Analysis of Noncovalent Bonding in *Mycoplasma* Membranes. Kinetics of Solubilization in Sodium Dodecyl Sulfate and Lithium Diiodosalicylate Solutions†

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ABSTRACT: The kinetics of solubilization of aqueous suspensions of *Acholeplasma laidlawii* membranes have been investigated by light scattering in a stopped-flow apparatus. Sodium dodecyl sulfate and lithium diiodosalicylate were used as solubilizers. In the range 16–30° the rate constant for solubilization by lithium diiodosalicylate decreased with increasing temperature. No such anomaly was found when sodium dodecyl sulfate was employed as a solubilizing agent. Above 33° the lithium diiodosalicylate rate constant sharply increased with temperature. A full set of activation parameters were reported, but most significant was the finding that in the 16–30° temperature range the entropy of activation amounts to −108 eu. Sedimentation and diffusion coefficients for bacitracin, bovine serum albumin, ovalbumin, and myoglobin indicated that these proteins are polymerized in lithium diiodosalicylate solution. Their intrinsic viscosities were also much higher than in pure water or dilute salt solution. A positive correlation between high helical content and individual protein solubility in lithium diiodosalicylate was also noted. These data were discussed relative to noncovalent bonding in membranes.

We have been concerned with the analysis of noncovalent bonding in biological membrane structure by means of chemical kinetics. In this connection, the following interesting facts were found in mycoplasmal membrane solubilization studies using the aliphatic, anionic detergent, sodium dodecyl sulfate (Auborn *et al.*, 1971). The solubilization process exhibited an appreciable activation energy (~10 kcal/mol). Moreover, micelles of sodium dodecyl sulfate combined directly with the membranes (without dissociation into monomers) with approximately the same activation energy as did monomers. Light-scattering studies revealed that the room temperature disaggregation process was not complete, but that small aggregates averaging about 70,000 molecular weight remained. These aggregates could be further dissociated by heat treatment (boiling for 5–10 min) to about half the above average molecular weight. Relative to bonding in membranes, then, it would be of interest to learn more about the sodium dodecyl sulfate–membrane-activated complex and to find out something concerning the noncovalent bonds that resisted complete disaggregation in sodium dodecyl sulfate solution.

In this paper we have continued the sodium dodecyl sulfate studies but have concentrated primarily on the compound LiI_2sal.[1] This compound has been important for use in membrane solubilization since the report of Marchesi and Andrews (1971) that LiI_2sal could be utilized during the isolation of a glycopeptide fraction from red cells. Robinson and Jencks (1965) had previously reported that LiI_2sal is an effective peptide solubilizing agent. It is unlikely that LiI_2sal could function as a strong detergent as does sodium dodecyl sulfate, and its aromatic character would suggest that its mode of entry into the membrane would be quite different. It therefore becomes interesting to compare the activated complex formed by LiI_2sal with that of sodium dodecyl sulfate during the solubilization of membranes and to study the products of LiI_2sal solubilization as we have previously those of sodium dodecyl sulfate.

Materials and Methods

Membranes from *Acholeplasma laidlawii*[2] (strain A) were prepared by osmotic lysis (Choules and Bjorklund, 1970). Washed membranes were suspended in redistilled, demineralized water and protein concentrations were determined spectrophotometrically following dilution of aliquots in 2-chloroethanol (Choules and Bjorklund, 1970). The pH of a 1-mg/ml aqueous membrane suspension was typically 7.2.

To prepare membrane lipids, membranes were acetone extracted three times, and extracts were combined and dried under nitrogen. This treatment removes more than 90% of the lipids (J. Maniloff, private communication). To prepare lipid suspensions, mixtures of lipid and water were sonicated with a Bronson Sonifer Model 875, until suspension was complete, then diluted to 1 mg/ml for use in kinetic studies. Protein suspensions were made simply by washing the wet acetone precipitate several times with water and finally, stirring into water suspension.

Reagents. 3,5-Diidosalicylic acid was obtained from Aldrich Chemical Co. and was twice recrystallized from methanol. The lithium salt of this acid (LiI_2sal) was prepared by titration with aqueous lithium hydroxide solution and then recrystallized from water. Typically, a 0.2 M solution of the LiI_2sal so prepared had a pH of 7.7.

Sodium dodecyl sulfate (99% pure) was obtained from Sigma Chemical Co. and was used without further purification (Auborn *et al.*, 1971). A 100 mM solution had a pH of 9.2. These sodium dodecyl sulfate and LiI_2sal solutions were filtered through 0.2 μ pore size Sartorious filters.

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[1] Abbreviations used are: LiI_2sal, lithium diiodosalicylate; cmc, critical micelle concentration.

