Biochimica et Biophysica Acta, 599 (1980) 436—447

BBA 78768

STRUCTURAL CHANGES IN ($Na^+ + K^+$)-ATPase ACCOMPANYING DETERGENT INACTIVATION

LELAND D. POWELL * and LEWIS C. CANTLEY

Department of Biochemistry and Molecular Biology, Harvard University, Cambridge, MA 02138 (U.S.A.)

(Received October 15th, 1979)

Key words: Detergent inactivation; Digitonin; ($Na^+ + K^+$)-ATPase; Oligomer

Summary

Structural changes in the purified ($Na^+ + K^+$)-ATPase accompanying detergent inactivation were investigated by monitoring changes in light scattering, intrinsic protein fluorescence, and tryptophan to β-parinaric acid fluorescence resonance energy transfer. Two phases of inactivation were observed using the non-ionic detergents, digitonin, Lubrol WX and Triton X-100. The rapid phase involves detergent monomer insertion but little change in protein structure or little displacement of closely associated lipids as judged by intrinsic protein fluorescence and fluorescence resonance energy transfer. Lubrol WX and Triton X-100 also caused membrane fragmentation during the rapid phase. The slower phase of inactivation results in a completely inactive enzyme in a particle of 400 000 daltons with 20 mol/mol of associated phospholipid. Fluorescence changes during the course of the slow phase indicate some dissociation of protein-associated lipids and an accompanying protein conformational change. It is concluded that non-parallel inhibition of ($Na^+ + K^+$)-ATPase and *p*-nitrophenylphosphate activity by digitonin (which occurs during the rapid phase of inactivation) is unlikely to require a change in the oligomeric state of the enzyme. It is also concluded that at least 20 mol/mol of tightly associated lipid are necessary for either ($Na^+ + K^+$)-ATPase or *p*-nitrophenylphosphatase activity and that the rate-limiting step in the slow inactivation phase involves dissociation of an essential lipid.

---

* Present address: Johns Hopkins University School of Medicine, Baltimore, MD.

Abbreviations: PA-microsomes, purified eel electroplax ($Na^+ + K^+$)-ATPase with incorporated β-parinaric acid; SDS, sodium dodecyl sulfate; CMC, critical micelle concentration; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

Introduction

The Na^+- and K^+-stimulated adenosine triphosphatase, ($Na^+ + K^+$)-ATPase maintains cellular ion gradients by transporting Na^+ and K^+ across the plasma membrane. It is an intrinsic membrane protein and has been purified from a number of tissues (for reviews see Refs. 1—3). Preparations containing a 100 000 dalton peptide (α) and a 40 000 dalton glycopeptide (β) are capable of active transport in lipid vesicles [4,5]. The ATP hydrolysis site is on the α chain and the role of the β chain is unknown. The smallest detergent solubilized and active enzyme appears to contain two α subunits and at least two β subunits with associated lipid [30,31].

Recently there has been much work toward understanding protein-lipid and protein subunit interactions on membrane transport systems. Conformational interactions between proteins and lipids have been clearly demonstrated in lipoproteins [6]. The role of membrane protein subunits and their interactions have been well characterized in the thermophilic bacterial ATPase the subunits of which have been successfully dissociated, purified and reconstituted to form an active protein pump [7]. However, attempts at separating and reconstituting the subunits of the ($Na^+ + K^+$)-ATPase have been unsuccessful thus far.

Detergents have been used extensively to purify the ($Na^+ + K^+$)-ATPase and to characterize its structure. Ottolenghi [8] showed that although complete delipidation with deoxycholate results in complete loss of hydrolysis activity, relipidation completely restores activity. Clark [9] and Winter [10] showed that, when treated with the non-ionic detergents, Triton X-100 or digitonin, the enzyme is solubilized into a particle containing one α and one or two β subunits but with no ATPase activity. Non-ionic detergents have also been shown to inhibit the ($Na^+ + K^+$)-ATPase activity more readily than some partial activities (including the *p*-nitrophenylphosphatase activity) under certain conditions [11—14]. These results suggest some partial activities may exist in smaller oligomeric states of the ($Na^+ + K^+$)-ATPase.

In this study we use light-scattering, fluorescence and gel filtration techniques to monitor structural changes in the ($Na^+ + K^+$)-ATPase accompanying detergent inactivation. The eel electroplax enzyme (purified to approx. 85% homogeneity in the absence of detergents [15]) is used and the structural changes are monitored in the same time range and at the same detergent and protein concentrations used in the inactivation measurements.

