Solubilized Tetrodotoxin Binding Component from the Electroplax of *Electrophorus electricus*. Stability as a Function of Mixed Lipid–Detergent Micelle Composition†

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ABSTRACT: In an attempt to provide a description of the molecular components responsible for the propagated action potential, we have investigated the solubilization of the voltage-sensitive sodium channel from the electroplax membranes of *Electrophorus electricus*. This channel is similar to those from nerve and muscle membranes and may be quantified by the binding of radiolabeled tetrodotoxin. When electroplax membranes were treated with Lubrol-PX, a tetrodotoxin binding protein was released in soluble form. The solubilized protein was extremely unstable to storage, elevated temperature, mild fractionation procedures, and elevated detergent concentrations. Loss of binding activity, measured at 18 °C, followed essentially a first-order exponential dependence on time, and the apparent first-order decay constant (k_r) was measured as an index of instability. In the initial extract k_r was a quantitatively reproducible function of the ratio of endogenous lipid phosphate to detergent. This ratio could be altered by (1) increasing the amount of detergent while holding the final concentration of extract constant, (2) dilution of extract into buffer containing a constant final concentration of detergent, or (3) addition of high concentrations of detergent but in combination with variable amounts of a lipid preparation from electroplax membranes. In all cases k_r depended only on the final ratio of lipid phosphate to detergent. When this molar ratio fell below about 0.07, the protein became enormously unstable. Experiments with pure lipids indicated that not all species were equally effective for stabilization. It has been concluded that the pure detergent micelle does not reproduce the stabilizing environment encountered in the native membrane but that this can be achieved by including low molar ratios of certain pure lipids such as phosphatidylcholine. The extreme instability observed at low lipid–detergent ratios indicates that the protein may not be easily purified or characterized unless mixed lipid–detergent micelles are employed.

One of the important strategies of membrane biochemistry involves the use of nondenaturing detergents to disassemble membranes by the mild disruption of noncovalent lipid–protein and lipid–lipid interactions. Solubilized components may then be isolated and their molecular properties studied alone or in combination with other membrane components. It is often the case, however, that solubilization of membrane-bound proteins with detergents results in loss of stability or catalytic activity. Presumably, the distribution of the protein in the native bilayer provides important interactions affecting protein conformation which are not always adequately reproduced by the detergent micelle. The present study describes investigations which were carried out in order to find a way to overcome such a problem with a solubilized intrinsic membrane protein of a type which is in part responsible for the electrical excitability of nerve and muscle cells.

The early sodium currents which characterize the propagated action potential of nerve and muscle are mediated by a voltage-sensitive ion conductance channel which is selectively permeable to sodium. Since the introduction and application of the voltage clamp (Cole, 1949; Hodgkin & Huxley, 1952a,b), the biophysical and biochemical characterization of the channel have become increasingly detailed [for reviews see Landowne et al. (1975), Keynes (1975), Armstrong (1975), and Hille (1978)]. Conductances for a single channel have been estimated to be in the range of 1–10 pS, corresponding to transport rates as high as 5×10^6 ions/s [Hille (1978) and references therein]. In view of this extremely rapid turnover, the activated channels are thought to form pores through which ions diffuse according to their electrochemical potential. The channels discriminate among the monovalent alkali cations in favor of Li^+ and Na^+, indicating the presence of a site responsible for ion-permeation selectivity. The channel behaves as though it exists in open or closed permeability or "gating" states which are regulated by mechanisms sensitive to the voltage gradient across the membrane.

Biochemical studies of the sodium channel have been made possible through the use of two extremely specific neurotoxins, tetrodotoxin (TTX)[1] and saxitoxin. These molecules bind reversibly, with high affinity (K_d = 1–10 nM), to a site accessible only from the outside of the cell membrane, to block sodium conductance (Narahashi et al., 1967; Hille, 1968; Cuervo & Adelman, 1970; Ulbricht & Wagner, 1975). The toxins appear to block the sodium ion permeation pathway without affecting structures involved in channel gating (Armstrong & Bezanilla, 1973, 1974; Keynes & Rojas, 1974). Experiments with radiolabeled toxins have demonstrated mutually competitive binding of the toxins to a single class of high-affinity sites which are present only in membranes containing TTX-sensitive sodium channels (Hafeman, 1972; Colquhoun et al., 1972; Henderson & Wang, 1972; Benzer & Raftery, 1973; Levinson, 1975a,b; Reed & Raftery, 1976; Ritchie et al., 1976). Comparison of data from these binding studies with independent physiological investigations (determination of K_d, channel densities, single-channel conductances, and the chemical characteristics of the site of toxin action) has provided strong evidence that the toxins bind only to the physiologically defined sodium channel, with a stoi-

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[1] Abbreviations used: TTX, tetrodotoxin; PMSF, phenylmethanesulfonyl fluoride; GSH, glutathione; IAA, iodoacetamide; EDTA, ethylenediaminetetraacetic acid.

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chiometry of one toxin binding site per channel (Hille, 1975; Narahashi, 1974; Keynes, 1975).

Several lines of evidence indicate that the sodium channel is largely or entirely a protein structure. Enzymic proteolysis of membranes from the unmyelinated axons of gar olfactory nerve (Benzer & Raftery, 1972) or from eel electroplax (Reed & Raftery, 1976) destroyed TTX binding. Protease treatment of the interior aspect of the squid giant axon membrane resulted in the loss of the inactivation gating mechanisms (Rojas & Armstrong, 1971; Armstrong et al., 1973; Rojas & Rudy, 1976). In addition, the target size of the TTX binding component in membranes from eel electroplax and from piglet cortex, estimated by the method of irradiation inactivation, revealed molecular weights in the range of 230 000–240 000 (Levinson & Ellory, 1973; Levinson, 1975b). This large size supports the contention that the channel structure is a macromolecule.

