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Solubilized ($Na^+ + K^+$)-ATPase from shark rectal gland and ox kidney – an inactivation study

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The bi-exponential time-course of detergent inactivation at 37°C of $C_{12}E_8$-solubilized ($Na^+ + K^+$)-ATPase from shark rectal glands and ox kidney was investigated. The data for shark enzyme, obtained at detergent/protein weight ratios between 2 and 16, are interpreted in terms of a simple model where the membrane bound enzyme is solubilized predominantly as (alpha-beta)$_2$ diprotomers at low detergent concentrations and as alpha-beta protomers at high $C_{12}E_8$ (octaethyleneglycoldodecylmonoether) concentrations. It is observed that the protomers are inactivated 15-fold more rapidly than the diprotomers, and that the rate of inactivation of both oligomers is proportional to the detergent/protein ratio. Inactivation of kidney enzyme was biexponential with a very rapid inactivation of up to 40% of the enzyme activity. The observed rate of inactivation of the slower phase varied with the detergent/protein ratio, but the inactivation pattern for the kidney enzyme could not readily be accommodated within the model for inactivation of the shark enzyme. The rates of inactivation at 37°C were about the same in KCl and NaCl, i.e., in the $E_2(K)$ and $E_1 \cdot Na$ forms, for both enzymes.

Introduction

Detergent solubilization is used extensively for physicochemical characterization of integral membrane proteins, as well as a first step in reconstitution of ion-transporting enzymes into lipid vesicles. A detailed examination of the effect of detergent on the structure and activity of the solubilized protein is therefore necessary.

In recent years there has been considerable interest in the effect of the nonionic detergent octaethyleneglycoldodecylmonoether ($C_{12}E_8$) on the activity and structure of the ($Na^+ + K^+$)-ATPase [1–13]. The molecular size of the solubilized enzyme has been studied in detail with ($Na^+ + K^+$)-ATPase from kidney [2–6, 9–12] and from shark rectal glands [1,7,8,13]. It is generally agreed that the α subunit (M_r 106 000) and the β subunit (M_r 40 000) appear in equimolar amounts in the solubilized particles, which therefore are referred to as $\alpha\beta$ protomers, $(\alpha\beta)_2$ diprotomers etc. Although solubilized and fully active ($Na^+ + K^+$)-ATPase can be obtained with $C_{12}E_8$, there is an inactivating effect of the detergent, particularly at high temperatures and high detergent/protein ratios.

The purpose of the present paper is dual. Firstly, since the shark enzyme is predominantly diprotomeric at low detergent/protein ratios and protomeric at high ratios [8], an investigation of the kinetics of inactivation at different $C_{12}E_8$/protein ratios was undertaken. The goal was to achieve

Abbreviations: ($Na^+ + K^+$)-ATPase, the Na^+- and K^+-activated adenosine triphosphatase; $C_{12}E_8$, octaethyleneglycoldodecylmonoether; $C_{12}E_{10}$, polyoxyethylene 10-laurylether; CDTA, *trans*-1,2-cyclohexylenedinitrilotetraacetic acid; alpha, the 106 kDa catalytic subunit; beta, the 40 kDa glycoprotein.

information about the protomer-diprotomer relationship through a kinetic analysis. The second purpose was to compare the inactivation kinetics of the solubilized shark and kidney enzyme in an attempt to explain the conflicting reports on the structure and stability of the solubilized enzymes [1–8,10,12,13].

The results presented below are interpreted in terms of a relatively simple model for the effect of $C_{12}E_8$ on the solubilized shark enzyme: The enzyme is diprotomeric and inactivated slowly at low detergent/protein ratios and is inactivated 15-fold more rapidly at high ratios, where it is protomeric.

In contrast to this, the inactivation of the kidney enzyme followed a more complex pattern, with no 'simple' interpretations of the available data being possible at present.

The inactivation patterns seems to be the same for the two major conformations ($E_1 \cdot Na$ in Na^+ and $E_2(K)$ in K^+) in the shark enzyme as well as in the kidney enzyme at 37°C.

