Detergent Inactivation of Sodium- and Potassium-Activated Adenosinetriphosphatase of the Electric Eel†

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ABSTRACT: The stability of the sodium- and potassium-activated adenosinetriphosphatase (Na,K-ATPase) of the electric eel, *Electrophorus electricus*, was studied in five detergents in an effort to establish conditions for reconstitution of this membrane protein into defined phospholipids. The Na,K-ATPase activity of purified electric organ membranes as well as the ATPase is stable for at least 1 month of storage at 0 °C in the absence of detergents. At low concentrations of detergents, the enzyme is also stable for several days, but irreversible inactivation occurs rapidly as the detergent concentration is further increased. This inactivation begins at well-defined threshold concentrations for each detergent, and these concentrations generally occur in the order of the detergent critical micelle concentrations. Increasing the concentration of the electric organ membranes causes a linear increase in the inactivation threshold concentrations of Lubrol WX, deoxycholate, and cholate. The onset of inactivation evidently occurs when the mole fraction of detergent associated with the membrane lipids reaches a critical value in the narrow range of 0.2–0.4, in contrast to the large differences in the bulk concentrations of these detergents. The eel Na,K-ATPase is more sensitive to detergents than the sheep kidney enzyme.

Cells normally have high potassium and low sodium concentrations inside, while outside the cell these relative concentrations are reversed. These concentration gradients are maintained by the integral transmembranous protein Na,K-ATPase,[1] which pumps three sodium ions out for two potassium ions in, using ATP as the energy source (Dahl & Hokin, 1974; Jorgensen, 1975; Skou, 1975). While the Na,K-ATPase is ubiquitous, one of the richest sources is the electric organ of the eel, *Electrophorus electricus*. For isolation of this enzyme, detergents are required for solubilization, although detergent exposure can often lead to inactivation. During the course of establishing appropriate conditions for substituting defined phospholipids for the endogenous lipids using detergents, we encountered difficulties with inactivation of the eel Na,K-ATPase. The purpose of this paper is to report the results of a systematic study of the stability and inactivation of the eel enzyme by detergents, which was carried out to determine the conditions under which the protein remains active after exposure to detergents. The detergents chosen represent those widely used for membrane protein isolation or lipid substitution (Helenius & Simons, 1975; Tanford & Reynolds, 1976). They fall into three classes: (1) the ionic bile salts with rigid steroid structures and fairly high cmc values (>1 mM) (Small, 1971); (2) the nonionic polyethylene oxide derivatives of hydrocarbons, usually chemically heterogeneous and with low cmc values (Helenius & Simons, 1975); and (3) the nonionic alkyl glucosides, chemically homogeneous and with high cmc values for the shorter hydrocarbon chain lengths (Baron & Thompson, 1975; Shinoda et al., 1961).

The results show that this enzyme is quite stable in the absence of detergents and that there is an unanticipated well-defined threshold detergent concentration above which the eel Na,K-ATPase is irreversibly inactivated. For the eel ATPase, this interesting phenomenon is general regardless of the chemical structure of the detergents. The inactivation by detergents can be avoided by working within well-defined limits of detergent/lipid ratios. A general rationale is described for determining these limits in order to provide conditions under which the eel enzyme can tolerate long periods of exposure to detergents.

Experimental Section

Materials. Imidazole (Sigma Chemical Co.) was recrystallized twice from benzene. Na_2ATP from yeast (Sigma Chemical Co.) was neutralized with sodium hydroxide to pH 7.2–7.4 and stored frozen as a 100 mM solution. Silicotungstic acid was obtained from J. T. Baker Chemical Co. Amberlite XAD-2 resin (Supelco, Inc.) was washed with chloroform–methanol (1:1 v/v) and crushed in a mortar to increase the effective surface area. Thin-layer chromatography was performed on silica gel F-254 plates (E. Merck Co.) and column chromatography on silicic acid (Bio-Sil CW from Bio-Rad Laboratories) or neutral aluminum oxide (J. T. Baker Chemical Co.). Reagent grade chloroform was distilled before use for column chromatography. Other reagents were analytical grade, and water was doubly distilled.

Detergents. Cholic acid (Aldrich Chemical Co.) was recrystallized 3 times from 95% ethanol, and sodium cholate was prepared by neutralizing cholic acid with sodium hydroxide to pH 7.4–7.6. Sodium deoxycholate (Nutritional Biochemicals Corp.) was recrystallized twice from 100% ethanol. Lubrol WX (polyethylene oxide adduct of cetyl and stearyl alcohol with an average degree of polymerization of 17, and a nominal molecular weight of 1000, from Supelco, Inc.) was recrystallized from diethyl ether, which removed the water-insoluble impurities. The purified Lubrol WX also gave a neutral solution in water (the crude detergent gave solutions with pH 8–9) and ran as a single spot (R_f 0.5–0.6) on thin-layer chromatography developed with chloroform–methanol–25% ammonia–water (100:15:1:1). Tween 80 (polyethylene oxide adduct of sorbitan monooleate with an average degree

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[1] Abbreviations used: Na,K-ATPase, sodium- and potassium-activated adenosinetriphosphatase; cmc, critical micellar concentration; STED buffer, 0.25 M sucrose, 1 mM Tris, 1 mM EDTA, and 0.5 mM dithiothreitol (pH 7.3); medium I, 2.5 mM ATP in the STED buffer; medium II, 25% (v/v) glycerol, 0.2 M KCl, 5 mM dithiothreitol, and 50 mM KEDTA (pH 7.0).

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of polymerization of 20 and a nominal molecular weight of 1300, from Sigma Chemical Co.) was purified by column chromatography on silicic acid by eluting with 2 and 5% methanol in chloroform. The fraction eluted with 5% methanol was considered as purified Tween 80 and was dried by rotary evaporation followed by an exhaustive desiccation under vacuum. It differed from the crude Tween 80 in giving a neutral solution in water (instead of pH 4–5), not interfering with the phosphomolybdate extraction after the silicotungstic acid precipitation in the enzyme assay, and containing the two main components out of the original five when run on thin-layer chromatography under the same conditions as for Lubrol WX. Octyl glucoside was synthesized by the method of Keana & Roman (1978).