Materials and Methods

Materials. Lubrol WX, digitonin, Triton X-100, ATP (Grade 2) and *p*-nitrophenylphosphate were from Sigma Chemical Co. β-Parinaric acid was a gift from Anil Lala (Harvard University) and was purified of oxidation products by the procedure of Fraley et al. [16] and stored under N_2 at $-70°$ in a methanol solution. All other reagents were of highest quality commercial grades. Deionized, distilled water was used for all solutions. Digitonin stock solutions were periodically heated briefly to keep the detergent in solution. The critical micelle concentration (CMC) of Lubrol WX was determined by changes in the 250 nm light scattering in an SLM 4000 fluorometer with no filters on the

emission side. The CMC of Triton X-100 was determined as described by Clarke [9].

($Na^+ + K^+$)-ATPase preparation. The ($Na^+ + K^+$)-ATPase was prepared from the electric organ of the electric eel by a sonication and sucrose gradient procedure as previously described [15]. The preparations were more than 80% pure as judged by gel electrophoresis and had specific activities of 15—20 μmol/mg per min at 37°C. Where specified in Results, the canine kidney ($Na^+ + K^+$)-ATPase (prepared by the procedure of Jørgensen [17]) was used. Protein was determined by the procedure of Lowry et al. [18] in the presence of 0.1% sodium dodecyl sulfate.

Preparation of PA-microsomes. Microsomes of eel electroplax tissue were labeled with β-parinaric acid by sonication. A sample of β-parinaric acid (33 μg) in methanol was evaporated to dryness under N_2 and 20 μl of dimethylformamide added, followed by 0.4 ml of purified microsomes (4.4 mg/ml protein). The dimethylformamide was necessary to prevent the β-parinaric acid from sticking to the glass. Sonication was performed with a Branson W 350 Sonifier, equipped with the cup horn attachment, level 4 intensity, 10 min duration, at 24—26°C. As a control, 0.4 ml of microsomes was sonicated with 20 μl of dimethylformamide alone. These two preparations of tissue will be referred to as the PA-labeled and control microsomes, respectively. The amount of β-parinaric acid used was sufficient to ensure that A_{280} of the PA-labeled microsomes was three times that of the control microsomes. The specific activities of the two preparations were measured relative to untreated enzyme. Both the *p*-nitrophenylphosphatase and ATPase activities of the control microsomes were 96% of untreated microsomes; both activities were 64% of untreated microsomes for the β-parinaric acid labeled enzyme. Inhibition by long-chained unsaturated fatty acids of the ATPase has been reported [19].

Activity measurements. ($Na^+ + K^+$)-ATPase activity was monitored in a Cary 15 recording spectrophotometer using the coupled assay of Barnett [20]. Assays were at 37°C and contained 1.4 mM phospho*enol*pyruvate, 0.26 mM NADH, 10 μg/ml each of pyruvate kinase and lactate dehydrogenase, 100 mM NaCl, 20 mM KCl, 20 mM Hepes-triethylamine (pH 7.4), 5 mM $MgCl_2$, 3.7 mM ATP, 1 mM dithiothreitol and 2 mM norepinephrine (to complex any residual vanadate [21]). In the absence of detergent these assays were linear for more than 20 min. Detergents were added subsequent to the ($Na^+ + K^+$)-ATPase, and activities at various times after addition were determined from tangents to the product vs. time curve. The detergents added did not affect the coupling enzymes. Detergent and protein concentrations are given in the figure legends. Experiments involving high protein concentrations were performed in an assay buffer lacking Mg^{2+}, and activities at various times after detergent addition were determined by diluting the mixture into a complete assay buffer and measuring initial velocities within 30 s.

p-Nitrophenylphosphatase activities were monitored at 410 nm in a Cary 15 spectrophotometer thermostatically maintained at 37°C. All assays contained 25 mM KCl, 20 mM Hepes-triethylamine (pH 7.4), 5 mM $MgCl_2$, 2.5 mM *p*-nitrophenylphosphate, 1 mM dithiothreitol, and 2 mM norepinephrine. Raising the ionic strength to 125 mM KCl to mimic that in the ($Na^+ + K^+$)-ATPase assay had no significant effect on the time course of detergent inactiva-

tion. Time-dependent activities were measured as described for the $(Na^+ + K^+)$-ATPase assay. Detergent and protein concentrations are given in the figure legends.

Fluorescence and light scattering. All fluorescence and light-scattering measurements were made on either an SLM 4000 spectrofluorimeter or a Farrand Mark I spectrofluorimeter, each equipped with a cell-holder thermostatically maintained at 37°C. Light scattering was monitored with excitation and emission at 400 nm. Protein fluorescence was excited at 290 nm and monitored at 340 nm. The excitation shutter was closed between measurements to prevent heating. All fluorescence and light-scattering measurements were made in 100 mM NaCl, 20 mM KCl, 20 mM Hepes-triethylamine (pH 7.4), 5 mM $MgCl_2$, 50 μM ATP and 1 mM dithiothreitol (i.e., the $(Na^+ + K^+)$-ATPase assay buffer without the coupling system).