Materials and Methods

Preparation of shark enzyme

$(Na^+ + K^+)$-ATPase is prepared from rectal glands of *Squalus Acanthias* as previously described, omitting saponin from the preparative procedure [14]. The specific $(Na^+ + K^+)$-ATPase activity of the membrane bound enzyme in this preparation is about 1100 μmol/mg protein per h. The purity is about 50% with respect to the α and β peptides (determined by polyacrylamide gel electrophoresis in SDS), and the phosphorylation capacity is about 2.0 nmol/mg protein. Activity measurements and protein determination of the membrane bound enzyme is performed as previously described [14].

Preparation of kidney enzyme

$(Na^+ + K^+)$-ATPase is prepared from ox kidney using a modification of the SDS procedure developed by Jørgensen [15]. The kidney microsomes are treated with SDS in the presence of 3 mM ATP for 30 min at 23°C and subjected to differential centrifugation as described for the shark enzyme (see Ref. 14). The specific activity of the membraneous kidney enzyme is about 1000 μmol/mg protein per h with a purity of about 45%. The $(Na^+ + K^+)$-ATPase activity and protein in determined as previously described for the shark enzyme [14].

Procedure for solubilization

The $(Na^+ + K^+)$-ATPase is solubilized using $C_{12}E_8$ in the following manner.

1 vol. of $C_{12}E_8$ in water is added to 3 vol. of enzyme in buffer at the desired temperature (4°C in Figs. 1–3 and 37°C in Figs. 4–7 and 9), giving a final buffer medium of 30 mM histidine/150 mM NaCl/1 mM CDTA (pH 7.0) at 37°C. The final protein concentration was 1.0 mg/ml for shark enzyme and 0.6 mg/ml for kidney enzyme. The detergent/protein ratio is varied as described in the legends to figures with the term 'ratio' indicating the total concentration (g/l) of $C_{12}E_8$ (bound plus free) divided by the protein concentration (g/l). Solubilization is instantaneous in the sense that the solution clarifies immediately when the detergent is added.

The amount of activity or protein solubilized is determined after centrifugation for 1 h at 100000 $\times g$ in a Beckman Airfuge. Solubilization of the enzyme is defined as $(Na^+ + K^+)$-ATPase activity remaining in the supernatant after the centrifugation.

When the extent of inactivation by detergent at 37°C is to be measured, an aliquot (e.g., 0.15 ml) is withdrawn, and transferred to 0°C and diluted with 0.6 ml cold 30 mM histidine (pH 7.0). At all detergent/protein ratios, this dilution and lowering of temperature stopped the inactivation immediately (see Fig. 5 below).

The membrane bound enzymes (both shark and kidney) were stable for at least 1 h at 37°C in the absence of $C_{12}E_8$. The inactivation at 37°C is therefore due solely to an effect of $C_{12}E_8$.

The supernatant enzyme for both kidney and shark enzyme is 90–100% pure with respect to the content of α and β peptides (not shown). The patterns of inactivation described in the text are therefore solely due to an effect of $C_{12}E_8$ on pure solubilized $(Na^+ + K^+)$-ATPase.

Measurement of $(Na^+ + K^+)$-ATPase and K^+ phosphatase activity of solubilized enzyme

Shark enzyme. The enzymatic activities are de-

termined at 23°C in order to minimize the inactivating effect of detergent at higher temperatures. About 20 μg protein is transferred to a 1 ml test solution comprising (final concentrations) 30 mM histidine (pH 7.0 at 23°C)/130 mM NaCl/20 mM KCl/4 mM $MgCl_2$/3 mM ATP for the ($Na^+ + K^+$)-ATPase assay, and for the K^+-*p*-nitrophenylphosphatase assay, 30 mM histidine (pH 7.0 at 23°C)/150 mM KCl/20 mM $MgCl_2$/10 mM *p*-nitrophenyl phosphate. The test solution also contains 1 mg $C_{12}E_{10}$/ml, which buffers the excess $C_{12}E_8$ that is transferred at the high detergent/protein ratios [7]. Hydrolysis of ATP or *p*-nitrophenylphosphate is linear with time at least up to 5 min under these conditions (for the shark enzyme). A test time of 3 min is routinely used.