[2] Formerly called *Mycoplasma laidlawii* (strain A).

TABLE I: Sedimentation and Diffusion Coefficients for Proteins in 0.2 M LiI_2sal at 20°.

Protein	Sp Vol (cm^3/g) in LiI_2sal	Diffusion Coef, D (sec^{-1}), 10^{-7}		Sedimentation Coef s (sec^{-1}), 10^{-13}		Mol Wt in LiI_2sal[b]	Mol Wt in H_2O[a]
		In LiI_2sal	In H_2O	In LiI_2sal	In H_2O[a]		
Bacitracin	0.689	4.9		2.57		45,600	1,450
Bovine serum albumin	0.735	2.17	6.0 (7.4)[c]	6.8	4.22–4.73 (5.0)[c]	330,300	69,000
Myoglobin	0.74	2.34	10.3	5.9	2.01	273,900	17,800
Ovalbumin							
Band 1	0.740	1.94	7.8	5.7	3.55–3.66	314,300	43,000
Band 2				7.8			
Band 3				12.8			

[a] Molecular weights, sedimentation coefficients, and diffusion coefficients in water obtained from Sober (1968). [b] Calculated from the relation $M = RTS/[D(1 - \bar{V}\rho)]$. [c] Confirmatory measurement made in water in our laboratory to verify the accuracy of our measurements in LiI_2sal.

Proteins. Ovalbumin and bacitracin were obtained from Schwarz/Mann, bovine serum albumin was obtained from Sigma Chemical Co., and myoglobin from Calbiochem. Weighed amounts of protein were dissolved in 0.2 M LiI_2sal (pH 7.5) and 0.2% dithiothreitol from Sigma Chemical Co., and then heat-treated for 5 min in boiling water (~96°).

Kinetic Experiments. Mixing experiments were performed in a Durrum-Gibson stopped-flow spectrophotometer equipped with a fluorescence attachment as described by Auborn *et al.* (1971). With this equipment it was possible to rapidly (within 5 msec) mix membrane suspension with an equal volume of either LiI_2sal or sodium dodecyl sulfate solution. Membrane solubilization resulted in a decrease in scattered white light intensity. This was followed by means of the fluorescence attachment coupled with an oscilloscope. Correction was made for the large refractive index contribution of the LiI_2sal in solution.

Calculations. Initial reaction velocities were derived from the extrapolated initial down slopes of the oscilloscope traces (see Figure 1). The rate constants were calculated from the following relation

$$\frac{-dc}{dt} = k_r[\text{membrane}]^a[LiI_2sal]^b \quad (1)$$

where c = concentration of membrane aggregate measured by light scattering, t = time, k_r = experimental rate constant, and superscripts a and b are the respective reaction orders (also experimentally determined).

Activation energies E^* were obtained from the equation

$$E^* = RT^2\frac{d \ln k_r}{dT} \quad (2)$$

where R = gas constant and T = absolute temperature.

Ultracentrifuge Experiments. Sedimentation velocity experiments were performed with a Beckman Model E analytical ultracentrifuge equipped with schlieren optics. Protein (2–10 mg/ml) in 0.2 M LiI_2sal was placed in a sample cell equipped with a double-sector centerpiece and sapphire windows. Samples were then centrifuged in an An-D rotor at 51,000 rpm at 20°. Diffusion coefficients in LiI_2sal were determined by synthetic boundary sediementation (Bowen, 1970; Chervenka, 1970). The accuracy of both the sedimentation and diffusion coefficients was verified by running bovine serum albumin in dilute aqueous buffer as noted in Table I. Partial specific volumes of the respective proteins were determined through the use of a 5-ml pycnometer. Three to five trials were performed for each protein, and a precision of ±0.5 μl was obtained.

Viscometry. For viscosity measurements, five Cannon–Fenske (1944) semimicro viscometers were employed, each with a separate timer. Each viscometer had approximately a 1-ml sample volume and a flow time between 220 and 300 sec with water. Flow capillaries were 0.013-mm i.d. and were obtained from Wilmad Glass Co., Inc., Buena, N. J. The viscometers were suspended in a circular water bath by means of vertical slotted mounts which kept them rigidly in the same position for each experiment. The bath was maintained at 25 ± 0.02°. The viscometers were calibrated with demineralized, distilled water that was further filtered through a carbon absorption column to remove organic material. Flow times were repeated four times with each viscometer, then the viscometer was cleaned in chromic acid cleaning solution, rinsed with dilute ammonia, demineralized distilled water, and then alcohol, and dried by suction. This process (flow time measurement and washing procedure) was repeated four times with each sample solution so that each data point reported here represents an average derived from 16 measurements.