There have been remarkably few reports on studies of the biochemical isolation and characterization of the sodium channel. Henderson & Wang (1972) and Benzer & Raftery (1973) have reported the detergent solubilization of the TTX binding component from gar olfactory nerve. The solubilized preparations demonstrated binding characteristics similar to those of the original membranes and were extremely sensitive to enzymic proteolysis. The protein sedimented as approximately a 9S particle and was eluted from a gel sieving column as a molecule somewhat larger than β-galactosidase. Further attempts at the purification of the toxin binding site were prevented by the extreme lability of the preparations.

An account of the isolation of the TTX binding component which is a part of the voltage-sensitive sodium channel from the electroplax of *Electrophorus electricus* has recently been presented (Agnew et al., 1978). Electroplax membranes were solubilized with the nonionic detergent Lubrol-PX, followed by fractionation with ion-exchange and gel exclusion chromatographic procedures. As was the case with gar olfactory nerve preparations, one of the obstacles to be surmounted was the extreme lability of the detergent-solubilized protein. Here we present evidence that an important determinant of the binding site stability is the ratio of lipid to detergent in the mixed micelles with which the protein is dissolved. The data strongly suggest that, when dissolved in the pure detergent, the protein is so unstable as to prohibit its further study. However, stabilized preparations may be made through the use of mixed lipid–detergent micelles. Experiments with pure lipids indicated that not all species tested were equally useful for stabilization. However, it was found that micelles containing low molar ratios of phosphatidylcholine or phosphatidylethanolamine could be used to keep the protein in a stable, soluble form suitable for purification or biochemical characterization. A preliminary account of these findings has been presented elsewhere (Agnew & Raftery, 1977).

Experimental Section

TTX, obtained citrate free from Sankyo Chemical Co., was tritiated by the Wilzbach procedure and purified from radioactive contaminants by ion-exchange chromatography (Benzer & Raftery, 1972). The purified toxin was approximately 65% pure as determined by the frog sciatic nerve bioassay procedure (Levinson, 1975a).

Lubrol-PX (Imperial Chemical Industries) was made up in 10% (w/v) solutions which were deionized with a mixed-bed resin [Bio-Rad AG 501-X8 (D)]. The resin was removed by filtration and solutions were stored frozen. The detergent is a C_{12}–C_{14} polyunsaturated alkyl poly(ethoxyethanol), and the molecular weight used in calculations reported here was 604 (Tanford & Reynolds, 1976).

Medium-sized (approximately 1 m) specimens of *E. electricus* were obtained from Ardsley Aquariums (Ardsley, NY). Animals were killed by hypothermia and the main electric organs stored frozen at −80 °C. To prepare membrane fragments, frozen organs were trimmed of connective tissue, minced, and homogenized in five volumes of potassium phosphate buffer (50 mM, pH 7.6), in a VirTis 60 apparatus (40 000 rpm, 45 s). Connective tissue clumps were removed by filtration through cheesecloth, and the filtrate was pelleted at 40000*g* for 1 h. The pellet was resuspended in 5–10 volumes of buffer with a glass homogenizer and recentrifuged at 100000*g* for 1 h in a Beckman 35 rotor. The pellets were then resuspended by addition of 2 mL of buffer/g (wet weight) of membranes, followed by homogenization in a glass, motor-driven homogenizer. Membrane suspensions were stored frozen at −80 °C.

Detergent extracts were prepared by adding Lubrol-PX to a final concentration of 1% (w/v) to a freshly thawed membrane suspension, followed by vigorous homogenization in a motor-driven glass homogenizer. The mixture was sedimented at 100000*g* for 1 h in a Beckman 65 rotor. The supernatant was removed with a Pasteur pipette and stored in an ice bath.

TTX binding in detergent extracts was measured as described by Agnew et al. (1978). Concentrated toxin ([^{3}H]TTX or [^{3}H]TTX and unlabeled TTX) was added to an aliquot of extract and allowed 10 min at 0 °C for binding equilibration. Of this, 0.20 mL was layered over a 1.5-mL Sephadex G-50 (fine) column made in the barrel of a 3-mL plastic syringe and given a few seconds to sink in. The column was suspended in a culture tube, placed in an MSE GT4 centrifuge (angle rotor), and accelerated at 1000*g* min^{-1} to 1200*g*. The radioactivity in the fluid collected in the culture tube was measured by standard scintillation counting methods. Unbound TTX was fractionated and retained by the stationary phase of the gel under these conditions. Because of the slow rate of dissociation of toxin from solubilized binding sites (Agnew et al., 1978), recovery of specific protein–toxin binding complex was essentially quantitative. All binding assays were performed at ice bath temperature, with control duplicates containing excess unlabeled toxin, to ensure that only specific toxin binding was measured. To measure total binding sites in a sample, toxin concentrations were adjusted so that free [[^{3}H]TTX] was approximately 150 nM.

Protein assays were by the method of Lowry et al. (1951). Lipid phosphate was measured by a modified Fiske SubbaRow technique (Radin, 1969; Dittmer & Wells, 1969).

Total unfractionated eel lipids were prepared from electroplax packed membranes by the method of Radin (1969). Pure phospholipids were obtained from PL-Biochemicals. Dipalmitoylphosphatidylethanolamine and dipalmitoyl- and distearoylphosphatidylcholine were the gift of Dr. S. I. Chan.