Gel filtration. A column of Sepharose 4B (1.4 × 44 cm) was prepared and run at room temperature in the buffer used for fluorescence measurements plus 0.015% Lubrol WX. Calibration was achieved using the following markers (followed by method of detection): *Limulus* hemocyanin (A_{280}); ferritin (horse, A_{420}); catalase (enzymic assay); lactate dehydrogenase (enzymic assay); bovine serum albumin (A_{280}). Blue dextran (A_{610}) and AMP (A_{259}) served as void and total markers, respectively. All runs of solubilized ATPase included catalase and AMP as well. Flow rate was 10—12 ml/h, 1.4-ml fractions were collected.

Phospholipid determination. Quantitation of protein-bound phospholipid before and after Lubrol solubilization was achieved by the ashing method of Ames and Dubin [22]. Unsolubilized microsomes were ashed directly. Solubilized microsomes were separated by centrifugation at 40 000 rev./min for 2.5 h in a Sorval TV 685 vertical rotor through step gradients consisting of 0.6 ml of 70% sucrose, 1 M KCl; 0.6 ml of 50% sucrose, 1 M KCl; and 2.7 ml of 25% sucrose. The solubilized protein, which collected on the lower interface, was separated from the rest of the gradient. Both portions were brought to the same volume with H_2O and extracted repeatedly with chloroform/methanol (2 : 1); the washes were evaporated to dryness and ashed as above.

Resonance energy transfer. The absorbance of β-parinaric acid overlaps the fluorescence emission of tryptophan so that at short distances, excited state energy may be transferred from tryptophan to β-parinaric acid by a resonance effect as described by Förster [23]. This transfer results in quenching of tryptophan fluorescence and the efficiency (E) of this quenching is related to the distance (R) between the donor and acceptor by the relationship:

$$E = 1 - F_{D-A}/F_D = (R_c/R)^6/[1 + (R_c/R)^6] \quad (1)$$

where F_{D-A}/F_D is the ratio of donor (tryptophan) fluorescence in the presence and absence of acceptor (β-parinaric acid) and R_c is the critical transfer distance at which $E = 0.5$.

The distance (R_c, in Å) between donor (tryptophan) and acceptor (β-parinaric acid) for 50% quenching of donor fluorescencs is a function of the spectral overlap of the two fluorophores (J), a transition dipole orientation factor (K^2), the quantum yield of tryptophan in the absence of β-parinaric acid

(Q), and the refractive index of the medium (n), as related by:

$$R_c = (9.79 \cdot 10^3) \cdot (JK^2Qn^{-4})^{1/6} \quad (2)$$

where J is determined by:

$$J = \int F(\lambda)\epsilon(\lambda)\lambda^4 d\lambda / \int F(\lambda) d\lambda \quad (3)$$

where $F(\lambda)$ and $\epsilon(\lambda)$ are the fluorescence intensity of the donor and extinction coefficient of the acceptor, respectively, at wavelength λ. The integrals can be evaluated by summing over λ at 10-nm intervals. Based on the calculations of Sklar and Hudson [24], R_c = 24—28 Å for tryptophan-β-parinaric acid transfer assuming $K^2 = 2/3$.

Results

The effects of the non-ionic detergents, Lubrol WX and digitonin, on ($Na^+ + K^+$)-ATPase and *p*-nitrophenylphosphatase activities of the purified eel electroplax enzyme were studied. In all cases, the loss of activity could be described as a double-exponential decay (Fig. 1). In the case of Lubrol WX, ($Na^+ + K^+$)-ATPase and *p*-nitrophenylphosphatase activities decayed in parallel over the entire time course under all detergent and protein concentrations studied. However, digitonin inhibited the *p*-nitrophenylphosphatase activity more than the ATPase activity during the rapid phase of inactivation (Fig. 1B). This result is in contrast with results using the canine kidney enzyme where we found, in agreement with Winter [13], that ($Na^+ + K^+$)-ATPase activity is inhibited more rapidly than *p*-nitrophenylphosphatase activity. The preferential inhibition of the electroplax *p*-nitrophenylphosphatase activity by digitonin could be