Specific viscosity η_{sp} is calculated from the following relation

$$\eta_{sp} = \frac{\eta' - \eta}{\eta} \quad (3)$$

where η' = viscosity of the protein solution and η = viscosity of the solvent alone.

Intrinsic viscosity $[\eta]$ is independent of concentration c since it is determined from the following relations.

$$[\eta] = \lim_{c\to 0}\frac{\eta_{sp}}{c} = \lim_{c\to 0}\frac{\eta' - \eta}{\eta c} \quad (4)$$

Results

Stopped-Flow Data. The light-scattering intensity of membranes in water exhibited a linear concentration dependence in the range 0–1 mg/ml. Membrane solutions remained visibly turbid after treatment with LiI_2sal, but the light scattering in-

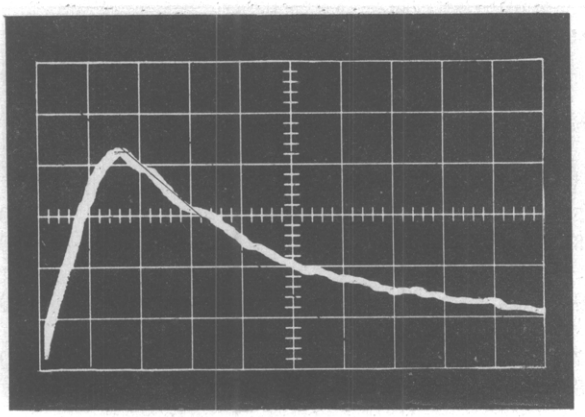

FIGURE 1: Light-scattering stopped-flow experiment. Equal volumes of 1 mg/ml of *A. laidlawii* membranes with 0.4 M LiI_2sal in water at 25°, pH 7.8. Vertical scale: 500 mV/division. Horizontal scale: 5 msec/division. Two successive experiments are shown superimposed. Initial slopes were taken from the descending portion of the curve just to the right of the maximum which occurs at about 8 msec.

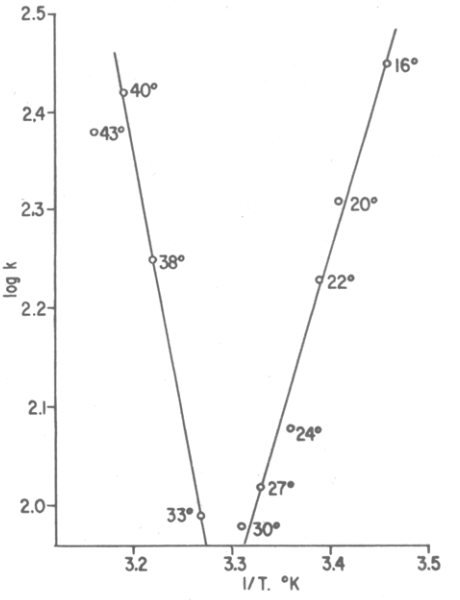

FIGURE 2: Plot of the logarithm to the base 10 of the mycoplasmal membrane solubilization initial rate constant in the presence of LiI_2sal (measured by the light-scattering stopped-flow technique), versus reciprocal absolute temperature of the aqueous sample suspension.

tensity in the stopped-flow apparatus was insignificant. A typical oscilloscope trace is shown in Figure 1.

Kinetics. With reference to LiI_2sal solubilization of mycoplasmal membranes we obtained the striking results shown in Figure 2. In the range 16–30° the rate constant decreases with increasing temperature. We had previously found no such anomaly when sodium dodecyl sulfate was employed as a solubilizing agent. A sharp reversal is seen between 30 and 33° and the rate constant is observed to increase in the range 33–40°. Above 40° membrane denaturation interfered with the light-scattering measurements. The reaction order with respect to membranes was ~1.5 and with respect to LiI_2sal ~2. These reaction orders did not change significantly with temperature.

This experiment has been repeated several times with similar results each time. Neither the membranes alone nor the LiI_2sal solutions alone produce relaxation curves when diluted with water in the stopped-flow apparatus in the temperature range 16–40°. Polystyrene particles also gave no relaxation effects with LiI_2sal solutions in this temperature range. The results were not affected by the age of the LiI_2sal solutions nor by the age of the membranes, although membrane preparations were always discarded after 4–5 days of storage in the cold. There are no endopeptidases in *Mycoplasma* membrane preparations (Choules and Gray, 1971).