Results

Solubilization of Membrane Components from Electroplax Packed Membranes. When prepared as described under Experimental Section, 0.1–0.2 g (wet weight) of packed membranes were recovered per g of eel organ. The bulk of the pelleted material was connective tissue, as the pellets could be greatly reduced by treatment with collagenase (S. R. Levinson, unpublished experiments), and only about 10–20 μmol of lipid phosphate (7–15 mg of phospholipid) was found per g of membranes. This preparation was a rich source of TTX binding sites, containing 250–500 pmol/g of membranes. This is somewhat greater than the number of α-bungarotoxin

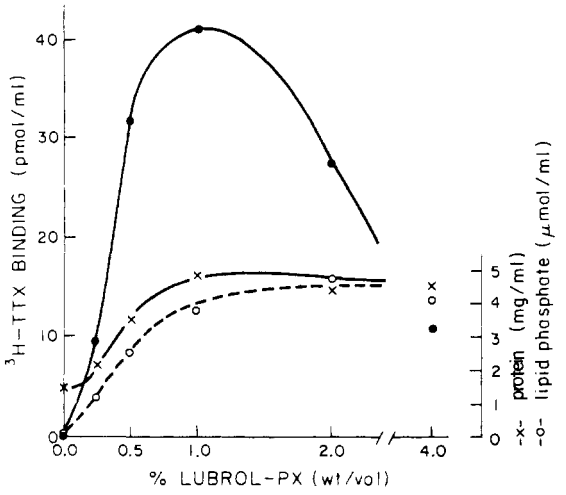

FIGURE 1: Solubilization of the TTX binding component, protein, and phospholipids with Lubrol-PX. Aliquots of a freshly thawed membrane suspension were mixed with increasing concentrations of Lubrol-PX. Each sample was homogenized in a glass homogenizer, in an ice bath, followed by centrifugation for 1 h at 100000g in a Beckman 65 rotor. Supernatants were removed, stored briefly in ice, and assayed for TTX binding, protein, and lipid phosphate as described under Experimental Section.

binding sites (Raftery et al., 1971) and is in agreement with values reported by Levinson (1975a,b). The data presented in Figure 1 illustrate the solubilization of packed membranes with Lubrol-PX. Aliquots of a suspension of the membranes were mixed with increasing concentrations of the detergent, homogenized in a glass homogenizer, and centrifuged at 100000g for 1 h in a Beckman 65 rotor. The supernatants were assayed for TTX binding, protein, and phospholipid as described under Experimental Section. Optimal solubilization of the TTX binding component was observed at about 1% detergent, at which point 50–60% of the sites originally measurable in the membranes were recovered in the supernatants. Extracts employed in these studies contained 40–100 pmol of TTX binding sites, 3–5 μmol of lipid phosphate, and 3–6 mg of protein per mL.

Effects of Detergent on Stability. The solubilized toxin binding component was much more labile than the membrane-bound protein. Whereas membranes retained binding capacity for several weeks when stored at 4 °C, the solubilized extract was rapidly inactivated. The loss of activity was on occasion somewhat biphasic, approximately 50% being lost after 24 h, followed by a slower decay over several days. At room temperature all activity was generally lost after several hours. Together with this instability to storage an even more pronounced lability to mild fractionation procedures was observed. All activity was lost during ion-exchange chromatography and during gel filtration, even when these were completed in a few hours at 4 °C. Stability was not improved by a variety of agents tested, including PMSF, GSH, IAA, EDTA, Ca^{2+}, glycerol (10% w/v), or sucrose (10% w/v) (J. S. Brabson, unpublished experiments).

It seemed logical that the loss of stability resulted from the change in environment of the protein from that of the native membrane bilayer to that of the detergent–lipid mixed micelle of the crude extract. We therefore further examined the effects of detergent on stability of the initial extract. Decay of binding was measured at 18 °C to shorten the time for experiments. Under these conditions the loss of binding was approximately a first-order exponential function of time for more than 90% of the time course, though in some instances there was slight evidence for biphasic decay.

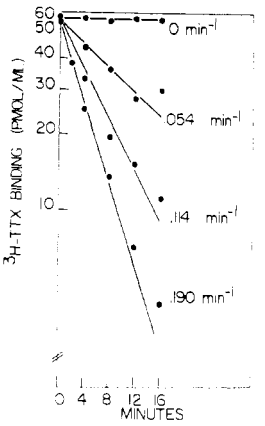

FIGURE 2: Effect of added detergent on stability of a soluble extract. A 1% Lubrol-PX extract was prepared from a freshly thawed membrane suspension as described under Experimental Section. To 0.35-mL aliquots of the extract was added 0.15 mL of potassium phosphate buffer (50 mM, pH 7.6) containing increasing concentrations of Lubrol-PX. Four series of tubes were prepared with final lipid to detergent ratios of 0.192, 0.064, 0.048, and 0.038 μmol of lipid phosphate per μmol of Lubrol-PX. Each tube was equilibrated for 10 min in ice and then placed in a water bath at 18 °C for the indicated times. The incubations were stopped by plunging the tubes into melting ice and immediately adding saturating amounts of [^{3}H]TTX. After 10 min each sample was assayed in duplicate for TTX binding. The numbers next to each line indicate k_r as defined by eq 1, the highest lipid to detergent ratio corresponding to the lowest value for k_r.

The experiment presented in Figure 2 illustrates the effect on stability when the detergent concentration was elevated while the extract concentration was held constant. The apparent first-order decay constant, k_r, is given by eq 1 where

$$k_r = 1/t \ln (B_0/B) \quad (1)$$

t is time at 18 °C and B_0 and B are the binding measured at time zero and time t, respectively. It was observed that k_r increased sharply as detergent concentrations were raised.