Lipids. Lipids were extracted from electric organ membranes and washed by the method of Bligh & Dyer (1959), and the polar lipid fraction was prepared by column chromatography on silicic acid (Kates, 1972). The main phospholipid components were tentatively identified by two-dimensional thin-layer chromatography (Brotherus & Renkonen, 1974) and quantitated by phosphorus analysis as phosphatidylcholine (43%), phosphatidylethanolamine (33%), phosphatidylserine (18%), phosphatidylinositol (4%), and sphingomyelin (1%). Phosphatidylcholine was purified from hens' eggs essentially by the method of Singleton et al. (1965). Soybean phospholipids (asolectin, from Associated Concentrates) were purified by acetone precipitation according to Kates (1972), but using EDTA instead of $MgCl_2$, followed by partitioning in the Bligh–Dyer solvent system and washing 4 times with the upper phase solution. Phosphatidylcholine was isolated from soybean lipids by column chromatography on aluminum oxide (Renkonen, 1962) and ran as a single spot on thin-layer chromatography. Approximately 0.1% (by weight) of butylated hydroxytoluene (Sigma Chemical Co.) was added to the lipids during solvent partition and after column chromatography, and the solutions were handled under a nitrogen atmosphere to avoid peroxidation. The absence of significant lipid peroxidation was confirmed by ultraviolet spectrophotometry (Klein, 1970). The lipids were stored in chloroform–methanol (4:1) under a nitrogen atmosphere at −20 °C.

Na,K-ATPase Preparations. The electric tissue of *E. electricus* was stored at −60 °C until needed. The electric organ Na,K-ATPase was purified by using Lubrol WX, aminoethylcellulose chromatography, and ammonium sulfate precipitation (Dixon & Hokin, 1978) and stored in 0.25 M sucrose, 1 mM Tris, and 1 mM EDTA, pH 7.3, at −60 °C.

A membrane fraction rich in Na,K-ATPase was prepared from the electric tissue. A piece of the frozen tissue (150 g) was hammered into powder, slurried in 150 mL of 0.25 M sucrose, 30 mM imidazole, 1 mM EDTA, 1 mM ATP, and 0.5 mM dithiothreitol (pH 7.5), and, after complete thawing, forced through holes (1-mm diameter) in a large stainless steel French press [see Kyte (1971)] chilled in an ice bath. All subsequent steps were performed at 0–5 °C. The slurry was diluted with 150 mL of the same buffer and centrifuged in a Sorval SS34 rotor at 12000 rpm for 10 min, yielding a pellet that contained 95% of the Na,K-ATPase activity. The pellet was resuspended in 150 mL of the same buffer and homogenized in a Teflon–glass Potter–Elvehjem homogenizer with a loose-fitting pestle (clearance of 0.3 mm). The homogenate was centrifuged at 10000 rpm for 10 min, and the turbid supernatant was saved. The pellet was rehomogenized in 150 mL of the same buffer and centrifuged at 8000 rpm for 10 min. The combined supernatants were centrifuged in a Beckman SW27 rotor at 27000 rpm for 60 min. The pellets were rehomogenized in 40 mL of the same buffer and centrifuged at 27000 rpm for 120 min. The resulting pellet was homogenized in 40 mL of a hypotonic buffer (Kuriki & Racker, 1976) consisting of 1 mM ATP, 1 mM EDTA, and 0.5 mM dithiothreitol (pH 7.5), frozen in a dry ice–acetone bath, and stored overnight at −60 °C. After being thawed, the solution was layered on 15 mL of 0.25 M sucrose, 1 mM Tris, 1 mM EDTA, and 0.5 mM dithiothreitol, pH 7.3 (STED buffer), and centrifuged at 27000 rpm for 90 min in the SW27 rotor. The pellet was resuspended in 4 mL of the STED buffer (protein concentration ~20 mg/mL) and stored at −60 °C. The Na,K-ATPase activity of these membranes ranged from 10 to 20 μmol of P_i min^{-1} mg^{-1} when measured under maximally stimulated conditions in Tween 80 (see Figure 1), and the recovery was 20–50% of the total activity. The polypeptides of Na,K-ATPase (Dixon & Hokin, 1974) were the most prominent bands in the sodium dodecyl sulfate (NaDodSO$_4$)–polyacrylamide gel electrophoresis of the membranes. The purified Na,K-ATPase used had an activity of 24 μmol of P_i min^{-1} mg^{-1} under the same assay conditions. The microsomes from the sheep kidney medulla were prepared by the method of Kyte (1971), were kindly donated by Teresa Odom, University of Oregon, and were stored at −20 °C. The Na,K-ATPase activity of the kidney microsomes was 1.1 μmol of P_i min^{-1} mg^{-1} when maximally stimulated by cholate. Na,K-ATPase activity of all the preparations was stable for at least 1 year under the storage conditions described.

Na,K-ATPase Activity Assay. Enzyme activity was assayed by the method of Post & Sen (1967), modified in order to avoid detergent interference and to simplify the phosphomolybdate measurement. The assay volume of 0.5 mL contained 120 mM NaCl, 20 mM KCl, 4 mM ATP, 4 mM $MgCl_2$, 30 mM imidazole buffer (pH 7.0), and the indicated concentrations of detergents. The reaction at 37 °C was initiated by the addition of 10–25 μL of the enzyme preparation (equivalent to 0.1–0.4 μg of purified Na,K-ATPase protein) and quenched after 20 min by the addition of 1 mL of ice-cold 3% silicotungstic acid in 0.5 M perchloric acid. The precipitate formed in the presence of Lubrol WX and Tween 80 was removed by centrifugation at 0–5 °C in order to avoid interference with the phosphomolybdate extraction, and the phosphate assays were continued with a 1-mL aliquot of the supernatant. After addition of 1.0 mL of 60 mM sodium molybdate in 2.25 M NaCl, the phosphomolybdate was extracted into 2.5 mL of butyl acetate (Post & Sen, 1967). The absorbance of the butyl acetate phase was measured at 310 nm (Wahler & Wollenberger, 1958), where the molar absorptivity of phosphomolybdate was found to be 26.0 ± 0.4 (SD) mM^{-1} cm^{-1} (13 independent assays). The reaction rate was linear with time and enzyme concentration. In the presence of 0.2 mM ouabain the rate of ATP hydrolysis by the electric organ membranes was less than 2% of the total activity. Since it was not affected by any of the detergents in preliminary experiments, the ouabain-resistant ATPase was usually neglected. The ouabain-resistant ATPase activity of the sheep kidney microsomes was ~24% of the total and was not significantly affected by the detergents used. The activities reported for the sheep enzyme represent the differences between the rates of ATP hydrolysis in the absence and presence of 0.2 mM ouabain.