Experiments were also done to determine the effect of pH on the initial rate of disappearance of membrane turbidity in LiI_2sal. In the pH range 7.0–10.0, the initial rate was the same within experimental error (Table II). Since all kinetic experiments were performed well within this pH range it is unlikely that the unusual kinetic data were due to small changes in pH with temperature.

Activation Parameters. A clarification of the kinetic results can be obtained from a careful study of Table III. The entropy term is expressed as $-T\Delta S$ so as to clearly show the magnitude of its contribution to the free energy. (Note the extremely high, unfavorable entropy contribution to the free energy of activation in the case of LIS between 16 and 30°.)

The positive entropy contribution abruptly reverses between 30 and 33° as does the negative enthalpy contribution. These results are highly suggestive of a phase change either in the water surrounding the activated site or within the membrane itself. This will be discussed later.

The kinetic data with sodium dodecyl sulfate (Table III) are more normal. Both the entropy and enthalpy terms make positive contributions to the free energy of activation. The terms are not much different for sodium dodecyl sulfate concentra-

TABLE II: pH Dependence of Initial Rate of Membrane Solubilization by LiI_2sal at 25.6°.

pH	dc/dt
7.0	6.1
7.5	6.87
8.0	7.25
8.5	5.01
9.0	6.24
10.0	6.02

TABLE III: Activation Parameters Obtained from the Temperature Dependence of Membrane Solubilization Rates.

	Sodium Dodecyl Sulfate		LiI_2sal	
	Below cmc	Above cmc	16–30°	33–44°
ΔG^* (kcal)[a]	+16.9	+18.8	+14.5	+14.3
ΔH^* (kcal)[b]	+9.4	+9.6	−18.0	+24.1
ΔS^* (eu)[c]	−25.3	−30.7	−108.0	+32.5
$-T\Delta S^*$ (kcal)	+7.5	+9.2	+32.5	−9.8

[a] Calculated from $\Delta G^* = -RT \ln K^* = -RT \ln [(h/kT)k_r]$, where $k_r \equiv$ experimental rate constant and the other symbols have their usual meaning. [b] $\Delta H^* = E^* - RT$, where $E^* =$ experimental activation energy. [c] $\Delta S^* = (\Delta H^* - \Delta G^*)/T$.

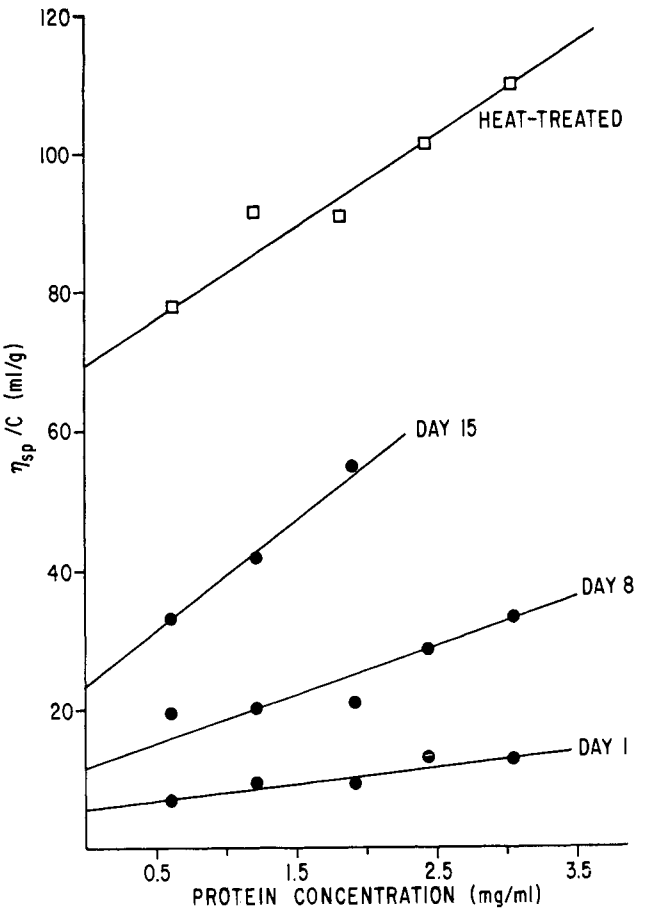

FIGURE 3: Plot of reduced viscosity versus protein concentration for ovalbumin in 0.2 M LiI_2sal including 0.2% dithiothreitol (pH 7.6). Dark circles refer to unheated ovalbumin at various times following LiI_2sal treatment. Heat treatment was for 5 min at boiling temperature (96°).

tions above and below the critical micelle concentration (cmc) although there may be a small unfavorable entropy contribution due to sodium dodecyl sulfate micelle formation above the cmc. It is important to note that these ΔG^* values for solubilization by sodium dodecyl sulfate (Table III) are relatively large for a physical process.