Inasmuch as detergent was present at levels well above the critical micellar concentration of the pure Lubrol-PX (0.005% w/v; Tanford & Reynolds, 1976), the effect of added detergent should be to increase the extent of the micellar "phase" in which amphipathic membrane components were dispersed. Thus, it seemed likely that high detergent concentration caused the dilution from one another of elements whose association was required for stability. More specifically, dilution of endogenous lipids with added detergent might result in decreased binding site stability. If this were true, it would be predicted that k_r would be a function of the ratio of lipid to detergent within the micellar phase.

The experiment illustrated in Figure 3A was performed to show the effect of varying the ratio of endogenous lipid to detergent without changing the final detergent concentration. Samples of a 1% Lubrol-PX extract were diluted 1:2, 1:3, and 1:4 into buffer containing 1% detergent, followed by the measurement of k_r (18 °C). The data show that k_r increased sharply as the ratio of endogenous lipid to detergent was lowered, even though the actual final concentration of detergent was constant. When samples were diluted to the same extent in buffer alone (Figure 3B) so that the ratio of lipid to detergent was unaltered, k_r was not affected.

The extracts employed in the experiments illustrated in Figures 2 and 3A were assayed for total lipid phosphate, and the values for k_r from both experiments were plotted as a function of the final ratio of lipid phosphate to detergent. The data from the two experiments fell on the same curve (Figure

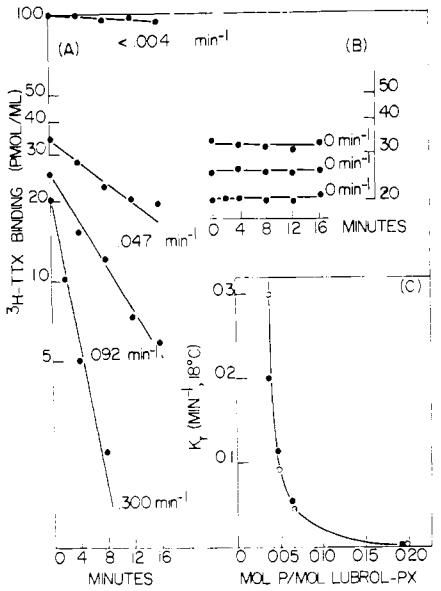

FIGURE 3: (A) Effect of dilution into buffer containing a constant concentration of detergent. A 1% Lubrol-PX extract was made, and a series of culture tubes was prepared containing 0.5 mL of the extract or 0.5 mL of the extract diluted 1:2, 1:3, or 1:4 into potassium phosphate buffer (50 mM, pH 7.6) containing 1% Lubrol-PX. Each tube was incubated for 10 min in ice and then placed in a water bath at 18 °C for the time indicated. Incubations were stopped by placing the tubes in an ice slurry and adding saturating concentrations of [^{3}H]TTX. After 10 min each tube was assayed in duplicate for TTX binding. The lipid to detergent ratio in the initial extract was 0.197 μmol of lipid phosphate/μmol of Lubrol-PX. The numbers to the side of each curve give the values for k_r. (B) Effect of dilution into buffer containing no detergent. Samples of the extract used in Figure 3A were diluted 1:2, 1:3, or 1:4 into potassium phosphate buffer (50 mM, pH 7.6) containing no detergent and were worked up exactly as in Figure 3A. The lipid to detergent ratio was not varied, and the amount of TTX binding in each dilution is the same as was observed for time = 0 in the corresponding samples of experiment 3A. (C) Relationship of k_r to lipid detergent ratio. The values measured for k_r in experiments illustrated in Figures 2 and 3A were plotted as a function of the final ratio of lipid phosphate to detergent. Solid symbols (●) are data from Figure 2; (○) are data from Figure 3A. The curve was fit by eye.

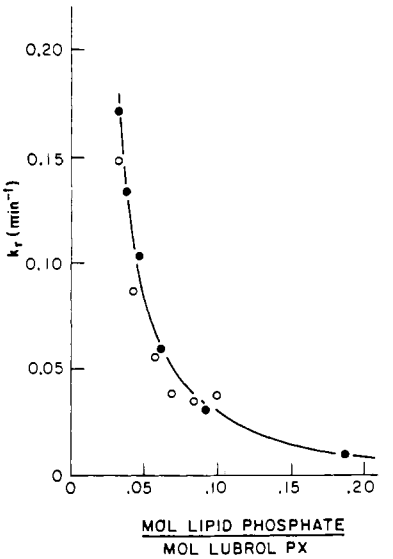

FIGURE 4: Stabilizing properties of total lipids derived from electroplax membranes. Control curve (●): samples of a 1% detergent extract (0.333 mL each) were mixed with increasing concentrations of Lubrol-PX (in 0.167 mL). Highest final detergent concentration was 4%. Each sample was equilibrated for 10 min in ice and then exposed for 15 min to 18 °C. Samples were removed to an ice slurry and saturating [^{3}H]TTX was added. After 10 min samples were assayed in duplicate for [^{3}H]TTX binding. Values of k_r for each sample were calculated from eq 1 and plotted as a function of the final lipid phosphate to detergent ratio. The curve was fit by eye. Experimental points (○): to samples of extract (0.333 mL) was added 0.167 mL of 10% Lubrol-PX (final detergent concentration 4%), in which had been dissolved increasing amounts of a total lipid preparation derived from electroplax membranes. Samples were worked up as for the control curve. Values for lipid phosphate to detergent were calculated from the sum of endogenous and added lipid phosphate.

3C) although in the first case the concentration of detergent and in the second the concentration of endogenous lipid had been varied.