Kidney enzyme. The $C_{12}E_8$-solubilized kidney enzyme is tested as the shark enzyme, but since $C_{12}E_{10}$ has an inactivating effect at the concentrations used for the shark enzyme, $C_{12}E_{10}$ was omitted from the test solution. For this reason, only a narrow range of $C_{12}E_8$ concentrations (40–80 μg/ml) can be tolerated in the test solution (at 23°C), see Fig. 2 below.

Protein determination

The amount of protein, solubilized with $C_{12}E_8$ was determined using the Peterson modification [16] of the procedure of Lowry et al. [17], using albumin as reference.

Materials

$C_{12}E_8$ is obtained from Nikko Chemicals, Tokyo, and $C_{12}E_{10}$ from Sigma.

Results and Discussion

Control experiments

The kinetics of inactivation by $C_{12}E_8$ of the enzyme activities are followed at 37°C in order to give a reasonably rapid rate of inactivation at $C_{12}E_8$/protein ratios between 2 and 16 for the shark enzyme.

To avoid problems with instability and protein determinations after solubilization in $C_{12}E_8$ we chose to add the detergent to the protein at 37°C, with no removal of nonsolubilized residues, i.e., the membrane preparation was solubilized but supernatant enzyme was not prepared (see Methods). It is therefore essential to measure how much of the enzyme activity is solubilized at a given $C_{12}E_8$/protein ratio. The following set of control experiments has therefore been performed. In these experiments (Figs. 1–3) the temperature at the solubilization step was kept low (4°C) to avoid inactivation as much as possible.

Fig. 1 shows the solubilization of shark ($Na^+ + K^+$)-ATPase activity and protein when the $C_{12}E_8$/protein ratio is varied. At detergent protein ratios above 2–3 all the ($Na^+ + K^+$)-ATPase activity is solubilized, and about 60% of the protein remains in the supernatant after centrifugation. This is carried out at 4°C, and even at this low temperature a partial inactivation (30–40%) is observed at detergent/protein ratios of 12–16.

Fig. 2 shows the similar experiments using membrane bound kidney enzyme solubilized with $C_{12}E_8$ as described in Materials and Methods. Here the ($Na^+ + K^+$)-ATPase is fully solubilized at ratios of about 1 mg detergent per mg protein and a large decrease in activity at 4°C is observed at detergent/protein ratios above 4–5 (note the different horizontal scaling in comparison with Fig. 1).

We have previously determined the optimum conditions for measurement of solubilized shark ($Na^+ + K^+$)-ATPase [7]. Since the kidney ($Na^+ +$

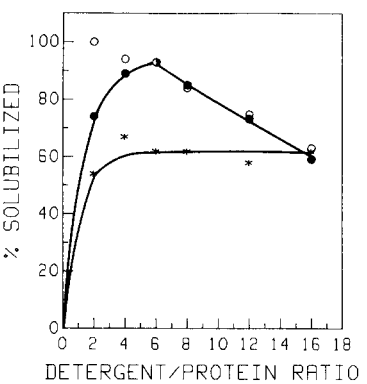

Fig. 1. Solubilization of shark ($Na^+ + K^+$)-ATPase activity. This figure shows solubilization of the ($Na^+ + K^+$)-ATPase activity (closed circles) and protein (*) at detergent/protein weight ratios of from 2 to 16. The protein content and enzyme activity in the supernatant is measured after 3 h at 4°C as described in Materials and Methods, and is given in percent of total protein (1 mg/ml) or maximal ($Na^+ + K^+$)-ATPase activity. For comparison is also given the ($Na^+ + K^+$)-ATPase activity of the whole sample before centrifugation (open circles).