Incubation of Na,K-ATPase in Detergents. Two media were used to study the stability and inactivation of the enzyme in detergents. Medium I was adapted from the low ionic strength buffer, fortified with ATP, that has been reported to protect Na,K-ATPase against Lubrol WX (Dixon & Hokin, 1974).

It consisted of 2.5 mM ATP in the STED buffer and was used with Lubrol WX and Tween 80. A glycerol-based medium with a high potassium content (medium II) was used with deoxycholate (Kyte, 1971; Lane et al., 1973; Ottolenghi, 1975) as well as with cholate and octyl glucoside. It consisted of 25% (v/v) glycerol, 0.2 M KCl, 5 mM dithiothreitol, and 50 mM KEDTA (pH 7.0). The Na,K-ATPase preparations (0.2–5 mg of protein per mL) were incubated at 0 °C in 100–150 μL of the above media containing various detergent concentrations. Aliquots of 10–25 μL were taken at indicated times and diluted at 0 °C into the same medium so that 10–25 μL of the diluted enzyme suspensions contained sufficient amounts of enzyme for assay of the activity, which was done 10–15 min after the dilution. The individual assays were supplemented with the detergent used so that all assays in the same experiment contained identical detergent concentrations, which were within the optimum regions established earlier (Figure 1).

In one set of experiments, the reversibility of detergent inactivation by added phospholipid was tested. Purified soybean phospholipids were sonicated at 0 °C under nitrogen at a concentration of 5.5 mg/mL in the appropriate medium (I or II). Twelve microliters of the lipid suspension was added to 10-μL aliquots of the detergent-treated electric organ membranes (1 mg of protein per mL). The added lipids (66 μg) represented a 10-fold excess over the endogenous phospholipids of the membranes. The mixtures were incubated at 0 °C for 15 min, diluted with the appropriate incubation medium, and assayed for activity after another 15 min at 0 °C. The controls were treated in the same fashion, except for omission of the added lipid.

In another set of experiments, phospholipids were added together with cholate. Aliquots containing 1–6 μmol of various lipids in small vials were thoroughly dried down under nitrogen. To each vial was added 75 μL of 53.3 mM sodium cholate in 1.33-fold concentrated medium II; the tubes were flushed with argon, capped, mixed, and sonicated in a low-power bath sonicator at 0 °C for an hour. Electric organ membranes (25 μL at 4 mg/mL) were added, giving final concentrations of 1 mg of protein per mL, 40 mM cholate, and 10–60 mM phospholipid. After incubation under argon at 0 °C for 1 day, aliquots were diluted and assayed for enzyme activity.

Removal of Lubrol WX. Electric organ membranes (3 mg of protein, 2.9 μmol of phospholipid) were incubated for 15 min (0 °C) in 0.4 mL of 30 mM Lubrol WX. The sample was centrifuged through a buffered linear sucrose gradient containing 50 μM Lubrol WX, which is in the vicinity of the cmc (Helenius & Simons, 1975) to prevent premature aggregation of the protein during early stages of detergent removal. Fractions containing the protein were pooled and dialyzed at 4 °C for 8 days against four changes of STED buffer, containing crushed XAD-2 resin, and then concentrated by dialysis under reduced pressure. TLC of lipid extracts (from 80 μg of protein) demonstrated that the Lubrol content was well below 1 μg/80 μg of protein.

Other Methods. Protein was analyzed after precipitation with trichloroacetic acid (Jorgensen, 1974b) by the Lowry method, as given by Bailey (1967) and making the reaction mixture 0.1% in sodium dodecyl sulfate. Samples that contained Lubrol WX were kept on a boiling water bath for 5 min after the addition of trichloroacetic acid in order to ensure the complete precipitation of the Na,K-ATPase. Phospholipids were analyzed after extraction (Bligh & Dyer, 1959) by a scaled-down version of the butyl acetate extraction method of Lowry & Tinsley (1974). Polyacrylamide gel electrophoresis followed the method of Fairbanks et al. (1971). Critical

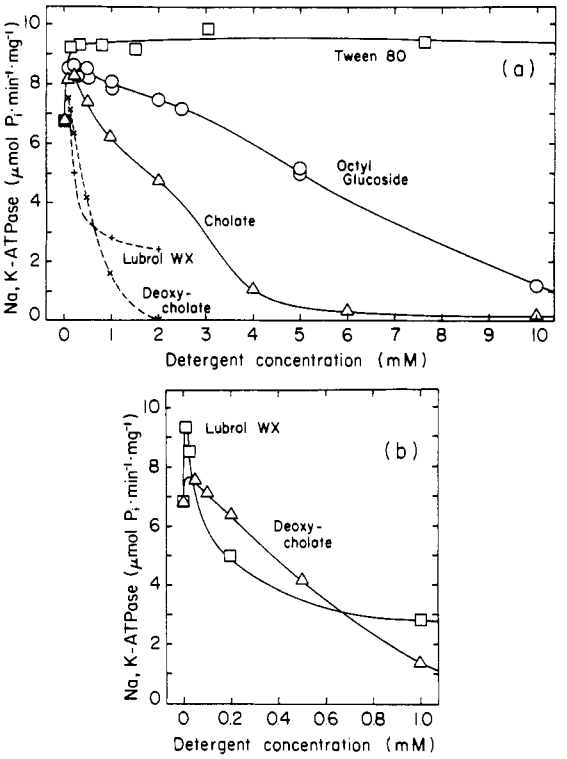

FIGURE 1: Effect of detergents on the activity of Na,K-ATPase. The ATPase reactions were started by adding 25 μL of electric organ membranes (1 μg of protein, 0.8 nmol of phospholipid) into the assay medium containing the indicated amounts of detergents (final concentrations in 0.5 mL final volume) in a 37 °C water bath. The zero-detergent point represents the average of all the detergent-free assays, the 90% confidence interval being between 6.1 and 7.5 μmol of P_i min^{-1} mg^{-1} (n = 12). Figure 1b shows the data for Lubrol WX and deoxycholate on a fivefold expanded concentration scale.