The next set of experiments examined the effects of detergent presented in combination with low amounts of a preparation of total lipids from electroplax membranes (Figure 4). A total lipid extract was prepared from packed membranes as described under Experimental Section. The control curve was obtained by addition of increasing concentrations of Lubrol-PX to a 1% detergent extract, followed by measurement of k_r (18 °C) at each final ratio of total endogenous lipid phosphate to detergent. The highest final detergent concentration was 4%. A sharp increase in k_r was again noted as the molar ratio declined below 0.07. The experimental curve was obtained by addition of detergent to a final concentration of 4% but in combination with increasing amounts of membrane lipids. The decline in k_r with the addition of lipids showed a close quantitative agreement with the control, dilution curve. Thus, the added membrane lipids protected the binding site from the elevated detergent concentration. It should be noted that in experiments in which the lipids were not properly dissolved in the detergent (turbid solutions) the effectiveness of the lipids was greatly decreased. [^{3}H]TTX binding in all instances could be completely displaced with unlabeled toxin, and no binding could be detected in samples containing electroplax lipids and detergent alone.

From these experiments we concluded that stability was to a critical extent determined by the ratio of lipid to detergent in the mixed micelles. Values for k_r were a quantitatively reproducible function of this ratio, and, when the molar ratio of endogenous lipid phosphate to detergent fell below about 0.07, k_r increased sharply.

Lipid Specificity. The major lipid species from eel electroplax are phosphatidylethanolamine, phosphatidlycholine, phosphatidylinositol, phosphatidylserine, and cholesterol (Trams & Hoiberg, 1970). Experiments similar to the one illustrated in Figure 4 were performed with commercially available pure samples of these lipids to search for evidence of a specific lipid requirements. The data in Figure 5A show that phosphatidylethanolamine (chicken egg) was essentially as effective, per equivalent of phosphate, as endogenous lipid. Further experiments (Figure 5B) showed that phosphatidylcholine, from chicken egg and from pig brain, was about equally effective and nearly as effective as endogenous lipid. Phosphatidylinositol (soybean) (Figure 5C) produced no stabilization under the conditions tested. Phosphatidylserine (pig brain) (Figure 5C) consistently produced a biphasic effect, providing stabilization up to the point where the added lipid represented about 60% of the total phospholipid present. However, above this level the protein was greatly destabilized.

The results presented in Figure 5C, illustrating the effects of the neutral lipid cholesterol, suggest that the stabilization afforded by endogenous lipids is likely not due to the phospholipid components alone. This is of interest because it may be noted that electroplax lipids were at least as effective, per equivalent of phosphate, as pure phosphatidylethanolamine and phosphatidylcholine, although the electroplax lipids contain