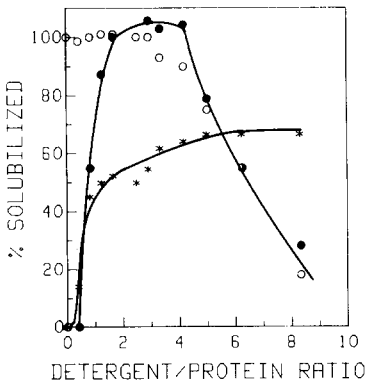

Fig. 2. Solubilization of kidney $(Na^+ + K^+)$-ATPase activity. Symbols and experimental conditions are as given in Legend to Fig. 1, except that the total protein concentration (100%) is 0.6 mg/ml.

K^+)-ATPase in the solubilized state seems more labile [2–4, 10], it seemed appropriate to define the conditions under which an optimum activity could be measured.

Unfortunately, the 'buffering' agent $C_{12}E_{10}$ [7] inactivates the kidney enzyme much more than the shark enzyme, and is therefore not useful in the test solution. The only thing to do is therefore to carefully control the $C_{12}E_8$ concentration in the test solution.

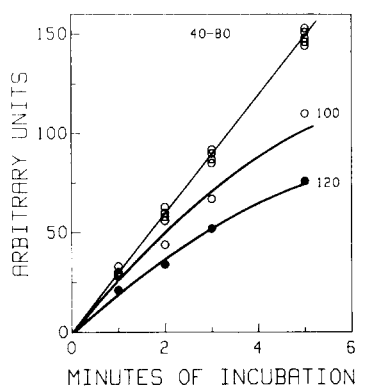

Fig. 3. Production of inorganic phosphate as a function of time at different $C_{12}E_8$ concentrations. Kidney enzyme was solubilized as given in Materials and Methods at a $C_{12}E_8$/protein ratio of 2. An aliquot was diluted with 4 vol. of histidine 30 mM (pH 7.0 at 23°C). The $(Na^+ + K^+)$-ATPase was tested at 23°C with the incubation times indicated. The test solution contained $C_{12}E_8$ to give final $C_{12}E_8$ concentrations (shown by the curves) in the test solution ranging from 40 to 120 μg/ml (including the contribution of 24 μg/ml from the enzyme suspension). There was no difference in the activity measured when the $C_{12}E_8$ concentration in the test solution was between 40 and 80 μg/ml. All steps were carried out at 4°C except for the $(Na^+ + K^+)$-ATPase test.

Fig. 3 shows the effect of $C_{12}E_8$ in the test solution when kidney enzyme, solubilized with $C_{12}E_8$ at a ratio of 2 mg detergent/mg protein is tested at 23°C (with 10 μg protein/ml in the test solution). The phosphate liberation is linear at detergent concentrations (final concentration in the test solution) of 40–80 μg per ml. A deviation from linearity, i.e., inactivation in the test solution, is observed at higher detergent concentrations. This limits the range of detergent concentrations that can be used in the inactivation experiments, since the detergent concentration in the test solution can not exceed 80 μg/ml.

Inactivation of solubilized shark enzyme at 37°C

Fig. 4 shows, on a logarithmic scale, the $(Na^+ + K^+)$-ATPase activity of shark enzyme as a function of time spent at 37°C at different $C_{12}E_8$/protein ratios. After a given time, an aliquot is diluted at 4°C and the $(Na^+ + K^+)$-ATPase activity is subsequently measured as given in Materials and Methods. Under all conditions