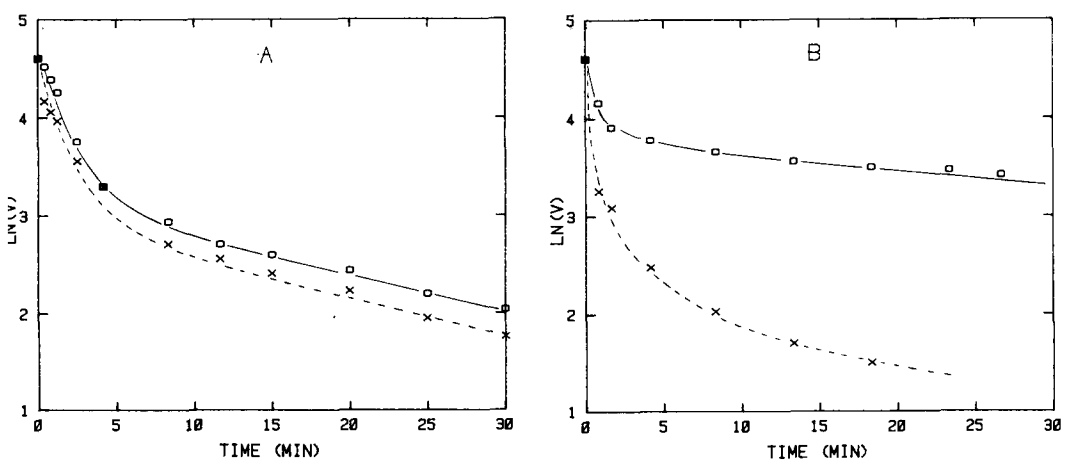

Fig. 1. Two phases of ($Na^+ + K^+$)-ATPase and *p*-nitrophenylphosphatase inactivation induced by non-ionic detergents. (A) ln of ($Na^+ + K^+$)-ATPase activity (□) or *p*-nitrophenylphosphatase activity (x) as a function of time following addition of 0.24% Lubrol WX to the purified eel electroplax enzyme. Both activities prior to detergent addition were assigned the value 100. The enzyme concentration was 4 μg/ml and assays were performed at 37°C as described in Materials and Methods. The inactivation curves were unaffected by changing the Lubrol WX concentration from 0.009 to 0.24%. (B) The same experiment as A except in the presence of 0.018% digitonin instead of Lubrol WX. The inactivation curves were unaffected by changing the digitonin concentration from 0.0014 to 0.02%.

prevented by adding 20% glycerol to the reaction medium (data not presented); however, glycerol had very little effect on the stability of the $(Na^+ + K^+)$-ATPase activity with the detergents investigated. These results indicate that although digitonin under some conditions causes a preferential inhibition of one activity over the other, this preference depends on the enzyme source and is not produced by Lubrol WX.

The time course of the detergent inactivation was studied over a wide range of detergent and protein concentrations. At Lubrol WX concentrations below the CMC (CMC = 0.002% for Lubrol WX under these conditions), the rate of the rapid phase of decay for both activities is proportional to the detergent concentration. The same concentration-dependent inactivation was found for Triton X-100 which at concentrations below the CMC (CMC = 0.02% under these conditions) produced an inactivation time course similar to that of Lubrol WX (data not shown). Varying the digitonin concentration (0.0014—0.02%) had little effect on the decay rates. The rates of the slow phase of decay for both ATPase and *p*-nitrophenylphosphatase activities were similar for all three detergents investigated and were independent of detergent concentration over a wide range of concentrations from below to well above the CMC *. When the protein concentration was less than approx. 10 μg/ml so that an insignificant fraction of added detergent became complexed with the microsomes, the inactivation was independent of the protein concentration. Thus, although the rapid inactivation phase depends on the detergent used and its monomeric concentration, the slow phase appears to result from some process which, although induced by detergent, is remarkably insensitive to the detergent used or its micelle concentration and which is also independent of protein concentration.

In order to understand the structural changes occurring in the ATPase containing microsomes during detergent inactivation, light scattering was monitored under the same conditions as the inactivation experiments (Table I). All detectable changes in light scattering were completed during the rapid phase of inhibition for all three detergents. Lubrol WX and Triton X-100 caused large decreases in light scattering, suggesting that fragmentation of microsomes occurs with only partial loss of activity during the rapid phase. However, digitonin caused an increase in light scattering which could not be explained by the detergent alone, suggesting that larger aggregates may actually form during the rapid inactivation phase.

The intrinsic protein fluorescence was also monitored as a probe of structural changes accompanying inactivation (Fig. 2B). Exposing the enzyme to 0.2% SDS produces a 30% decrease in tryptophan fluorescence during the mixing time. Adding Lubrol WX to the enzyme under conditions similar to those in the inactivation experiment resulted in an 18% decay in protein fluorescence with a rate constant similar to that of the slow inactivation phase. Digitonin, at concentrations which caused complete inhibition of *p*-nitrophenylphosphatase activity, did not affect protein fluorescence. Absorption problems prevented the use of Triton X-100 in this experiment. Thus, only the

* The relative amplitudes of the fast and slow phases varied considerably with detergent concentration and at high Triton X-100 concentrations the rapid phase resulted in complete inhibition.

TABLE I

EFFECTS OF DETERGENTS ON LIGHT SCATTERING OF PURIFIED EEL ELECTROPLAX ($Na^+ + K^+$)-ATPase MICROSOMES

Light scattering was measured as described in Materials and Methods. The detergent solutions were centrifuged to remove dust. The detergents were added to 5 μg/ml of enzyme where indicated and the scattering measured 90 s later. All changes were complete in 90 s. The temperature was 37°C and the buffer was the same as in Fig. 1.