micellar concentrations of octyl glucoside and cholate were determined by using the method of Waggoner et al. (1968), based on the changes in the electron spin resonance spectrum of the spin-label 2,2,6,6-tetramethyl-4-piperidinyl-1-oxy octanoate when it associates with detergent micelles.

Results

Optimum Detergent Concentrations for the Assay of Na,K-ATPase Activity. The rate of ATP hydrolysis by Na,K-ATPase in microsomal preparations is known to be stimulated by low concentrations of detergents in the assay medium, whereas high concentrations decrease the reaction rate (Jorgensen & Skou, 1971; Jorgensen, 1975; Nakao et al., 1973; Swanson et al., 1964). The knowledge of these concentration optima in the assay is essential for the interpretation of the effects of incubation of Na,K-ATPase at higher detergent concentrations. Therefore, the effect of detergent concentration on the rate of ATP hydrolysis by the electric organ membranes was determined for each of the detergents (parts a and b of Figure 1). Low concentrations of all detergents increase the reaction rate sharply. In Tween 80 the rate remains high up to 15 mM (the highest concentration tested). All other detergents have fairly well-defined optimum concentration regions, after which the enzyme activity is progressively inhibited. The optimum regions are characteristic for each detergent and are listed in Table I. It should be noted that the positions of the optima and the rates of subsequent inhibition may depend on the assay conditions, e.g., on the membrane concentration in the assay, and on the incubation time.

Inactivation of Na,K-ATPase When Incubated at Higher Detergent Concentrations before Assay. The effect of deter-

Table I: Optimum Detergent Concentrations for the Na,K-ATPase Assay of Electric Organ Membranes[a]

detergent	concn (mM) in the assay at: max act.	80% of the max	50% of the max
octyl glucoside	0.1–0.5	3	6
cholate	0.05–0.2	1	2
deoxycholate	0.05[b]	0.3	0.6
Lubrol WX	0.01[b]	0.05	0.3
Tween 80	0.05–15	[c]	[c]

[a] The same membrane concentration (2 μg of protein per mL) was used in all assays. [b] Based on one point in the optimum region. The lower limit of the region may be a factor of 2 smaller. [c] The activity in Tween 80 was over 80% of the maximal value at all concentrations tested.

Table II: Composition of the Incubation Mixtures (Figure 2) at the Threshold Concentrations of Detergents[a]

detergent	threshold concn (mM)	cmc (mM)	detergent/protein (mg/mg)	detergent/phospholipid (mol/mol)
octyl glucoside[b]	10	22[e] (25[f])	2.9	14
cholate[b]	15	12[e] (3-20)[g]	6.5	21
deoxycholate[b]	2.5	1–5[g]	1.0	3.5
Lubrol WX[c]	0.2[d]	0.02–0.06[h]	0.2	0.21
Tween 80[c]	<1.5	~0.01[h]	<2	<2

[a] Membrane concentration, 1 mg of protein per mL; incubation time, 1 day (threshold concentration is defined in the text). [b] Medium II. [c] Medium I. [d] Crude Lubrol WX gives a slightly higher threshold concentration (~0.3–0.4 mM). [e] This study. [f] Shinoda et al. (1961). [g] Small (1971). [h] Helenius & Simons (1975).

gent concentration on the stability of Na,K-ATPase was studied in two media that are known to protect the enzyme against inactivation by either Lubrol WX (medium I) or deoxycholate (medium II). The activity was assayed at various times of incubation of the electric organ membranes, and care was taken to dilute the samples down to the noninhibitory concentration regions of the detergents in the final assay volume. Figure 2 shows the results for short (10–15 min) and long (5–8 days) incubation times.

In the absence of detergent the eel enzyme is quite stable in both incubation media, retaining ~90% of its initial activity even after 1 month of storage at 0 °C. All of the detergents have qualitatively similar effects on the stability of Na,K-ATPase. The enzyme is stable (or even seems to be slightly stimulated) in the presence of the detergents up to fairly well-defined concentrations, beyond which the full activity is not restored by dilution. In the following we use the term *threshold concentration* to indicate the highest detergent concentration in which the Na,K-ATPase is stable for extended periods (days). The threshold concentration for Lubrol WX fell below the region chosen for the first experiment (Figure 2e) and was subsequently determined at the surprisingly low level of 0.2 mM (Figure 2f). In Tween 80 the activity initially stayed high (tested after 15 min of incubation; Figure 2d) but then began to decrease even at the lowest concentration used (1.5 mM). The compositions of the incubation mixtures at the threshold concentrations are summarized in Table II.

The rates of inactivation are complex. In general, a large drop of activity occurs rapidly (in 10–15 min; see Figure 2), followed by a slow rate of inactivation. Semilogarithmic plots of the activity for each detergent during a period of 30–40 days demonstrated that the decay rate was slower than a first-order reaction. This is in contrast to the findings for the detergent-induced inactivation of rhodopsin (Stubbs & Litman, 1978) and the sodium channel protein (Agnew & Raftery, 1979).