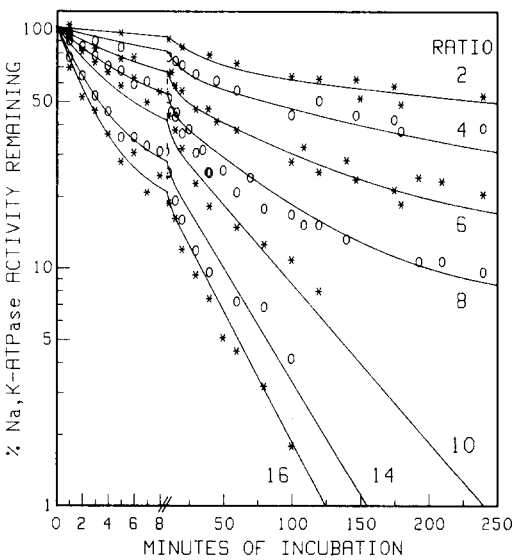

Fig. 4. Inactivation of shark $(Na^+ + K^+)$-ATPase activity. After the indicated times of incubation at 37°C, the enzyme was diluted and cooled to 4°C, and the $(Na^+ + K^+)$-ATPase activity – measured at 23°C – is shown on the logarithmic scale. The protein concentration at 37°C was 1 mg/ml and the detergent concentration was varied between 2 and 16 mg/ml, see the figure, with alternating symbols of * and O. The solid lines are drawn according to Eqn. 3 (see the text). The data points shown are the average of 3–5 independent experiments.

the phosphate liberation in the test solution was linear with time at 23°C (see Materials and Methods).

It is essential to these experiments that the inactivation by $C_{12}E_8$ is quenched immediately by dilution and lowering of temperature. A control experiment (see Fig. 5) shows that the reaction with $C_{12}E_8$ which leads to inactivation was completely stopped by the dilution and lowering of temperature. In Fig. 5 is shown an experiment in which enzyme at detergent/protein ratios of 4, 8 or 16 are kept at 37°C for 2 min and then transferred to 4°C as described. The $(Na^+ + K^+)$-ATPase activity is thereafter measured as a function of time spent at 4°C after dilution. Clearly, the inactivation by $C_{12}E_8$ is quenched, since there is no subsequent inactivation at 4°C at either ratio.

Qualitatively, the inactivation of the $(Na^+ + K^+)$-ATPase activity (Fig. 4) can be seen to occur in a rapid phase followed by a considerably slower phase. The amplitude of rapid phase also depends on the $C_{12}E_8$/protein ratio (note the expanded time scale for the initial part of the inactivation in Fig. 4).

In Fig. 6 the inactivation of the K^+-phosphatase activity is shown, and the phosphatase activity is seen to decrease in the same fashion as the $(Na^+ + K^+)$-ATPase activity (at least at the lower detergent/protein ratios).

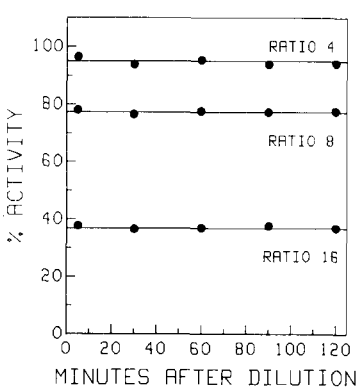

Fig. 5. Quench of detergent inactivation by dilution and lowering of temperature. Shark $(Na^+ + K^+)$-ATPase was incubated at 37°C as given in Legend to Fig. 4. After 2 min an aliquot was diluted with 4 vol. of ice-cold 30 mM histidine (pH 7.0) and kept at 4°C for the times indicated on the figure. The $(Na^+ + K^+)$-ATPase activity was subsequently measured at 23°C. The detergent/protein weight ratio was varied between 4 and 16 as indicated in the figure.

An explanation for the biphasic inactivation

If the solubilized enzyme at a given $C_{12}E_8$ concentration consisted of a homogeneous population of particles – and the inactivation was a 'simple' pseudo-first-order process – a monoexponential decay of activity is to be expected, which is not what is found in Fig. 4. A more complicated effect of the detergent on the $(Na^+ + K^+)$-ATPase is therefore to be expected.