Kinetic Studies with Separated Lipid and Protein from Membranes. It was desirable to know whether the anomalous kinetic behavior was attributable to attack by LiI_2sal on the protein or lipid portion of the *A. laidlawii* membrane. Accordingly stopped-flow experiments were performed with suspensions of separated membrane lipid and protein, respectively.

A better than 90% separation of the lipid and protein (as mentioned previously in the experimental section) was effected by acetone extraction. During the subsequent stopped-flow kinetic experiments the acetone extracted protein behaved qualitatively the same as did the native membranes with a somewhat lower rate of solubilization but similar temperature dependence.

Under similar conditions the extracted, resuspended lipid behaved differently than the protein fraction: apparently mechanical agitation during the rapid mixing broke up lipid aggregates into smaller micelles so as to give a reduction in light scattering capacity. On standing the lipid reaggregated so that after about 10 sec the intensity of scattered light returned to the starting value. The temporary lipid disaggregation also showed a positive temperature dependence in the range in which native membrane exhibited a negative dependence.

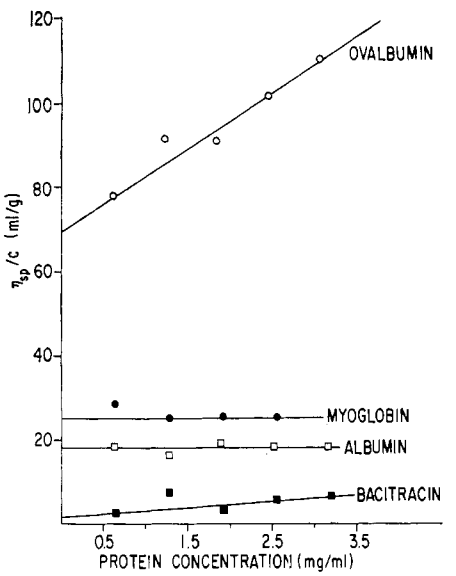

FIGURE 4: Plot of reduced viscosity versus protein concentration for various proteins dissolved in 0.2 M LiI_2sal including 0.2% dithiothreitol (pH 7.6). The mixtures were heat treated for 5 min at boiling temperature.

These stopped-flow experiments suggested the possibility that the lipid fraction, but not the protein fraction, was insoluble in the LiI_2sal solutions in the concentration range tested (up to 0.6 M LiI_2sal). Further evidence of this was obtained in another experiment in which membranes were dissolved in 0.2 M LiI_2sal at pH 7.4 and then centrifuged at 100,000*g* for 2 hr. By amino acid analysis about 94% of the protein remained in solution, but by spectral analysis over 90% of the carotenoid pigment was found in the clear, yellow sedimented material. A similar experiment involving ^{32}P labeling of membranes was ambiguous because a considerable portion (~50%) of the ^{32}P went into nucleic acid fragments associated with the membranes.

Viscosity Studies. When ovalbumin was dissolved in LiI_2sal the intrinsic viscosity continued to increase as a function of time (Figure 3). After heat treatment in boiling water (96°) the viscosity reached a higher stable value. Thus, it appeared that the heat treatment made it possible to attain a limiting value of the viscosity. Accordingly, the remaining proteins were heat treated before viscosity measurement.

Globular proteins in water are characterized by intrinsic viscosities in the range 3.3–4.0 (Tanford, 1961). Thus, it appeared (Figure 4) that most of the proteins tested were in an extended configuration or were polymerized. As we will show below in considering our analytical ultracentrifugation results there are strong indications that the proteins formed long linear polymers. We would not expect small aggregate formation (dimers, etc.) to greatly increase the intrinsic viscosity. This is considered in the next section also. In any case, the degree of asymmetry varied considerably from protein to protein. Similar results were obtained with membranes (Figure 5) although the interactions with LiI_2sal solution, undissolved lipid and other particulate material raised the apparent intrinsic viscosity. When the membranes were centrifuged at moderate speed (18,000 rpm) the partially clarified solution exhibited a much lower viscosity, and when fully clarified by high-speed centrifugation the viscosity was still lower, but still considerably above that for globular proteins. The unusual result was the strong upward trend of the intrinsic viscosity with dilution suggesting that the LiI_2sal-soluble membrane