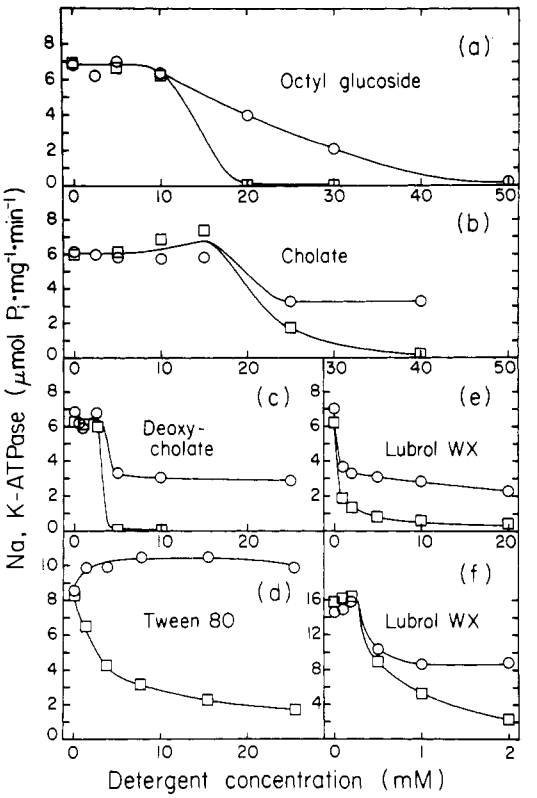

FIGURE 2: Stability of Na,K-ATPase in detergents. Electric organ membranes (1 mg of protein per mL, 0.7–0.9 mM phospholipid) were incubated at various detergent concentrations at 0 °C. Samples were diluted in detergent-free incubation media and assayed for ATPase activity. The final detergent concentrations in the assay medium were adjusted to within the optimal regions given in Table I. Tween 80 concentrations below 1.5 mM were not studied to find the putative threshold concentration range. Note that after 15 min of incubation, Tween 80 did not inactivate at any concentration, but after 6 h all concentrations tested showed some inactivation. Most of the experiments were conducted by using the same batch of electric organ membranes, except the last one (f), where another batch with a higher activity was used. Note that the concentration scale in (f) has been expanded 10-fold from that of the other figures. (○) Short incubation time (10 min, a–c; or 15 min, d–f); (□) longer incubation time (5 days, b and c; 7 days, a, d, and e; or 8 days, f).

When purified eel Na,K-ATPase was incubated in the presence of Lubrol WX under the same conditions as for the electric organ membranes (1 mg of protein per mL in medium I), no initial region of stability was observed and the inactivation began at the lowest concentration added (0.05 mM Lubrol WX). This is evidently largely due to the residual detergent in the purified enzyme. A Lubrol WX content of 0.3 mg/mg of protein (Dixon & Hokin, 1978) corresponds to a concentration of ~0.3 mM under the present conditions, which is only slightly higher than the threshold concentration (~0.2 mM) that we have observed for the microsomal Na,K-ATPase.

Irreversibility of the Detergent-Induced Inactivation. Detergent-inactivated Na,K-ATPase preparations from other species can often be reactivated by phospholipids [see Kimelberg (1976)]. To test this possibility, we added a 10-fold excess of sonicated soybean phospholipids to samples of electric organ membranes after 5 h of incubation in the detergents. The added phospholipids had no effect on reversing the inactivation at any detergent concentration.

In another experiment, no change in the activity of cho-

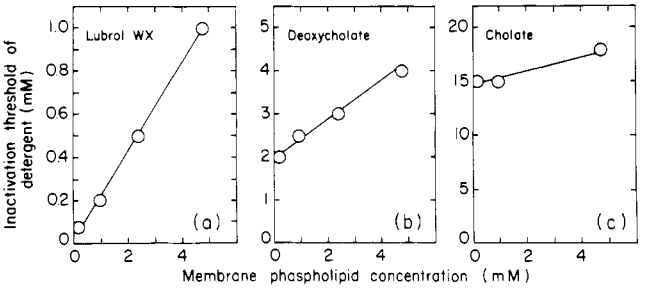

FIGURE 3: Dependence of the inactivation threshold concentration of three detergents on the concentration of membranes in the incubation mixture. Each point is based on an experiment similar to those in Figure 2, except that the electric organ membrane protein concentration was varied between 0.2 and 5 mg/mL (phospholipid concentration 0.19–4.75 mM) and the detergent concentration was sampled at smaller intervals. The incubation time was 1 day.

late-treated samples was observed after a 2-day period at 0 °C following dilution. This excludes the possibility that slow dissociation of this detergent from the membrane might occur with partial restoration of activity after some delay. Essentially complete removal of Lubrol WX from the detergent-inactivated membranes (down to the level of less than 1 molecule/140 000-dalton unit of Na,K-ATPase, corresponding to a concentration of 0.005 μM Lubrol WX or less in the final Na,K-ATPase assay) failed to reactivate the enzyme. No stimulation was achieved by adding sonicated soybean phospholipids to the Lubrol-free protein, which retained ~0.2 mg of phospholipid per mg of protein. Treatment of the purified eel Na,K-ATPase with Lubrol WX under the same conditions as those described for the electric organ membranes, using the same method of Lubrol removal, completely inactivated the enzyme, which retained 0.18 mg of phospholipid per mg of protein out of the original 0.26 mg/mg. Exhaustive dialysis was not in itself responsible for the loss of activity, since a sample of purified electric organ Na,K-ATPase dialyzed as a control retained 85% of its original activity.

Effects of Membrane Concentration. The threshold concentration of Lubrol WX, deoxycholate, and cholate was determined for widely varying concentrations of the electric organ membranes. In all cases the threshold concentration increases linearly with the membrane concentration (Figure 3). The slopes of the lines are within a factor of 3, whereas the intercepts are different by several orders of magnitude. The intercept for Lubrol WX is so close to zero that the threshold concentration is practically directly proportional to the membrane concentration, whereas for cholate the threshold at the highest membrane concentration tested (5 mg of protein per mL) is only ~20% higher than the intercept. Deoxycholate represents an intermediate case.