Conditions	Relative scattering
Microsomes (5 μg/ml)	1.00
0.03% Lubrol WX	0.14
0.005% Triton X-100	0.13
0.009% digitonin	0.18
Microsomes + 0.03% Lubrol WX	0.40
Microsomes + 0.005% Triton X-100	0.24
Microsomes + 0.009% digitonin	1.90

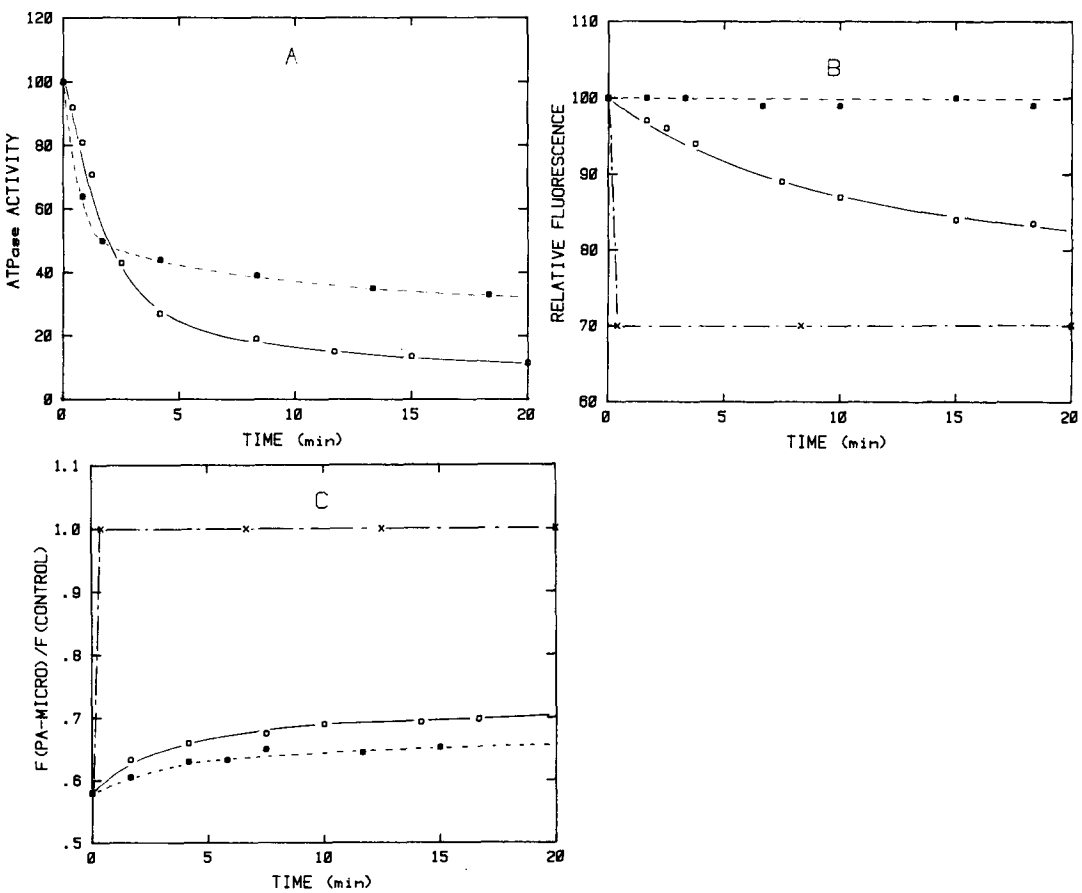

Fig. 2. A comparison of the time course of detergent inactivation with intrinsic protein fluorescence changes and β-parinaric acid-quenching of tryptophan fluorescence. All measurements were at 37°C. (A) Digitonin (■) and Lubrol WX (□) inactivation of ($Na^+ + K^+$)-ATPase activity. The same conditions as in Fig. 1. (B) The change in protein fluorescence following addition of digitonin (■), Lubrol WX (□) or sodium dodecyl sulfate (x). Excitation was at 290 nm and emission at 340 nm as described in Materials and Methods. The protein concentration was 6 μg/ml, digitonin was 0.016%, Lubrol WX was 0.033% and sodium dodecyl sulfate was 0.17% when present. (C) The ratio of tryptophan fluorescence of PA-microsomes to control microsomes following addition of digitonin (■), Lubrol WX (□), or sodium dodecyl sulfate (x) to each preparation. Excitation was at 290 nm and emission was 340 nm. In each case the protein concentration was 6 μg/ml and the detergent concentrations were the same as in B. The PA-microsome preparation is described in Materials and Methods.

slow phase of inhibition induced by Lubrol WX appears to involve a gross protein conformational change as judged by changes in tryptophan environment.