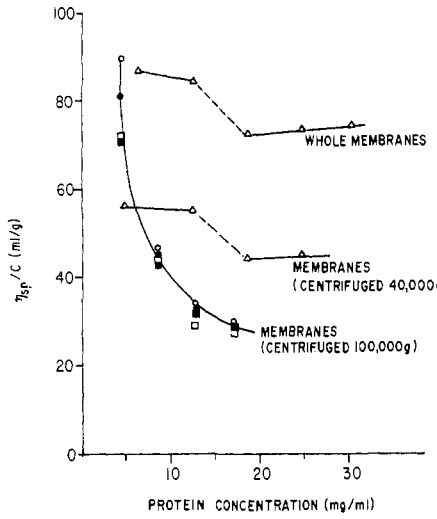

FIGURE 5: Plot of reduced viscosity *vs.* protein concentration for heat-treated membranes in 0.2 M LiI_2sal including 0.2% dithiothreitol (pH 7.6). The upper curve refers to membranes without centrifugation. The middle curve refers to membranes after centrifugation at 40,000*g* for 20 min. The lower curve refers to membranes after centrifugation at 100,000*g* for 1 hr. Variously shaped dots on the lower curve refer to different preparations on different days. Addition of sonicated membrane lipid to membranes after high-speed centrifugation had no effect. Protein concentrations were determined after centrifugation.

subunits became either greatly extended or formed linear polymers on dilution. It is also possible that at the higher concentrations aggregation occurs to yield more compact forms. Various treatments as indicated in Figure 5 had no effect on the viscosity increase with dilution. Reynolds and Tanford (1970b) also noted an increasing intrinsic viscosity with increasing dilution in the case of sodium dodecyl sulfate complexes of some proteins, but the effect was less pronounced.

Dithiothreitol changed the viscosity of the disulfide containing proteins but had no effect on myoglobin or membranes. Nevertheless, it was included in all viscosity solutions except water for consistency.

Analytical Ultracentrifuge Studies. Sedimentation and diffusion constants were measured in order to find out if the proteins in LiI_2sal solution were in the form of large aggregates or if the large values of intrinsic viscosity were due to extended monomers. Data were extrapolated to zero protein concentration in calculating both the sedimentation and the diffusion constants.

The sedimentation coefficients (Table I) in LiI_2sal were significantly higher than those of native proteins in water or dilute buffer.

Ovalbumin exhibited more than one band during sedimentation. Apparent diffusion coefficients were appreciably lower in LiI_2sal than in water indicating highly aggregated species in LiI_2sal. Molecular weights of these species are also shown in Table I.

Solubility in LiI_2sal vs. Native Protein Conformation. We were puzzled to find that a good many proteins were insoluble in LiI_2sal solution (Table IV). However, with several exceptions, we found a correlation between the amount of helical coiling and solubility. With reference to the optical rotatory dispersion data given in Table IV, a maximum a_0 value (100% random coil or disordered structure) would be about -500 to -600 and a maximum b_0 value (100% α helix) would also be about -500 to -600.

TABLE IV: Relation between Protein Helix Content and Solubility in LiI_2sal (Optical Rotatory Dispersion Data).[a]

	a_0 (Coil)	b_0 (α-helix)	Molecular Weight
Soluble in LiI_2sal			
Bovine serum albumin	−340	−310	67,000
β-Amylase		−195	152,000
Fibrinogen	−266	−214	320,000
γ-Globulin	−275	+35	160,000
Hexokinase		−325	95,000
β-Lactoglobulin	−169	−66	37,000
Myoglobin	+20	−387	18,000
Ovalbumin	−177	−198	43,000
Pepsin	−169	−190	33,000
Insoluble in LiI_2sal			
Carbonic anhydrase	−370	+10	30,000
α-Chymotrypsin	−349	−99	21,600
Chymotrypsinogen A	−436	−86	23,650
Deoxyribonuclease		−74	31,000
Glutamic dehydrogenase		−255	350,000
Glyceraldehyde-3-phosphate dehydrogenase	−150	−240	135,500
Lysozyme	−295	−150	14,000
Ovomucoid	−436	−86	14,000
Ribonuclease	−111	−101	12,500
Trypsin		−19	15,000

[a] All ORD data taken from Sober (1970) except for pepsin and ribonuclease derived from Urnes and Doty (1961).

Two of the soluble proteins, γ-globulin and β-lactoglobulin, are apparently low in α-helix content. Both γ-globulin and β-lactoglobulin are thought to contain a significant amount of β structure (Urnes and Doty, 1961; Townend *et al.*, 1969). It is possible that β structure performs the same function as α helix in rendering the protein soluble in LiI_2sal.

We do not understand why glutamic dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase are exceptional among the LiI_2sal-insoluble proteins. They appear to have large amounts of helical structure, but their unusual salt requirements for stability may be related to their insolubility in LiI_2sal.