Protection of the Na,K-ATPase Activity by Phospholipids. Although added phospholipids did not restore lost Na,K-ATPase activity, they did stabilize the enzyme against inactivation by cholate when mixed with the detergent before adding the electric organ membranes. This stabilization is similar to the observations of Agnew & Raftery (1979) with Lubrol PX and the tetrodotoxin-binding protein of the electric organ membranes. This protective effect was dependent on both the kind and the amount of added phospholipid. The polar lipid fraction from the electric organ membranes was the most efficient of the lipids tested, preserving the Na,K-ATPase activity close to the level of the untreated enzyme after 1 day of incubation in 40 mM cholate (approximately threefold higher than the threshold concentration in the absence of added lipid). Even after 8 days the enzyme still retained ~50% of the control activity in the presence of 20 mM or more of eel phospholipids in 40 mM cholate. Soybean phospholipids also have a considerable protective effect at equivalent concentrations. Phosphatidylcholine purified from eggs or from soybean phospholipids did not protect the enzyme activity at low concentrations, although at high concentrations (40–50 mM) the efficiency of the phosphatidylcholines approached that of the soybean phospholipid mixture. Repurification of the soybean lipids or egg phosphatidylcholine by column chromatography did not improve their efficiency.

Comparison of Eel Electric Organ and Sheep Kidney Na,K-ATPase. The electric organ Na,K-ATPase has been observed to be less stable in Lubrol WX than the enzyme from the rectal gland of the dogfish shark (Dixon & Hokin, 1974), suggesting that Na,K-ATPase from different sources may not have the same response to detergents. For a direct comparison under our conditions, the effect of cholate and octyl glucoside on the Na,K-ATPase activity and stability of sheep kidney microsomes was examined. The optimum detergent concentrations in the assay medium are similar for both enzymes. The sheep kidney Na,K-ATPase is less susceptible to inhibition by the detergents above the optimum concentrations than is the eel electric organ enzyme. The sheep kidney Na,K-ATPase is also more stable when incubated at high detergent concentrations (Figure 4) before dilution for assay. This difference is dramatic for cholate, where incubation in 20 mM cholate for 1 to 2 days almost doubles the activity of the sheep kidney enzyme, and even in 40 mM cholate the activity remains higher than that of the unincubated sample.

Discussion

Three Stages of Detergent Action on Na,K-ATPase. Despite the differences in structure and properties of the detergents studied, their effects on the Na,K-ATPase were qualitatively similar. With increasing concentrations of the detergents, three effects on the Na,K-ATPase activity of the electric organ membranes are observed. (1) Low concentrations stimulate the rate of ATP hydrolysis. (2) Intermediate concentrations reversibly inhibit the activity. (3) Incubation at higher detergent concentrations inactivates the enzyme irreversibly.

The stimulation by low detergent concentrations in the assay medium may be due to an increased permeability of a population of closed vesicles (Jorgensen & Skou, 1971; Jorgensen, 1974a,b, 1975). However, the marked stimulation of the kidney Na,K-ATPase by preincubation in cholate (Figure 4) is difficult to explain, since all assays were adjusted to the same final concentration (0.2 mM) of cholate, the concentration that had given the maximum enzyme activity in the assay of the untreated samples.

As the detergent concentration in the assay medium increases, the activity generally passes through an optimum and decreases at higher concentrations. The inhibition is reversible at intermediate detergent concentrations (compare Figures 1 and 2). This inhibition may involve an interaction between the detergent monomers and the enzyme since it begins at concentrations well below the cmc values of octyl glucoside, cholate, and deoxycholate. Tween 80 differs from the other detergents in that it does not inhibit the enzyme under the assay conditions used even at high concentrations (15 mM). This is a useful property since Tween 80 can be employed over a wide concentration range to stimulate the latent activity. However, this lack of inhibition over the period of the assay is apparently due to a slow rate of interaction with the enzyme since preincubation of the membranes in Tween 80 for 6 h or more substantially decreases the activity (Figure 2).

Incubation in higher concentrations of all detergents used inactivates the electric organ Na,K-ATPase irreversibly. The

onset of inactivation is sharp enough to allow a reasonably precise definition of the highest concentration (threshold concentration) at which the enzyme remains fully active even when exposed to the detergent for extended periods of time. The threshold concentrations are generally in the order of the cmc values of the detergents (Table II).

The nature of the changes responsible for detergent inactivation is not known. The inactivation is apparently not due to the persistence of protein–detergent association since removal of the detergent does not reverse the inactivation. Inactivation occurs before substantial delipidation of the protein is achieved, and added phospholipid is ineffective in restoring activity. The sigmoidal shape of the curves describing the activity vs. detergent concentration in the incubation mixture (Figure 2) indicates that the process is cooperative; i.e., several detergent molecules are involved in the inactivation of one unit of Na,K-ATPase [see Tanford (1970)].

Dependence of the Inactivation Threshold on Membrane Concentration. The mechanism of detergent inactivation of membrane enzymes at present is not well understood. The detergent may partition between the membrane phase, the aqueous phase, and any detergent micelles present. In view of the complexities of the system, it is interesting to note that the inactivation threshold is linearly related to the membrane lipid concentration (Figure 3). This can be accounted for by a variation[2] of the well-known partition model (Glasstone, 1946; Seeman, 1972; Kaufman, 1977; Hill, 1974; Zaslavsky et al., 1978). The expression for the threshold concentration C_D^*, as a function of the membrane lipid concentration C_L, is $C_D^* = X^*(1 - X^*)^{-1}C_L + (X^*/K)$, where X^* is the mole fraction of the detergent in the hydrophobic phase at the onset of inactivation (critical mole fraction), $K = P/C_A$, P is the lipid–water partition coefficient, and C_A is the relatively constant concentration of substances other than detergent in the aqueous phase (water, sucrose, glycerol, etc.; so $C_A \sim 50$ M). In Figure 3, the slope $X^*(1 - X^*)^{-1}$ gives the value of the critical mole fraction X^*, and this combined with the intercept (X^*/K) gives an estimate of the partition coefficient. The intercept itself gives the aqueous concentration of detergent in equilibrium with the hydrophobic phase. The calculated values of X^* are 0.2 (Lubrol WX), 0.3 (deoxycholate), and ~0.4 (cholate). The corresponding values of P are approximately 5×10^5, 7×10^3, and 1×10^3, respectively.