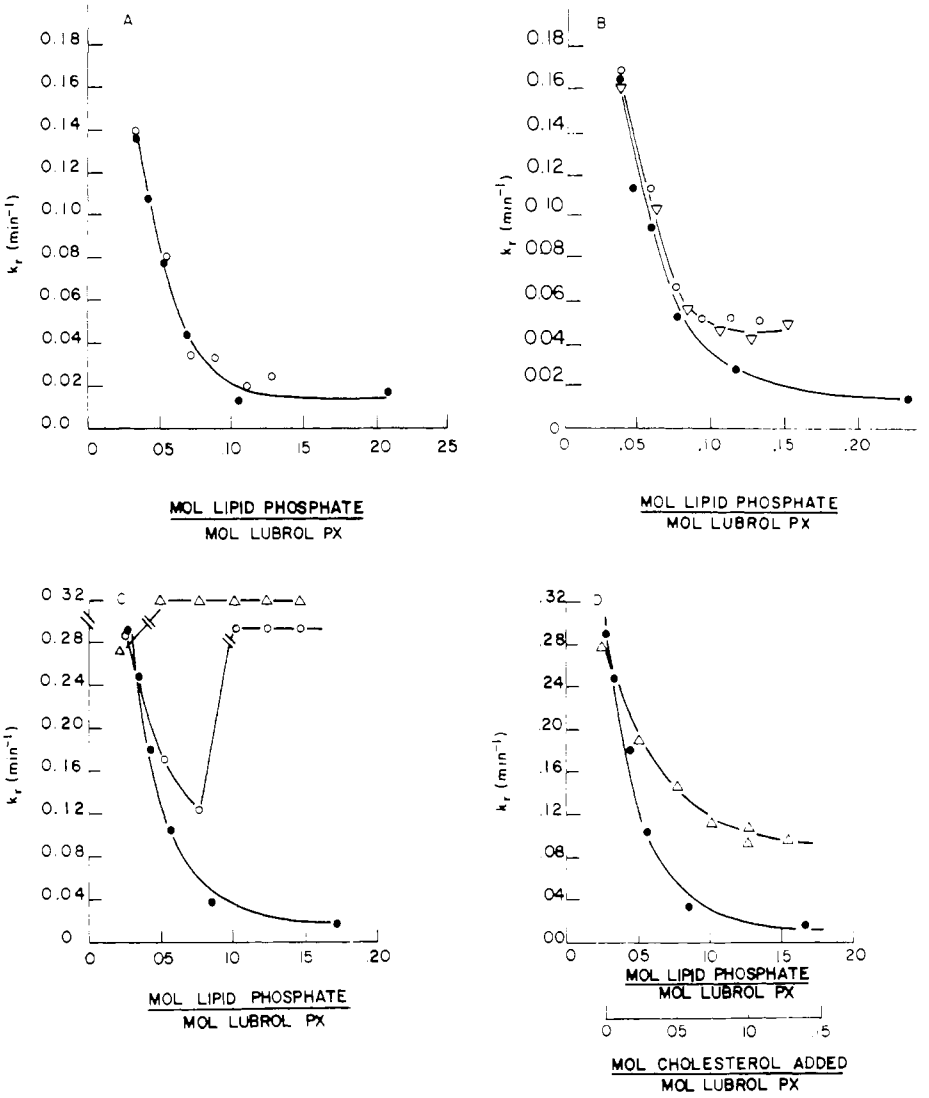

FIGURE 5: Stabilizing properties of pure lipid species. These experiments were performed as was the one in Figure 4, except that commercially available pure lipids were used. (●) represent control samples produced by dilution of endogenous lipids. Symbols were (A) (○) added phosphatidylethanolamine (chicken egg), (B) (○) phosphatidylcholine (pig brain) and (▽) phosphatidylcholine (chicken egg), (C) (○) phosphatidylserine (pig brain) and (△) phosphatidylinositol (soybean), and (D) (△) cholesterol (chicken egg).

a significant amount of phosphatidylserine and phosphatidylinositol which may not be particularly effective. This discrepancy might be ascribed to the contribution by cholesterol to stabilization.

Figure 6 shows results from experiments with synthetic phosphatidylethanolamine and phosphatidylcholines containing only saturated fatty acyl moieties. Dipalmitoylphosphatidylethanolamine was almost completely ineffective as a stabilization agent. Dipalmitoylphosphatidylcholine was more effective but not as effective as the biologically derived, unsaturated species. Distearoylphosphatidylcholine was even less effective than the dipalmitoyl derivative.

Taken together, the results from these studies suggest that the stability of the toxin binding component may be responsive to several physical parameters which describe the mixed micelle, such as relative concentrations of lipid and detergent molecules, net surface charge, hydrocarbon packing in the interior of the micelle, and perhaps overall micellar geometry. Of the phospholipids tested only the zwitterionic species were effective, whereas the anionic species were not. Although there may be some statistical differences between the fatty acyl moieties of all of these phospholipid species, it seems likely that the net charge on the polar head groups may determine the ability to stabilize. The biphasic response of phosphatidylserine suggests that there are two or more effects of this lipid. For example, supplementation with this species may be beneficial up to a point where a significant negative surface charge density is developed, after which the protein, if it is itself negatively charged at neutral pH, may not be able to properly orient in the micelle. Furthermore, stabilization by phospholipid appears to depend to some extent on the degree of fatty acyl unsaturation and on chain length, both factors which can be expected to affect hydrocarbon packing and perhaps the geometry of the mixed micelle. The effectiveness of cholesterol may also reflect changes in the organization of lipid packing within the micelle.

Temperature Dependence of k_r as a Function of Lipid/Detergent Ratio. When measured at 18 °C, the values for k_r seemed to increase almost discontinuously as the molar ratio of lipid to detergent fell much below 0.05. It was of interest to perform experiments to determine the dependence of k_r on temperature. Such experiments would provide information which might relate to the mechanism of stabilization. In addition, from a practical standpoint, it would be useful to provide an accurate assessment as to how important the stabilization might be, both for the design of a purification

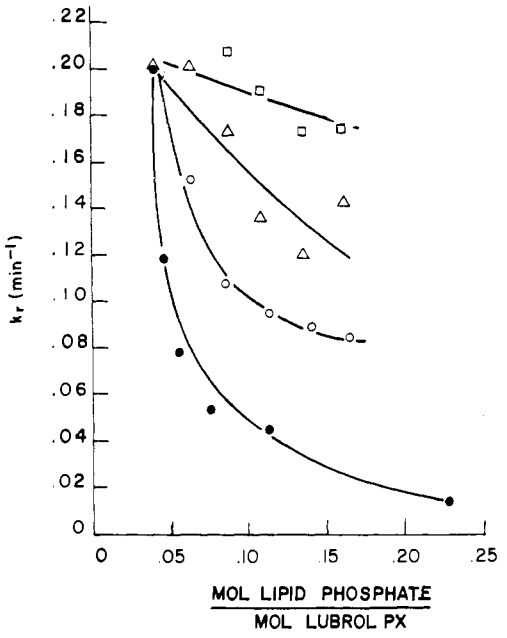

FIGURE 6: Stabilizing properties of synthetic phospholipids. The experiments were performed as was the one in Figure 4, except the lipids used for supplementation were (□) dipalmitoylphosphatidylethanolamine, (△) distearoylphosphatidylcholine, and (○) dipalmitoylphosphatidylcholine. (●) represent control samples produced by dilution of endogenous lipids.

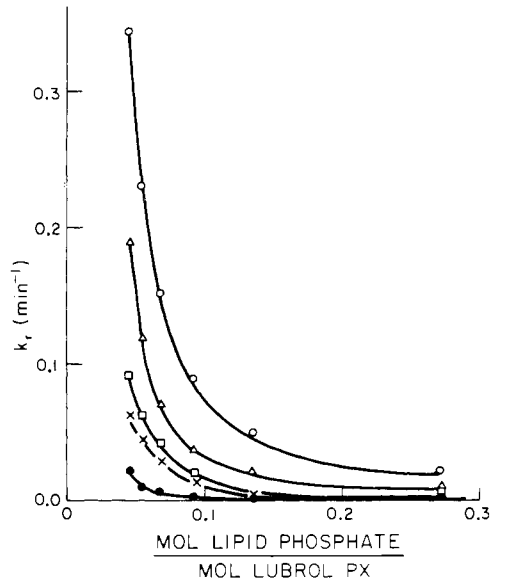

FIGURE 7: Temperature dependence of k_r at different lipid/detergent ratios. Each curve was produced by dilution of endogenous lipids with Lubrol-PX, just as for the control curve in experiment 4. Values for k_r were then measured at 20 °C [8-min incubations (○)], 15 °C [8-min incubations (△)], 12.5 °C (×)], incubations (□)], 10 °C [10-min incubations (×)], and 5 °C [14-min incubations (●)].

protocol and for the design of experiments to study the molecular characteristics of the purified protein.