Discussion

Sodium Dodecyl Sulfate Studies. Reynolds and Tanford (1970a) report on the basis of their binding studies and other considerations that the binding of sodium dodecyl sulfate to protein is primarily hydrophobic in nature and suggest that the protein–sodium dodecyl sulfate complex may bear some resemblance to a micellar system (Reynolds and Tanford, 1970b). These ideas are also in agreement with the concepts of Kauzmann (1959) concerning hydrophobic bonding and its importance in protein structure. It might also be noted that since the sodium dodecyl sulfate is aliphatic in character, dispersion forces would tend to favor association of the hydrophobic portion of the sodium dodecyl sulfate with aliphatic amino acid residues within the hydrophobic interior of the protein. Aromatic regions might be relatively unassociated with sodium dodecyl sulfate and hence, proteins linked through *aromatic* interactions might be resistant to sodium dodecyl sulfate attack.

Further evidence for hydrophobic interaction is derived from the activation parameters. While it is true that a small unfavorable enthalpy contribution would result from salt linkage formation, one would also expect a small negative or actually favorable entropy contribution resulting from a strongly favorable unitary[3] entropy component (Kauzmann, 1959). The large enthalpy of activation observed (over +9 kcal) could more easily result from the unwinding of the sodium dodecyl sulfate molecule from its more compact configuration in water or close self-association in micellar form.

The entropy term is less easily explained, but it seems likely that for sodium dodecyl sulfate monomers a large contribution would come from the cratic[3] entropy (about +8 eu) resulting from the binding of the relatively free monomers in solution to the relatively fixed membrane. A large unitary contribution might result from the exchange of a fluid lipid environment for the sodium dodecyl sulfate chain to the relatively structured protein interiors within the membrane. These speculations depend on the involvement of a large portion of the sodium dodecyl sulfate carbon chain at one time in the membrane solubilization activation step.

The results of our studies with sodium dodecyl sulfate have the following significance relative to the analysis of noncovalent bonding in these membranes. (1) The high efficiency with which most of the noncovalent bonds were broken suggests that the bulk of the bonding (both lipid–lipid, lipid–protein) is simple aliphatic, hydrophobic bonding. (2) Most interesting, however, is the finding that some (presumably protein–protein) bonds are highly resistant to the powerful hydrophobic bond-breaking ability of sodium dodecyl sulfate. Future work will place major emphasis on finding out what these sodium dodecyl sulfate resistant bonds are. (3) The activation parameters are consistent with the idea that sodium dodecyl sulfate first enters the membrane with the insertion of much of its hydrocarbon chain at one time. Disaggregation follows, rapidly, when sufficient sodium dodecyl sulfate is attached.

Lithium Diiodosalicylate Data. Perhaps the most striking result of our LiI_2sal studies is the strongly negative temperature dependence of the solubilization rate in the range 16° to about 30°. We had previously found no such anomaly in the log k_r *vs.* $1/T$ plot of sodium dodecyl sulfate solubilization of mycoplasmal membranes. A clarification of this result can be obtained from a careful study of Table III. Note the extremely high, unfavorable entropy contribution resulting from LiI_2sal solubilization in the low temperature range. By the process of elimination (Kauzmann, 1959) such a high entropy value suggests that not one, but several, hydrophobic residues are exposed to water during the activation step. The entropy results from the ordering of water around the newly exposed hydrophobic residues. At higher temperatures this ordering of water presumably breaks down, and the solubilization kinetics then become more normal. It is possible that the sharp shift in kinetics is attributable to a phase change within the membrane. However, the lipid transition temperature for these membranes most likely lies above 35°. Melchior *et al.* (1970) concluded that the cells try to regulate their lipid composition so that the transition temperature is slightly below the growth temperature (in this case 37°). Our own lipid analysis (unpublished) shows that most of the component fatty acids from the present strain grown in the present media are saturated and average about 15–16 carbons in length. This would give a relatively high transition temperature (Steim *et al.*, 1969).

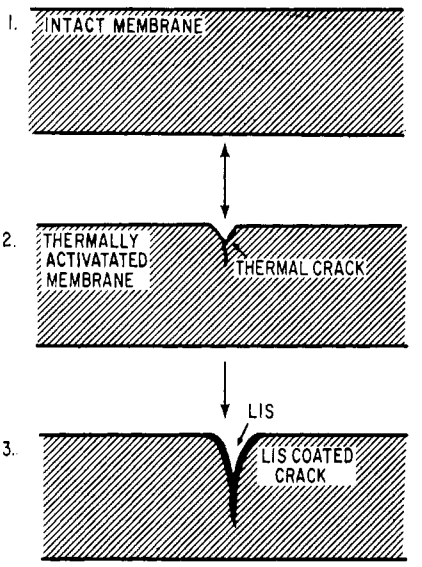

FIGURE 6: Schematic representation of membrane (cross-hatched) undergoing solubilization by a combination of thermal effects and the presence of LiI_2sal in an aqueous suspension of *A. laidlawii* membranes. The double-headed arrow between the postulated first and second stages denotes a reversible equilibrium.