All inactivation curves at a given $C_{12}E_8$/protein ratio could be fitted by two exponentials (together with – at the lowest detergent/protein ratios – a small constant activity). This can be expressed as follows:

$$\mathrm{Act}(t) = M \cdot \exp(-k_1' \cdot t) + D \cdot \exp(-k_2' \cdot t) + O \qquad (1)$$

A nonlinear least-squares method showed that both the observed rate constants (k_1' and k_2') and the amplitudes M, D and O varied with the detergent/protein ratio. A further analysis showed that – by choosing appropriate values for M, D and O – all the data could be fitted by a equation

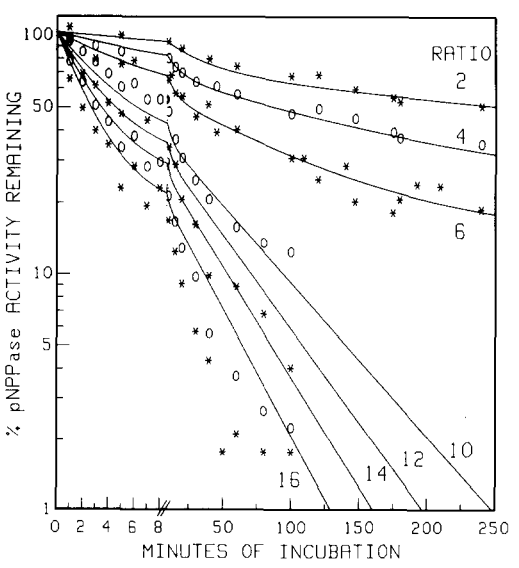

Fig. 6. Inactivation of shark *p*-nitrophenylphosphatase (pNPPase) activity. This is the same experiment as shown in Fig. 4, but the residual K^+-phosphatase activity is shown as a function of time. The solid lines are drawn according to the same equation as those in Fig. 4.

in which the observed rate constants were proportional to the detergent concentration (note that the protein concentration is constant (1 mg/ml) in these experiments), i.e.

$$k_1' = k_1 \cdot [C_{12}E_8] \text{ and } k_2' = k_2 \cdot [C_{12}E_8] \quad (2)$$

Note that the detergent concentration used in Eqns. 2 and 3 is the free concentration, i.e., total concentrations minus the bound detergent (about 0.8 mg/mg protein, Ref. 18).

The curves in Figs. 4 and 6 show enzyme activity as a function of time corresponding to the following equation:

$$\text{Act}(t) = M_D \cdot \exp(-k_1 \cdot [C_{12}E_8] \cdot t) + D_D \cdot \exp(-k_2 \cdot [C_{12}E_8] \cdot t) + O_D \quad (3)$$

where now only M, D and O depend on the detergent concentration (indicated by subscript D). The best fit (by eye) gave values for the rate constants k_1 and k_2 of values of 0.03 and 0.002 $\text{min}^{-1} \cdot (\text{mg free } C_{12}E_8/\text{mg protein})^{-1}$, respectively.

The values for M, D and O used for curve-fitting in Figs. 4 and 6 at each $C_{12}E_8$/protein ratio are given in Fig. 7 (the sum of the three equalling 100%, see Eqn. 3). Interestingly, the value for M increased with the detergent concentration, with a concomitant decrease in D.

The value for O was low, about 14% at 2 mg detergent/mg protein and decreased to 0% at detergent/protein ratios above 8. The activity represented by the quantity O – which is insensitive to detergent – could represent trace amount of non-solubilized protein (see, for example, Fig. 1), or higher oligomers that are inactivated very slowly by $C_{12}E_8$.

An interpretation of the results shown in Fig. 4

Since the solubilized $(Na^+ + K^+)$-ATPase may exist in several oligomeric states [2–6,8,18], it is attractive to interpret the quantities M and D as the amount of $\alpha\beta$ protomer (M) and $(\alpha\beta)_2$ diprotomer (D). In agreement with this – Fig. 7 – the amount of protomer increases as the $C_{12}E_8$/protein ratio is increased (see Ref. 18). The protomer is inactivated 15-fold more rapidly than the diprotomer ($k_1 = 15 \times k_2$, see Eqn. 3), and it is therefore much more difficult to measure an $(Na^+ + K^+)$-ATPase activity of enzyme in the protomeric state.

