (usually five washes, 10 vol. per wash). Labeled cells were used within three days.

The maleimide spin label *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)maleimide was synthesized by the method of Seidel *et al.*[17]. It was verified by the procedure of Sandberg *et al.*[18] that only sulfhydryl groups were labeled.

Protein was measured by the modified biuret method of Jacobs *et al.*[19]. Hemoglobin was determined by a pyridine hemochrome method[20] using $A_{557\,nm}=34.4$ $mM^{-1}\cdot cm^{-1}$ (ref. 21). All preparations used had a hemoglobin content of less than 0.5% of the total protein.

Membranes were treated with surfactant or neutral salt by addition of one volume of the solubilizing agent to one volume of a packed membrane suspension whose protein concentration was approx. 5 mg/ml. All reagents were at room temperature and pH 7.4. Before treatment with Triton X-100, the membranes were washed with 2 mM phosphate buffer, pH 7.4, to increase the solubilization[5]. The membrane suspensions were exposed to the solubilizing agents for 30 min at 20–24 °C. The protein which remains in the supernatant after centrifugation at $140000\times g$ for 90 min at 4 °C is defined to be soluble. Protein solubilization induced by dodecyl sulfate, deoxycholate, Triton X-100, and KSCN was measured as percent of initial protein which is present in the supernatant. Protein solubilization induced by guanidine·HCl was measured as percent of initial protein which remains in the pellet after it was washed 3 times with 20 vol. of 0.05 M NaCl to remove guanidine. Guanidine interferes with both the biuret and Folin assays at low and moderate concentrations, respectively.

Electron paramagnetic resonance spectra were recorded on a Varian E-9 spectrometer in standard aqueous cells at room temperature (20–24 °C). Experiments were conducted to find optimum instrument settings for microwave power and modulation amplitude. The following settings did not produce power saturation or line broadening: modulation amplitude, 3.2 G; microwave power, 50 mW and receiver gain, 320–1600.

The sources of the solubilizing agents are: Triton X-100, Sigma; sodium deoxycholate, Sigma; guanidine·HCl, Sigma and Mann; sodium dodecyl sulfate, Mann; and KSCN, Allied Chemicals.

RESULTS

The covalent attachment of the maleimide spin label to erythrocyte membranes yields EPR spectra which are identical to previous reports [18,22]. *N*-Ethylmaleimide, a sulfhydryl reagent similar to the maleimide spin label, labels essentially all of the erythrocyte membrane proteins[23,24] and blocks the attachment of the maleimide spin label to erythrocyte membrane proteins[1,18]. The EPR spectra show at least two different correlation times (corresponding to strongly immobilized and weakly immobilized probes) and suggest that the spin label occupies several types of sites, each with a different steric interference for rotational tumbling.

This experimental system is exposed to increasing concentrations of solubilizing agents. The line shape of the EPR spectrum of spin-labeled erythrocyte membranes depends on the concentration of dodecyl sulfate, deoxycholate, guanidine·HCl, and KSCN and is essentially independent of the concentration of Triton X-100. In Fig. 1A the relative signal height measured peak-to-trough of the $I = +1$ isotropic transition is plotted (see Stone *et al.*[25] for a similar analysis of EPR spectra) as a function of the concentration of solubilizing agent. Since the concentration of membrane protein is the same for each measurement, the relative signal height is proportional to the amount of weakly immobilized spin label and to the correlation time of the spin label. The maximum increase due only to a change in correlation time is less than a factor of approx. 2.3 which predicts a maximum relative signal height of 1.74. The observed maximum relative signal heights are: sodium dodecyl sulfate 2.4; sodium deoxycholate, 2.2; guanidine·HCl, 4.5; and KSCN, 1.2. The majority of the changes exceed the predicted maximum and suggest that the increase in relative signal height is due to the conversion of strongly immobilized sites to weakly immobilized sites. This observation is in agreement with the decrease in the amount of strongly immobilized probe.