We conclude that the LiI_2sal does not participate in the rate-determining step of membrane solubilization. This first step involves, instead, thermal activation of the membrane with exposure of new hydrophobic residues to the surrounding water. We visualize this as the development of a crack in the membrane. The proposed sequence of events is shown schematically in Figure 6. Note that the LiI_2sal is presumed to enter the crack and coat the exposed inner surface only after the crack has first developed by a thermal process. We postulate that the rate of solubilization decreases with increasing temperature in the range 15–30° simply because the number of thermal breaks steadily decreases due to the increase of hydrophobic bond strength with increasing temperature in this range (Kauzmann, 1959).

Our conclusions from the LiI_2sal studies are summarized as follows. (1) Of significance to the analysis of nonvalent bonding in membranes is the finding that membranes disaggregate to a high degree in a 0.2 M LiI_2sal solution with significant separation of lipid from lipoprotein. This suggests a greater structural significance to protein–protein bonding in membranes than current theories (Singer and Nicolson, 1972; Robertson, 1964; Vanderkooi and Green, 1971; Fox, 1972; Ji *et al.*, 1968) would explain. (2) The thermal activation studies suggest that LiI_2sal cannot enter the membrane hydrophobically as does sodium dodecyl sulfate but must wait for thermal breakage of these bonds before entry. This finding reaffirms the importance of hydrophobic bonding in membranes, but more important is the suggestion that some of the protein–protein bonds broken are not the ordinary hydrophobic linkages one might expect. The aromatic character of the LiI_2sal suggests instead that there is aromatic involvement in membrane bonding. (3) The viscosity studies may have a broader significance in protein chemistry. The fact that the proteins that were soluble in LiI_2sal solution were highly helical (to begin with), together with chemical considerations, suggest that the helical regions remain helical in LiI_2sal. Since LiI_2sal blanks out the relevant

[3] The cratic entropy refers to the entropy of *mixing* or of belonging to a solution. The unitary entropy refers to the inherent entropy of the solute molecule itself in association with its immediate surroundings—sometimes called the *contact* entropy (Kauzmann, 1959).

spectral regions, it is impossible to verify this speculation by optical rotatory dispersion or circular dichroism measurements.

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Flavine–Protein Interactions in Flavoenzymes. Effect of Chemical Modification of Tryptophan Residues upon Flavine Mononucleotide Binding and Protein Fluorescence in *Azotobacter* Flavodoxin†

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ABSTRACT: *N*-Bromosuccinimide easily oxidizes two of the four tryptophan side chains of *Azotobacter vinelandii* flavodoxin. The reaction occurs more rapidly and to a somewhat greater extent with the apoprotein than with the holoprotein. The titration follows a sigmoidal curve and the initial lag phase is essentially eliminated by blocking the single sulfhydryl group of the protein *via* reaction with dithiobis(nitrobenzoic acid). Oxidation of the tryptophans is associated with a loss of flavine mononucleotide (FMN) binding ability. Analysis of these results indicates that a single tryptophan residue is involved in binding. Titration of the decrease in tryptophan fluorescence of the apoprotein upon *N*-bromosuccinimide oxidation demonstrates that a single tryptophan is responsible for approximately 90% of the emission intensity. This correlates with the quenching of tryptophan fluorescence upon FMN binding to the native apoprotein. Oxidation of the tryptophans completely eliminates the near-uv circular dichroism bands and produces changes in the far-uv spectra which suggest conformational alterations. Blockage of the sulfhydryl group with dithiobis(nitrobenzoic acid) has no effect on FMN binding, but allows dithionite to reduce the bound FMN at a much faster rate to form the semiquinone.

Previous work from this laboratory (D'Anna and Tollin, 1971) has shown that approximately 90% of the tryptophan fluorescence of *Azotobacter* apoflavodoxin is quenched upon FMN binding. The quenching follows second-order kinetics with a rate constant identical with that for the flavine fluorescence quenching which also occurs upon binding. Similar observations have been made with a variety of other flavodoxins (D'Anna and Tollin, 1972; Mayhew, 1971), and thus this appears to be a general property of this group of flavoproteins. In view of these results and the recent demonstration, based on X-ray crystal structure determination, that a tryptophan side chain is in close proximity to the isoalloxazine ring of

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