Changes in protein-lipid interactions were studied by monitoring resonance energy transfer quenching of tryptophan fluorescence by the conjugated fatty acid, β-parinaric acid. This fluorescent molecule has been shown to interpolate into lipid bilayers [16,24]. Since the efficiency of this quenching decrease with a $(1/R)^6$ dependence (R = distance between tryptophan and β-parinaric acid, and 50% quenching occurs at a donor acceptor distance of 24—28 Å; see Materials and Methods), this measurement is a good indication of a change in the population of β-parinaric acid molecules near the protein. β-Parinaric acid was sonicated into ($Na^+ + K^+$)-ATPase microsomes to give 42% quenching of tryptophan fluorescence. The ratio of the protein fluorescence of PA-microsomes to control microsomes was measured following detergent addition under conditions similar to the inactivation experiments (Fig. 2C). SDS immediately restored both fluorescence intensities to the same level. However, Lubrol WX, under conditions which caused nearly complete inhibition of ATPase activity, only increased the protein fluorescence of the PA-microsomes from 58% of control microsomes to 71% of control microsomes and the time course of this change paralleled the slow phase of inactivation. Digitonin had an even smaller effect on the PA-microsomes but with a similar time course. These results indicate that the slow phase of inactivation involves removal of lipids (i.e., β-parinaric acid) which are in close proximity to the ($Na^+ + K^+$)-ATPase and indicate that much of the closely associated lipid remains bound to the protein when greater than 95% of the activity is gone.

In order to characterize further the structure of the Lubrol WX-treated protein, the PA-microsomes were treated with Lubrol WX under conditions

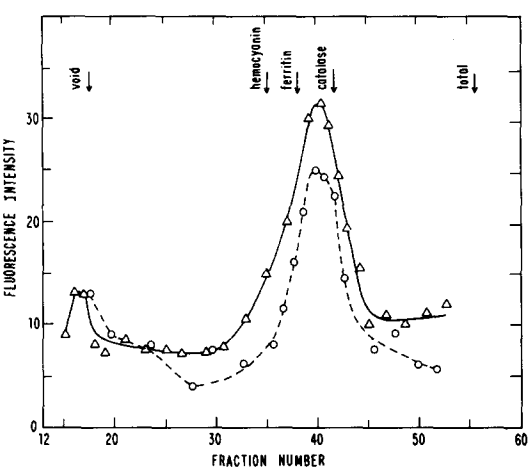

Fig. 3. The elution profile of protein (△) and β-parinaric acid (○) after passing Lubrol WX-solubilized ($Na^+ + K^+$)-ATPase or PA-microsomes through a Sepharose 4B column as described in Materials and Methods. Enzyme (approx. 0.2 mg/ml) was incubated with 5% Lubrol WX for 30 min at 37°C before adding to column. Protein was detected by 340 nm fluorescence (exciting at 290 nm) and β-parinaric acid was detected by 380 nm fluorescence (exciting at 329 nm). The arrows indicate elution peaks of marker proteins. The elution buffer is described in Materials and Methods.

resulting in greater than 95% inhibition of ATPase activity and then passed through a Sepharose 4B column equilibrated with 0.015% Lubrol WX. The detergent-to-protein ratio added to the column was similar to that in the inactivation experiment. The protein eluted between catalase and ferritin, suggesting a particle size of approx. 400 000 daltons (including associated lipid and detergent). Most of the β-parinaric acid also eluted with this peak, indicating that the Lubrol WX-inactivated enzyme still has tightly associated lipids in agreement with the results in Fig. 2C.

A phospholipid analysis was made on ($Na^+ + K^+$)-ATPase microsomes and on the Lubrol-solubilized and inactivated protein separated by a discontinuous sucrose gradient (see Materials and Methods). The initial ratio of phospholipid to enzyme was 112 mol/mol (assuming approx. 300 000 daltons for ($Na^+ + K^+$)-ATPase) and the solubilized, inactivated protein retained 20 mol/mol of associated phospholipid. Thus, as suggested above, a considerable fraction of phospholipid is tightly associated with the enzyme even after complete inactivation in detergent.

Discussion

The results presented here indicate that detergent inactivation of the ($Na^+ + K^+$)-ATPase occurs in two phases. The rapid phase is sensitive to the detergent used and its monomeric concentration and is accompanied by fragmentation of the membrane (when Triton X-100 or Lubrol WX is used) as judged by light-scattering changes. The kinetics of this inactivation are consistent with insertion of detergent monomers into the membrane. Similar losses of ($Na^+ + K^+$)-ATPase and *p*-nitrophenylphosphatase activities occur during this phase using Triton X-100 or Lubrol WX, however, digitonin preferentially inhibits one activity over the other, although the preference reverses with change in enzyme source. Neither Lubrol WX nor digitonin causes a large change in protein conformation or protein-lipid association (as judged by tryptophan fluorescence or tryptophan to β-parinaric acid energy transfer) during this rapid phase, making a change in the oligomeric structure of the protein unlikely.