The threshold concentration required to change the relative signal height is approx. 1.5 mM for anionic surfactants and is approx. 100 mM for the neutral salts. In the case of dodecyl sulfate, a second change occurs and the relative signal height decreases above 20 mM sodium dodecyl sulfate. A striking difference occurs with a nonionic surfactant; the signal height is independent of the concentration of Triton X-100.

The protein solubilization is shown as a function of concentration of solubilizing agent in Fig. 1B. Complete solubilization of membrane protein is achieved with dodecyl sulfate and deoxycholate. In contrast to the behavior of the anionic surfactant, neutral salts do not produce complete solubilization, and the concentration dependence

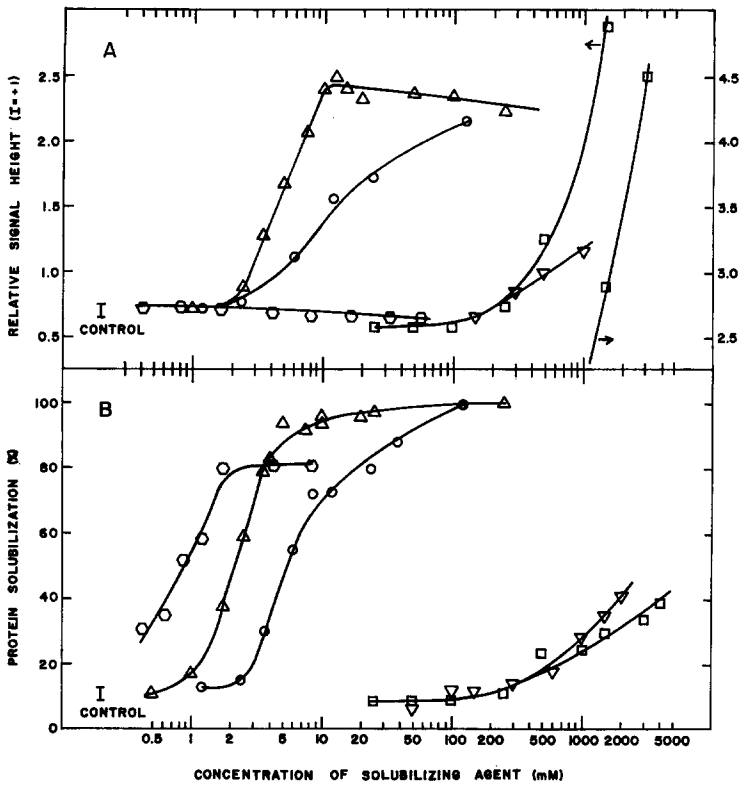

Fig. 1. Comparison of protein solubilization and conformational changes in spin-labeled erythrocyte ghosts as a function of the concentration of the solubilizing agent. The line shape changes in the EPR spectrum of the spin label is a function of the conformational change. The relative signal height is the peak-to-trough height of the $I = +1$ isotropic transition in the first derivative display normalized to unit gain. The protein solubilization is measured as the percent of initial protein which is present in the supernatant after centrifugation for 90 min at 140000 $\times g$ and at 4 °C. △, dodecyl sulfate; ⬡, Triton X-100; ○, deoxycholate; □, guanidine; ▽, KSCN. The bars, labeled "control", indicate the initial value in the absence of the solubilizing agent. The concentration of Triton X-100 was determined spectrophotometrically[40]. The protein concentration is about 5 mg mg/ml. The curves are drawn by eye.

of solubilization by both guanidine and KSCN are similar. Miller[5] obtained complete solubilization of erythrocyte membranes with Triton X-100 when the salt concentration was 5 mosM*. Since 12% of total membrane protein was lost during the first wash with 2 mM phosphate, the membranes were used without additional washing and hence had a final salt concentration of about 2 mM phosphate and 5 mM NaCl. This undoubtedly accounts for the maximum of 80% solubilization obtained. If Triton was added to membranes in 50 mM NaCl, as used for the other agents, then only about 35% solubilization was obtained; however, the maximum was reached at the same surfactant concentration, approx. 2 mM. Addition of 1 mM EDTA to the latter system decreased maximal solubilization, as described by Miller[5];

* The ideal milliosmolarity (mosM) was calculated by adding the concentrations of all ionizable species in the solution, neglecting deviation of the salts from ideal behavior.

however, it also resulted in a striking optical clearing, both of the supernatant and of the pellet resulting from centrifugation.