It seemed possible that the ratio of lipid to detergent could directly effect the energy of activation of the rate-limiting step for a conformational change resulting in loss of binding activity. The experiment reported in Figure 7 shows k_r measured at various values of lipid to detergent at 5, 10, 12.5, 15, and 20 °C. It is clear that there was a marked temperature coefficient for k_r, indicating a fairly low energy of activation. When the data were replotted according to the Arrhenius relationship, however (Figure 8), it was noted that the energy

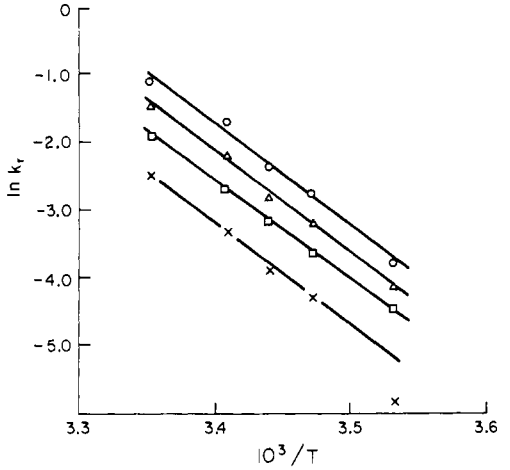

FIGURE 8: Arrhenius plot of k_r values obtained at different ratios of endogenous lipid phosphate to detergent. Values for k_r measured at the four lowest ratios of lipid to detergent in the experiment from Figure 7 were plotted according to the Arrhenius relationship ($\ln k_r = \ln A - E_a^*/RT$). The value estimated for E_a^* was approximately 29.7 kcal/mol and was not changed by alteration of the lipid/detergent ratio. The molar ratios of lipid phosphate to detergent were 0.045 (○), 0.054 (△), 0.068 (□), and 0.089 (×).

of activation was not a sensitive function of lipid/detergent ratio (cf. legend to Figure 8). The major increase in k_r at low lipid/detergent ratios appeared to reflect only an increase in the temperature independent preexponential term.

From this experiment it was determined that at 0.045 mol of endogenous lipid phosphate/mol of detergent, the half-life of the protein at 5 °C is only about 33 min. Thus, it seems likely that the binding component may not readily tolerate fractionation procedures which separate it from stabilizing lipids.

Discussion

We have found that the TTX binding component which is part of the voltage-sensitive sodium channel in the electroplax membranes from *E. electricus* could be solubilized with the nonionic detergent Lubrol-PX (Agnew et al., 1978). However, as had been reported for the TTX binding component from gar olfactory nerve (Henderson & Wang, 1972; Benzer & Raftery, 1973), solubilization caused the protein to become quite unstable to storage or biochemical manipulation. Loss of toxin binding activity, measured at 18 °C, demonstrated an approximately first-order exponential dependence on time. It was observed that the apparent first-order decay constant was a quantitatively reproducible function of only the ratio of endogenous lipid phosphate to detergent in the solubilized membrane extract. This was concluded from experiments in which the ratio was altered (1) by addition of high concentrations of detergent while holding the level of extract constant, (2) by dilution of extract into buffer containing a constant concentration of detergent, or (3) by the addition of high levels of detergent in combination with variable amounts of a lipid preparation derived from electroplax membranes. Thus, to keep the protein in a stable soluble form, it is apparently necessary to make use of mixed micelles containing a proportion of certain membrane lipids.

Experiments with commercially available pure lipids indicated that not all species were equally effective. The zwitterionic (at neutral pH) lipids phosphatidylcholine (egg or pig brain) and phosphatidylethanolamine (egg) were nearly as effective for stabilization as endogenous lipids. However, the anionic species phosphatidylinositol and phosphatidylserine

demonstrated no stabilization and partial stabilization, respectively. These results suggest that some specificity is conferred by the charge on the polar head group. Experiments with synthetic, saturated phosphatidylethanolamine and phosphatidycholines indicated that unsaturation of the fatty acyl moieties and shorter chain length correlate with improved stabilization. These results might suggest that increased hydrocarbon fluidity enhance stability. Interestingly, cholesterol, which tends to favor tight packing of hydrocarbon chains in bilayers (Lenaz, 1977), also improves stability.

There are perhaps two possible general mechanisms whereby micelle composition could affect stability. There could be, for instance, multiple binding sites on the protein for which lipid and detergent molecules compete with the result that lipid–protein complexes are more stable than detergent–protein complexes. Alternatively, variation of micelle composition may change general physical parameters of the microenvironment to which the protein is sensitive. If mechanisms involving multiple binding equilibria were invoked, determinants on the lipid could specify (1) the number of sites to which lipid could bind, (2) the relative affinity of the site for lipid compared to detergent, and (3) the energy of stabilization afforded by lipid binding. An alternative explanation for the experimental observations is that the effects of composition of micelle surface charge, local partitioning into lipid- or detergent-enriched domains, hydrocarbon fluidity, or micelle geometry may determine stability. Several well-characterized intrinsic membrane proteins have been shown to associate with an annulus of "boundary" lipid (Lenaz, 1977). This lipid is ordinarily not readily removed by solubilization procedures. However, it seems possible that, at elevated ratios of detergent to lipid, such a boundary, or perhaps at least second- and third-order layers of lipid, might be depleted, causing a loss of important stabilizing interactions.