The slow phase of inactivation is accompanied by a decrease in intrinsic protein fluorescence and also by a decrease in β-parinaric acid-quenching of protein fluorescence in the PA-microsomes. These observations suggest a removal of closely associated lipids from the protein and an accompanying protein conformational change. The kinetics of the slow phase are independent of detergent or protein concentration (in the range studied) and are consistent with a mechanism in which the rate-limiting step of inactivation is dissociation of a tightly bound lipid from the protein. After the slow phase of inactivation is complete, approx. 20 phospholipids still remain associated with the protein in a 400 000 dalton particle suggesting that complete loss of activity occurs when only a few of the tightly associated lipids are removed.

The ability of digitonin to preferentially inhibit one of the activities of the enzyme during the rapid phase of inactivation should be discussed in more detail. A number of agents have been shown to inhibit ($Na^+ + K^+$)-ATPase activity more readily than the *p*-nitrophenylphosphatase activity (see review by Glynn and Karlish [2]). We previously suggested that 7-chloro-4-nitrobenzo-

2-oxa-1,3-diazole preferentially inhibits ($Na^+ + K^+$)-ATPase activity over *p*-nitrophenylphosphatase activity because two sites on the enzyme could hydrolyze *p*-nitrophenylphosphate somewhat independently but both were required for ($Na^+ + K^+$)-ATPase activity [15]. This proposal agress with radiation inactivation experiments which show a target area for *p*-nitrophenylphosphatase activity smaller than that of ($Na^+ + K^+$)-ATPase activity [25]. Since several non-ionic detergents (including Triton X-100 [9] and digitonin [10]) have been shown to break any α-α associations of the enzyme and solubilize a protein particle of approx. 180 000 daltons, it is tempting to speculate that selective resistance of *p*-nitrophenylphosphatase activity to some detergents [12,14] is due to a *p*-nitrophenylphosphatase activity of the half-enzyme. The results presented here indicate that with the detergents we used, this is not the case. Although we found, in agreement with Winter [13], that digitonin preferentially inhibited the ($Na^+ + K^+$)-ATPase activity over the *p*-nitrophenylphosphatase activity with kidney enzyme, the reverse was true for the eel electroplax enzyme. In addition, the preferential inhibition occurred prior to any significant changes in tryptophan fluorescence or tryptophan to β-parinaric acid energy transfer making a change in the oligomeric state of the protein unlikely during this phase. The other detergents tried (Lubrol WX and Triton X-100) gave no preferential inhibition of ($Na^+ + K^+$)-ATPase activity over *p*-nitrophenylphosphatase activity for either the eel electroplax enzyme or the canine kidney enzyme (data not presented). It should be pointed out that the *p*-nitrophenylphosphatase activity does not require the full catalytic cycle and probably does not require the complete substrate binding site necessary for ATP hydrolysis [3]. Thus, a reagent or detergent which prevents a conformational change in the ATP turnover cycle or which partially obscures the ATP binding site may have little effect on *p*-nitrophenylphosphatase activity.

The change in tryptophan fluorescence of PA-microsomes with respect to control microsomes has been ascribed to a change in resonance energy transfer efficiency. One alternative explanation is that β-parinaric acid induces a conformational change in the protein to change the local environment of the tryptophan residues. Such a large environmental change is unlikely (notice the 42% quenching in Fig. 2C) and the concentration of parinaric acid used was insufficient to cause a significant inner filter absorbance, so the energy transfer assignment seems reasonable. The multiplicity of tryptophan and β-parinaric acid molecules involved in the energy transfer preclude any meaningful distance measurements, however, as pointed out in Results, significant transfer is only expected to β-parinaric acid molecules less than 30 Å from the tryptophan residues. It therefore is likely that changes in tryptophan fluorescence of PA-microsomes reflect movement of lipids closely associated with the protein.

The model for detergent inactivation presented here assigns the slow phase of inactivation to loss of lipid tightly associated with the protein. The fact that much lipid remains bound after complete inactivation (approx. 20 mol/mol) suggests that an annulus of at least 20 phospholipids is essential for any ($Na^+ + K^+$)-ATPase activity. The minimum stoichiometry for activity may be somewhat higher. These results are in agreement with those of Warren et al. [26] who found that the Ca^{2+}-ATPase of sarcoplasmic reticulum has approx. 30 tightly bound phospholipids which are essential for activity. Dean and