Concentrations of Triton X-100 above 49 mM showed an additional effect, in which intensity of the spin label signal decreased with time. This effect could be prevented by blocking residual membrane sulfhydryls with *N*-ethylmaleimide, and was presumably caused by a chemical reduction of the spin labels by the sulfhydryls[26]. The rate was sufficiently slow to be negligible (approx. 10% reduction over 24 h), but this effect could conceivably account for the slight decrease in weakly immobilized label seen with Triton X-100.

It should be noted that at the lowest concentration of Triton X-100 shown, 0.42 mM, there is significant solubilization of protein. While some of this solubilization may be due to the low ionic strength, it seems clear that the use of Triton X-100 as a lysing agent for red cells should be treated with caution.

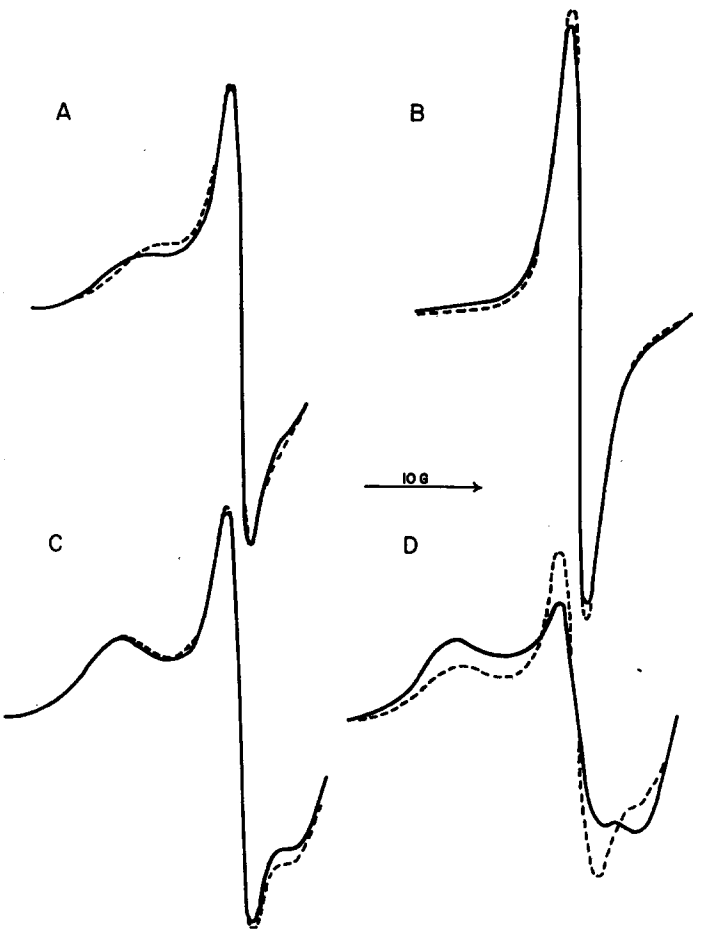

Fig. 2. Comparison of the EPR spectrum (low field region) of (——) supernatant and (– – –) pellet obtained from partially solubilized, spin-labeled erythrocyte membranes. The solubilizing agents are: A, 5 mM dodecyl sulfate; B, 3 M guanidine·HCl; C, 4.8 mM deoxycholate; D, 0.83 mM Triton X-100.