The critical mole fraction of detergent (X^*), representing the onset of irreversible inactivation, provides a basis for the comparison of the effects of detergents on Na,K-ATPase because it is independent of membrane concentration. It also factors out the effect of the partition coefficient, which reflects the general lipophilicity of the detergent. The critical mole fractions are similar for all three detergents in spite of the large differences in their partition coefficients and structures. This suggests that the mechanism of inactivation is similar in each case, in agreement with the similarity of the shapes of the stability vs. detergent concentration curves (Figure 2). It is interesting to note that this behavior is similar to Meyer–Overton rule for anesthetic action (Seeman, 1972; Kaufman, 1977), i.e., that the onset of anesthesia occurs over a narrow range of mole fractions in the membrane, irrespective of the structure of the anesthetic.

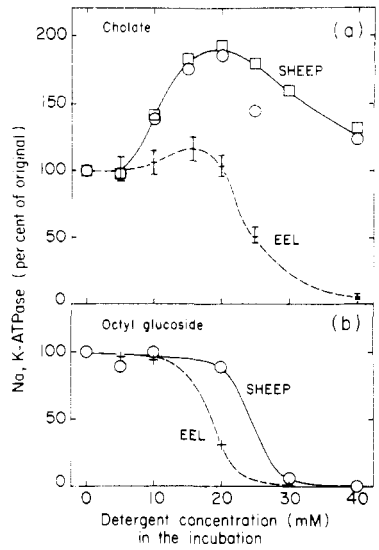

FIGURE 4: Comparison of the stability of the sheep kidney and eel electric organ Na,K-ATPase in cholate and octyl glucoside. The conditions for the kidney microsomal ATPase were the same as those in Figure 2, except that a protein concentration of 2 mg/mL (instead of 1 mg/mL) was used. This is not expected to change the effects of these two high cmc detergents significantly (see Figure 3). For the electric organ Na,K-ATPase incubated in cholate, the mean and range of three independent experiments are shown. The symbols refer to data points taken after 1 day of incubation except for □, where the incubation time was 2 days.

For the detergents, the effective partition coefficients are spread over 2 orders of magnitude. This reflects the large differences between the affinities of the detergents for the lipid phase. The partition coefficients are in the same relative order as the cmc values. Published data on the membrane–water partitioning of detergents are scant. Helenius et al. (1976) measured the binding of deoxycholate to the membrane of an animal virus. Their reported value for the partitioning of deoxycholate corresponds to a partition coefficient of $\sim 5 \times 10^3$, using our notation, and is in a good agreement with the value of 7×10^3 that we have estimated for the electric organ membranes.

Comparison of the aqueous concentrations of the detergents at the onset of inactivation (i.e., the intercept X^*/K in the equation and Figure 3) provides insight for the choice of conditions when using different detergents. The aqueous concentration of Lubrol WX is low, on the order of 20 μM. In practice, the membrane concentration is usually so high that $C_L X^*/(1 - X^*) \gg X^*/K$; i.e., a very little detergent remains in the aqueous phase. Therefore, the threshold concentration is almost proportional to the membrane concentration (Figure 3), and the amount of Lubrol WX used must be adjusted relative to the membrane concentration in order to avoid the inactivation of the Na,K-ATPase. Under the conditions used for the purification of the electric organ Na,K-ATPase (Dixon & Hokin, 1974, 1978; Perrone et al., 1975), the membrane concentration is very high (wet pellet) when the detergent is added, so that the high concentration of Lubrol WX does not cause irreversible inactivation.

In the case of cholate, the aqueous detergent concentration at the onset of inactivation is so high that $C_L X^*/(1 - X^*) \ll$

[2] The partition coefficient P is defined by $P = X_D^h/X_D^a$, where X_D^h and X_D^a are the mole fractions of the detergent in the hydrophobic phase (membrane plus any micelles present) and the aqueous phase, respectively. The total number of moles of detergent is $n_D = n_D^h + n_D^a = X_D^h(n_L + n_D^h) + X_D^a(n_A + n_D^a)$, where n_A is the number of moles of the other substances in the aqueous phase and n_L is the number of moles of phospholipid in the lipid phase. Substitution of $X_D^a = X_D^h/P$ and $n_L + n_D^h = n_L/(1 - X_D^h)$, followed by division by the volume of the system to convert the number of moles into concentrations C (in moles per liter), gives $C_D = X_D^h(1 - X_D^h)^{-1}C_L + X_D^h P^{-1}(C_A + C_D^a)$. A good approximation is that $C_D^a \ll C_A$ and $C_L \ll C_A$ (typically, C_D^a and $C_L < 20$ mM and $C_A \simeq 50$ M). Thus, $C_D = X_D^h(1 - X_D^h)^{-1}C_L + X_D^h P^{-1}C_A$. This equation is applicable over the full range of X_D^h, but in the text it is used to characterize the specific value (X^*) associated with the inactivation threshold.

X^*/K at all reasonable membrane concentrations. Therefore, the threshold concentration of cholate changes relatively slowly with membrane concentration (Figure 3), and for practical purposes it may be accurate enough to assume that the threshold concentration is independent of the membrane concentration. Deoxycholate is an intermediate case since the amounts of the detergent in the aqueous phase and in the membrane lipid phase are of the same order of magnitude at experimentally useful conditions.

The choice of detergent, therefore, will determine the importance of the control of the membrane (lipid) concentrations. The relationship between the effective partition coefficients and cmc values suggests a rule of thumb for the use of detergents with this enzyme. When the cmc is much lower than the membrane lipid concentration used, practically all detergent is expected to be in the membrane phase and the detergent/lipid ratio dominates the inactivation. If the cmc is much higher than the lipid concentration, the bulk of the detergent is in the aqueous phase and, therefore, the total detergent concentration largely determines the onset of the inactivation. When the cmc is in the region of the lipid concentrations employed, the inactivation depends both on the detergent and on the membrane lipid concentrations. In general, the safe limits for these variables can be estimated from standard curves like those in Figure 3.