Tanford [27] were able to maintain a fully active Ca^{2+}-ATPase by replacing these lipids with non-ionic detergents in the presence of glycerol. We were unable to observe ATPase or *p*-nitrophenylphosphatase activity in a delipidated ($Na^+ + K^+$)-ATPase even in the presence of glycerol; however, Ottolenghi [8] was able to fully restore ($Na^+ + K^+$)-ATPase activity to a completely delipidated preparation by readdition of exogenous phospholipids, indicating that no irreversible protein denaturation occurs during delipidation with detergents. In a more recent paper, Ottolenghi [28] has shown that low concentrations of phosphatidylcholine or phosphatidylethanolamine activate the *p*-nitrophenylphosphatase activity of delipidated enzyme prior to the ($Na^+ + K^+$)-ATPase activity. The interpretation was that clumps of lipid binding to one subunit were sufficient to activate the *p*-nitrophenylphosphatase but that both subunits need be occupied for ($Na^+ + K^+$)-ATPase activity. However, there is still no evidence that dissociated subunits carry out a *p*-nitrophenylphosphatase activity. Finally, it should be noted that no more than 90 mol/mol of phospholipid were needed for ATPase activity of phospholipase-treated enzyme [29] and that the fully active, detergent-solubilized ($Na^+ + K^+$)-ATPase used for molecular weight determinations had approx. 60 mol/mol of associated phospholipid [30,31].

Note added in proof (Received May 1st, 1980)

We have recently obtained strong evidence against interacting nucleotide sites on the ($Na^+ + K^+$)-ATPase [32]. This result questions the necessity of an $(\alpha\beta)_2$ structure for ATPase activity. Perhaps, as suggested by the results here, the failure to detect ATPase activity in detergent solubilized $\alpha\beta$ structures [9,10] is due to removal of essential lipid rather than an insufficient oligomeric structure.

Acknowledgement

This work was supported by Grant GM 26199-01 from the National Institutes of Health.

References

1 Skou, J.C. (1975) Q. Rev. Biophys. 7. 401—434
2 Glynn, I.M. and Karlish, S.J.D. (1975) Ann. Rev. Physiol. 37, 13—55
3 Robinson, J.D. and Flashner, M.S. (1979) Biochim. Biophys. Acta 549, 145—176
4 Hilden, S., Rhee, H.M. and Hokin, L.F. (1974) J. Biol. Chem. 249, 7432—7500
5 Goldin, S. (1977) J. Biol. Chem. 252, 5600—5642
6 Jackson, R.L., Morrisett, D.J., Sparrow, J.J., Seyrest, J.P., Pownall, H.J., Smith, L.C., Hoff, H.F. and Gotto, A.M. (1974) J. Biol. Chem. 249, 5314—5320
7 Kagawa, Y. (1978) Biochim. Biophys. Acta 505, 45—93
8 Ottolenghi, P. (1975) Biochem. J. 151, 61—66
9 Clarke, S. (1975) J. Biol. Chem. 250, 5459—5469
10 Winter, C.G. (1979) in Na, K ATPase Structure and Kinetics (Skou, J.C. and Norby, J.G., eds.), pp. 25—32, Academic Press, London
11 Wheeler, K.P. and Walker, J.A. (1975) Biochem. J. 146, 723—727
12 Wheeler, K.P., Walker, J.A. and Barker, D.M. (1975) Biochem. J. 146, 713—722
13 Winter, C.G. (1974) Ann. N.Y. Acad. Sci. 242, 149—157
14 Laing, S.-M. annd Winter, C.G. (1976) Biochim. Biophys. Acta 452, 552—565

15 Cantley, L.C., Gelles, J. and Josephson, L. (1978) Biochemistry 17, 418—425
16 Fraley, R.T., Jameson, D.M. and Kaplan, S. (1978) Biochim. Biophys. Acta 511, 52—69
17 Jørgensen, P.L. (1974) Biochim. Biophys. Acta 356, 36—52
18 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
19 Ahmed, K. and Thomas, B.S. (1971) J. Biol. Chem. 246, 103—109
20 Barnett, R.E. (1970) Biochemistry 9, 4644—4648
21 Cantley, L.C., Ferguson, J.H. and Kustin, K. (1978) J. Am. Chem. Soc. 100, 5210—5212
22 Ames, B.N. and Dubin, D.T. (1960) J. Biol. Chem. 235, 769—775
23 Förster, T. (1959) Discuss. Faraday Soc. 27, 7—17
24 Sklar, L.A. and Hudson, B.S. (1976) J. Supramol. Struct. 4, 449—465
25 Kepner, G.R. and Macey, R.I. (1968) Biochim. Biophys. Acta 163, 188—203
26 Warren, G.B., Houslay, M.D., Metcalfe, J.C. and Birdsall, N.J.M. (1975) Nature 255, 684—687
27 Dean, W.L. and Tanford, C. (1978) Biochemistry 17, 1683—1690
28 Ottolenghi, P. (1979) Eur. J. Biochem. 99, 113—131
29 DePont, J.J.H.H.M., van Prooijen-van Eeden, A. and Bonting, S.L. (1978) Biochim. Biophys. Acta 508, 464—477
30 Esmann, M., Skou, J.C. and Christiansen, C. (1979) Biochim. Biophys. Acta 567, 410—420
31 Hastings, D.F. and Reynolds, J.A. (1979) Biochemistry 18, 817—820
32 Smith, Zinn and Cantley, L.C. (1980) J. Biol. Chem., in the press