In Fig. 1, one can compare, simultaneously, the effect of concentration of solubilizing agent on both the relative signal height of the spin label and the solubilization of membrane protein. In the case of the neutral salts, guanidine·HCl and KSCN, the threshold for both events is the same. The anionic surfactants do not have a common behavior. Deoxycholate has a common threshold for both solubilization and conformationl change, while dodecyl sulfate reaches about 50% solubilization before the threshold of the conformational change is reached. In contrast, the nonionic surfactant, Triton X-100, solubilizes the membrane protein but does not appreciably change the relative signal height. Thus deoxycholate, guanidine·HCl, and KSCN have a parallel behavior between changes of relative signal height and solubilization; dodecyl sulfate solubilizes prior to a change in the relative signal height; and Triton X-100 solubilizes without a conformational change.

The dependence of relative signal height on concentration of solubilizing agent involves conformational changes both in the soluble protein and in the protein remaining in the membrane. Typical ESR spectra of the pellets and supernatants of centrifuged membrane suspensions which contained a mixture of unsolubilized and solubilized protein are shown in Fig. 2. They show that for dodecyl sulfate, deoxycholate and guanidine there is no significant difference between the EPR spectra of solubilized and unsolubilized proteins, and that the spectra of both are quite different from the spectra of untreated membranes. The proteins solubilized by 0.8 mM Triton X-100 have a significantly different EPR spectrum which shows an increased proportion of weakly immobilized spin label, when compared to that of the unsolubilized proteins. Since Triton X-100 did not produce a net conformational change, the data suggests that selective solubilization may initially separate proteins which have an increased proportion of weakly immobilized sites over strongly immobilized sites, compared to the membrane average. Dodecyl sulfate, deoxycholate, and guanidine·HCl produce conformational changes in both solubilized and unsolubilized proteins so that selective solubilization cannot be detected.

Since the conformational changes produced by dodecyl sulfate, deoxycholate, and guanidine·HCl are present in both soluble protein and protein in the membrane, a question arises about the number of conformational states which are present. In Fig. 3A, the superimposed spectra of spin-labeled membrane suspensions containing different sodium dodecyl sulphate concentrations have isoslopic points*. The existence of isoslopic points suggests that the spectral changes arise by conversion between two conformational states[27]. Comparable isoslopic points are observed for the other solubilizing agents with the exception of Triton X-100. The location of these isoslopic points is shown in Fig. 3B. Two sets of isoslopic points are observed for dodecyl sulfate; the low range occurs between 2.5 mM to 15 mM dodecyl sulfate, and the high range occurs between 10 mM and 250 mM dodecyl sulfate. The neutral salts, guanidine and KSCN, have isoslopic points at the same location. Deoxycholate has an isoslopic point near that of the low dodecyl sulfate point. The isoslopic points are different for each solubilizing agent and cluster into groups that correlate with the nature of the solubilizing agent. This may imply that each class of solubilizing agent solubilizes by a different mechanism.

* Isoslopic points are analogous to isosbestic points but the term is defined for the EPR spectrum which is a first derivative display of the absorption spectrum.