Relative Stability of Eel and Sheep Kidney Enzymes. The electric organ Na,K-ATPase appears to be unusually vulnerable to inactivation by detergents when compared to the enzyme from other sources. When tested under the same experimental conditions, our data show that the electric organ enzyme is considerably less stable than the kidney enzyme at higher concentrations of cholate and octyl glucoside. Deoxycholate concentrations of 5–14 mM have been used for the purification (Jorgensen et al., 1971; Kyte, 1971; Lane et al., 1973; Jorgensen, 1974b) and reversible delipidation (Tanaka & Strickland, 1965; Kimelberg & Papahadjopoulos, 1972) of Na,K-ATPase from the mammalian kidney and brain, as well as the enzyme from the shark rectal gland (Ottolenghi, 1975). Relatively high concentrations of Lubrol WX (typically 10–40 mM at a protein concentration of 2 mg/mL) can be used for the reversible partial delipidation of the kidney Na,K-ATPase (Palatini et al., 1972; Wheeler et al., 1975) and for the solubilization of the shark rectal gland enzyme (Hastings & Reynolds, 1979). All of these detergent levels are much higher than the threshold concentrations for the inactivation of the electric organ Na,K-ATPase.

A practical consequence of the exceptional detergent sensitivity of the electric organ Na,K-ATPase is that careful control of the amount of detergent is important for successful purification (Dixon & Hokin, 1978; Perrone et al., 1975). Our preliminary experiments (unpublished data) also demonstrate that cholate at concentrations below the inactivation threshold may be used for partial lipid substitution and partial lipid depletion of the electric organ Na,K-ATPase without reducing the enzyme activity.

In summary, the eel Na,K-ATPase is inherently stable but is exceptionally sensitive to detergent inactivation when compared to the sheep kidney enzyme. Low concentrations of detergents increase the observed enzymatic activity. Slightly higher concentrations result in reversible inactivation. At well-defined concentrations there is a threshold beyond which irreversible inactivation occurs. By controlling the membrane and detergent concentrations within the limits presented here, this inactivation can be avoided.

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Creatine Kinase. Nuclear Magnetic Resonance and Fluorescence Evidence for Interaction of Adenosine 5′-Diphosphate with Aromatic Residue(s)†

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ABSTRACT: The structural features of the purine binding site of creatine kinase (CPK) were explored by ^{1}H NMR spectroscopy at 360 MHz, using the measurement of "truncated driven nuclear Overhauser effects" (TOE). Irradiation of the adenine C-2 and C-8 proton resonances in the CPK–ADP complex by this technique resulted in the negative enhancement of a number of resonances of the protein (intermolecular NOE's). Two of the affected resonances coincide with the irradiation frequencies shown previously to induce negative intermolecular NOE's in the adenine C-2 proton resonance of bound ADP [James, T. L. (1976) *Biochemistry 15*, 4724]. The occurrence of several NOE's in the aromatic region between 6.5 and 8.0 ppm is compatible with the location of one or more aromatic side chains near the adenine ring in the CPK–ADP complex. Independent evidence for an interaction between the purine moiety of the coenzyme and aromatic amino acid chromophores comes also from quenching studies of protein fluorescence. Binding of nucleoside phosphates reduces tryptophan emission of CPK. The extent of quenching by ADP and GDP corresponds to the relative magnitudes of the Förster overlap integrals, thus suggesting a resonance transfer mechanism. Since the calculated critical Förster distance for resonance transfer between ADP and the affected tryptophanyl residues in CPK is not larger than 5 Å, at least one tryptophanyl residue must be located in the immediate vicinity of the purine binding site of CPK. The data are in accordance with our previous proposal that the coenzyme-induced Cotton effects at 260 nm arise from a dipole–dipole interaction of the adenine transition with a nearby aromatic oscillator.

The structure of the binding sites of CPK[1] for ADP/ATP and creatine phosphate/creatine has been under investigation for a long time. Chemical modification studies carried out in the presence and absence of coenzymes and substrates have identified a number of amino acid residues located at or near the active center of CPK. Thus, the reaction with certain thiol reagents resulted in derivatization of a single active site cysteine (Mahowald et al., 1962) whose proximity to the substrate binding sites was demonstrated by magnetic resonance techniques using a covalently attached spin-label (Taylor et al., 1971; McLaughlin et al., 1976). The existence of one essential lysyl residue per subunit was deduced from the reaction with dansyl chloride (Kassab et al., 1968). Similarly, the involvement of arginine was inferred from the effect of butadiene or phenylglyoxal on the nucleotide binding properties of CPK (Borders & Riordan, 1975). Independent support for the location of both lysine and arginine in the active site was recently obtained also by James & Cohn (1974) and by James (1976), who observed a negative intermolecular NOE between resonances assigned to lysine and arginine and that of the ADP C-2 proton of the coenzyme in the ^{1}H NMR spectra of the enzyme–coenzyme complexes. An indication for an involvement of histidyl residues was provided by chemical modification with diethyl pyrocarbonate. Thus, Pradel & Kassab (1968) reported modification of one histidyl residue per subunit with concomitant complete loss of enzyme activity.

Except for a report that the inactivation of the enzyme by iodination is accompanied by the modification of two to three tyrosyl residues per subunit, no chemical evidence is available for the presence of aromatic amino acid residues in the active site of CPK (Fattoum et al., 1975). There are, however, data on record that the optical absorption of the aromatics in the enzyme is perturbed when the binary enzyme–coenzyme or

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[1] Abbreviations used: CPK, creatine kinase (adenosine 5′-triphosphate:creatine *N*-phosphotransferase, EC 2.7.3.2); NOE, nuclear Overhauser effect; TOE, truncated driven nuclear Overhauser effect; TSP, sodium 3-(trimethylsilyl)[2,2,3,3-$^2H_4$]propionate; CD, circular dichroism; ORD, optical rotatory dispersion.

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