When the variation of k_r with temperature was compared at different lipid/detergent ratios, it was observed that the apparent energy of activation for k_r (approximately 29.7 kcal/mol) was not a sensitive function of the micelle composition. It has not yet been possible to measure the limiting stability of the completely delipidated protein. In the experiment illustrated in Figure 8, however, the half-life of the binding site measured at 5 °C, 0.045 mol of lipid phosphate/mol of Lubrol-PX, was approximately 33 min. Thus, it seems likely that even with stabilization afforded by occupying the binding site with TTX or saxitoxin (Agnew et al., 1978; S. R. Levinson and W. S. Agnew, unpublished experiments) it may not be possible to purify the protein in the absence of at least a low level of certain membrane lipids. For this reason the purification described by Agnew et al. (1978) was performed with buffers containing phosphatidylcholine (chicken egg)–Lubrol-PX (1:7) mixed micelles.

It may be that the use of mixed micelles for the characterization of intrinsic membrane proteins will be of general utility. In the case of the sodium channel, it seems possible that spectroscopic investigations with the purified protein, in micelles of completely defined composition, may provide direct information as to the interactions of the protein with membrane lipids. Because the voltage-sensitive elements of the native channel must exist within the membrane bilayer, such information may ultimately assist in deciphering channel function as well as structure.

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Electrostatic Effects in Hemoglobin: Hydrogen Ion Equilibria in Human Deoxy- and Oxyhemoglobin A†

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ABSTRACT: The modified Tanford–Kirkwood theory of Shire et al. [Shire, S. J., Hanania, G. I. H., & Gurd, F. R. N. (1974) *Biochemistry 13*, 2967] for electrostatic interactions was applied to the hydrogen ion equilibria of human deoxyhemoglobin and oxyhemoglobin. Atomic coordinates for oxyhemoglobin were generated by the application of the appropriate rigid rotation function to α and β chains of the deoxyhemoglobin structure [Fermi, G. (1975) *J. Mol. Biol. 97*, 237]. The model employs two sets of parameters derived from the crystalline protein structures, the atomic coordinates of charged amino acid residues and static solvent accessibility factors to reflect their individual degrees of exposure to solvent. Theoretical titration curves based on a consistent set of pK_{int} values compared closely with experimental potentiometric curves. Theoretical pK values at half-titration for individual protein sites corresponded to available observed values for both quaternary states. The results bring out the cumulative effects of numerous electrostatic interactions in the tetrameric structures and the major effects of the quaternary transition that result from changes in static solvent accessibility of certain ionizable groups.

The classical treatment of hydrogen ion titration curves of proteins regards the molecule as an impenetrable sphere on which amino acid residues are grouped into classes of intrinsically identical sites with their charges uniformly distributed over the surface (Linderstrøm-Lang, 1924). The elegant simplicity of this frequently used model is offset by its inability to yield electrostatic information about individual groups, their specific roles, and their interactions.

In the more realistic discrete-charge electrostatic theory (Tanford & Kirkwood, 1957; Tanford, 1957), the amino acid groups are point charges positioned at fixed sites on the surface of the protein or are buried a short distance within the interior of the molecule which is assumed to be a continuous medium of low dielectric constant. The theory was successfully tested on a variety of model compounds. An early application to hemoglobin was the calculation of electrostatic contributions to the free energy and enthalpy of acid dissociation of the iron-bound H_2O in human ferrihemoglobins A, S, and C; however, this calculation was limited to the mutual effect of two groups only, the iron atom and the amino acid concerned in the substitution (Beetlestone & Irvine, 1964). A full treatment of the hydrogen ion titration curve for tetrameric human hemoglobin was carried out by Orttung (1968, 1969, 1970), although the numerical work was prohibitive. Whereas previous treatments had required burial of the charges up to 1 Å into the low dielectric medium beneath the surface of the molecule, Orttung found that in order to obtain proper fit of the data it was necessary to place all charges at the surface of the hemoglobin molecule. A much more efficient iterative algorithm was developed by Tanford & Roxby (1972) who applied it in analyzing the hydrogen ion titration curve of lysozyme, all charges being assumed in this case to be buried at a uniform depth of 0.4 Å.

In an attempt to overcome the uncertainty over the burial parameter and to allow for the irregular surface of a real protein, Shire et al. (1974a,b, 1975) introduced a modification into the Tanford model whereby, for each individual group, the magnitude of electrostatic intramolecular interaction was reduced in direct proportion to the extent of the group's exposure to the solvent. The degree of exposure of each group is measured by its solvent accessibility parameter (Lee & Richards, 1971). On this new basis, it was shown that the discrete-charge model can be fruitfully employed to study several aspects of electrostatic effects in myoglobin. The theory correctly predicts the individual pK values, determined independently from proton NMR measurements, for histidine ionizations in 12 distinguishable myoglobin species including several histidine residues that are not present in the reference sperm whale myoglobin structure (Botelho et al., 1978). By use of appropriate crystallographic data, the theory has also been shown to account for the hydrogen ion titration behavior of human hemoglobin α chain as well as cytochrome c (Matthew et al., 1978b).

In the present work, the modified Tanford–Kirkwood theory is applied in a detailed study of electrostatic effects in hemoglobin. The generality of this approach is illustrated by the fact that all computations are based on the same consistent set of intrinsic pK values already used (Matthew et al., 1978b; Botelho et al., 1978), with the appropriate solvent accessibility parameter obtained from the known atomic coordinates (Matthew et al., 1978a). The atomic coordinates are derived from a 2.5-Å resolution electron density map of human deoxyhemoglobin (Fermi, 1975). Since corresponding crys-

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