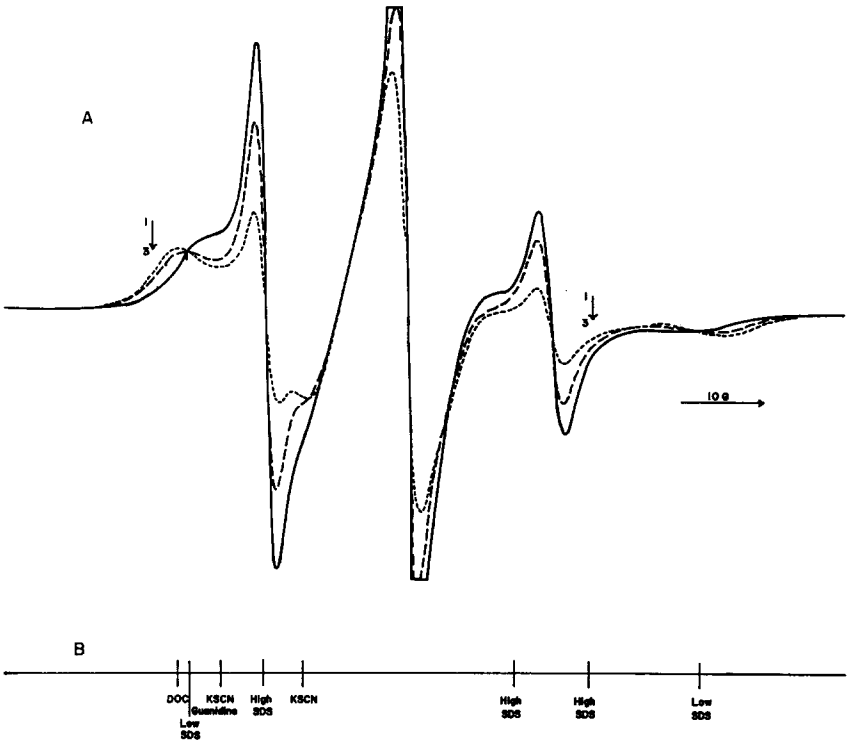

Fig. 3. Isoslopic points in the superimposed EPR spectra of spin-labeled erythrocyte membranes as a function of concentration of the solubilizing agent. In A, the amount of spin-labeled membranes and the control settings on the EPR spectrometer remain constant as the dodecyl sulfate concentrations range from 2.5 mM to 15 mM. The dodecyl sulfate concentrations are: Spectrum 1, 2.5 mM; Spectrum 2, 5 mM; and Spectrum 3, 15 mM. This concentration range of dodecyl sulfate is denoted as low SDS. At higher concentrations, another set of isoslopic points are observed (high SDS). In B the locations of isoslopic points are shown for deoxycholate (DOC), guanidine·HCl, KSCN, and dodecyl sulfate (high concentration range). The concentration ranges are: deoxycholate, 2.4 mM to 48 mM; guanidine·HCl, 1M to 3 M; KSCN, 0.1 M to 1.0 M; high dodecyl sulfate, 10 mM to 250 mM. The magnetic field axis is alligned with the spectra in A.

DISCUSSION

Sulfhydryl groups occur in all molecular weight classes of membrane proteins[23,24] and are probably distributed in all regions of the membrane occupied by protein[18]. The spin label probe detects conformational changes at all sites to which it is attached and is probably attached to all classes of membrane proteins. This assumption is supported by the presence of spin label in both the pellet and supernatant at partial solubilization by all surface active agents. In the case of Triton X-100 where no net conformational change occurred, addition of spectra of supernatant and pellet weighted by the protein percentage generated the spectrum of the suspension. This observation suggests that the amount of spin label is proportional to the amount of protein. Hence, the effects reported are certainly not due to changes in only a small fraction of the membrane proteins.

The relation between conformational change and solubilization is different for deoxycholate and dodecyl sulfate. Whereas the solubilization and conformational change as a function of deoxycholate concentration have coincident thresholds and similar monotonic shape, the solubilization and conformational change as a function of dodecyl sulfate concentration have different thresholds. The fortunate occurrence of this difference provides insight into the possible mechanism of the conformational change.

Several explanations can be advanced for the lag of conformational change behind solubilization by dodecyl sulfate. (1) Selective solubilization of membrane protein which has a small amount of spin label attached would limit detection of conformational change. However, the threshold for the conformational change is reached when approx. 50% of the protein is solubilized. The distribution of sulfhydryl groups among the classes of membrane proteins would disfavor the first explanation. (2) Bound phospholipid may protect the solubilized protein from conformational change. The threshold of the conformational change coincides with the critical micelle concentration of dodecyl sulfate. It is observed in our laboratory and reported by others[4] that surfactant micelles efficiently remove fatty acids bound to proteins. Thus it appears that, above the critical micelle concentration, micelles of dodecyl sulfate may remove some lipid from the protein[4,11]. Since the conformational change induced by dodecyl sulfate is the same in both the pellet and the supernatant at partial solubilization, it is necessary that the phospholipids are removed from both protein in the pellet and in the supernatant.

In the case of deoxycholate, two possible mechanisms can be advanced. (1) Deoxycholate binds to the protein and induces a conformational change. This mechanism would require that deoxycholate can reach the necessary binding sites of all proteins. Philippot[4] was unable to detect the binding of deoxycholate to membrane proteins while Rudman and Kendall[28] report the binding of approximately 2 moles deoxycholate per mole protein. (2) Alternatively micelles of deoxycholate may remove phospholipid essential for stability of the protein conformation. Lipids are readily incorporated into micelles of deoxycholate[4,29]. The critical micelle concentration of deoxycholate is comparable to the threshold for solubilization and conformational change[9,30]. Since the conformation of the protein in the membrane pellet is changed to the same extent as the soluble protein (see also Schneider and Smith[1]), the conformational change is not due to solubilization. This mechanism requires that phospholipid is removed both from the protein in the pellet and in the supernatant. Our results favor the second alternative and suggest that the mechanism of both anionic surfactants is similar. Since the topographical distribution of proteins is not known, cooperativity may exist to transmit conformational changes from surface proteins to interior proteins.

Unlike deoxycholate, dodecyl sulfate produces two distinguishable conformation changes. When the monomer concentration of dodecyl sulfate exceeds 0.8 mM, membrane proteins bind approx. 1.4 g dodecyl sulfate/g protein to form a saturated complex[2,3]. The secondary structure of this saturated complex is uncertain but ORD spectra and viscosity measurements suggest that the conformation has changed[31]. The threshold of the second conformational change observed by EPR exceeds the minimum concentration required to form the saturated complex. The EPR spectrum exhibits an intermediate immobilization in agreement with studies of other dodecyl

sulfate–protein complexes in our laboratory. The amount of intermediate immobilization appears to depend on the total dodecyl sulfate concentration. The existence of the unique isoslopic points for this second conformational change suggests a three-step conversion from the conformation of the native protein.

In contrast to the anionic surfactants, neutral salts do not solubilize the most hydrophobic membrane proteins, which are strongly associated with lipid[6,7,32]. Hence it is important to note that the unsolubilized protein remaining in the membrane undergoes the same conformational changes as those of the solubilized protein. Soluble proteins exposed to neutral salt solutions in the 1 M concentration range undergo conformational changes[33]. These conformational changes are explained by the dependence of solubility of amino acid residues on the salt concentration. The conformational changes observed in the membrane proteins may be due to this same mechanism. Since the topographical distribution of membrane protein is unknown, the data does not distinguish between the possibility of conformational changes transmitted by intermolecular interactions between surface proteins which are solvated and interior proteins or the possibility that a critical region of all proteins are solvated. The latter alternative is suported by the observation that the class of proteins not solubilized by neutral salts can be labeled by non-penetrating protein reagents[32].

The difference between anionic and nonionic surfactants is illustrated by the solubilization of acetylcholinesterase from erythrocyte membranes in the active form with Triton X-100 and in the denatured form with dodecyl sulfate[5,34,35]. The effect of Triton X-100 on spin-labeled erythrocyte membranes correlates with the preservation of enzymatic activity. The nature of the interaction of nonionic surfactants with membrane proteins is not understood. Triton X-100 remains bound to membrane protein after dialysis or gel filtration[5]. While the displacement of phospholipids from proteins solubilized by Triton X-100 has been observed[36], it is not known if the presence of phospholipid is necessary to stabilize the protein conformation. Lipid-free cytochrome oxidase preparations have been obtained with nonionic surfactants[37,38], and the absence of conformational change noted[39].

This laboratory is studying the interaction of nonionic surfactants with proteins in order to elucidate the mechanism of this interaction.

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