Following the interpretation given above, the effect of detergent on the membrane bound enzyme can be outlined as given in Fig. 8. As the enzyme is solubilized, the concentration of detergent (at a given protein concentration) determines the amount of protomer and diprotomer formed. Both are then inactivated, at different rates, with the observed rate constant being proportional to the detergent concentration. An equilibrium between the diprotomer and the protomer is not required (if there is an equilibrium, it must be slow on the time-scale of these experiments, since a rapid equilibrium between the protomer and the diprotomer would give a monoexponential decay of activity).

Recently published experiments by Hayashi and co-workers [19], in which the protomer and diprotomer are separated by HPLC, also suggest that the activity of the protomer is more labile than the diprotomer. The specific $(Na^+ + K^+)$-ATPase activity is 6-fold lower for the protomer than for the diprotomer when measured after HPLC in the presence of $C_{12}E_8$ (Table 2 in Ref. 19).

However, a definitive test of the hypothesis outlined in Fig. 8 is difficult to perform. The test would require first a good separation of the protomer and the diprotomer (this can probably only

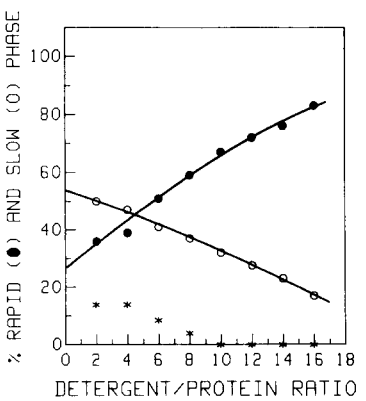

Fig. 7. Amplitudes of rapidly and slowly decaying enzyme activity. This figure shows the amplitudes of the rapid phase (M, solid circles) and slow phase (D, open circles) used for fitting the data in Figs. 4 and 6 with Eqn. 3. The residual constant activity (see Eqn. 3) is also shown (*).

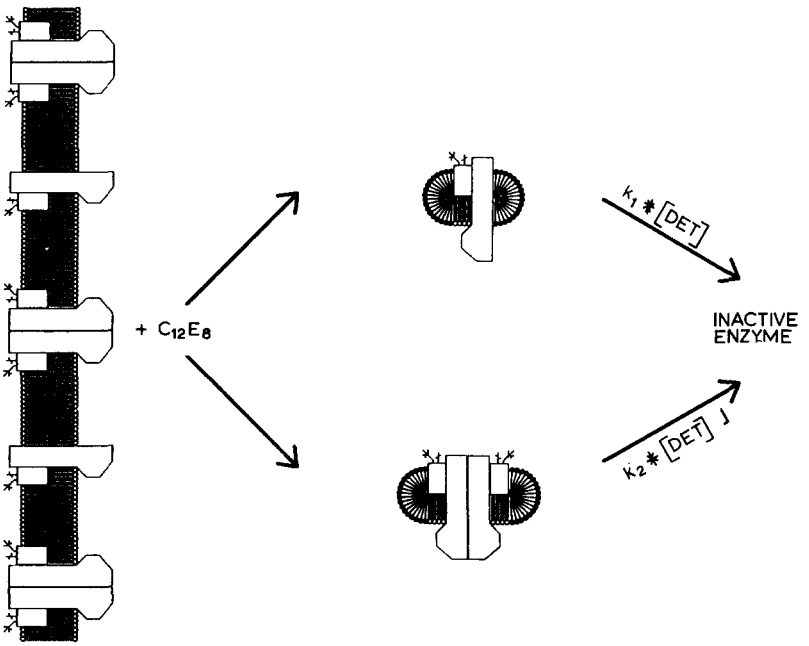

Fig. 8. Model for the effect of $C_{12}E_8$ on the membrane bound shark $(Na^+ + K^+)$-ATPase. $C_{12}E_8$ solubilizes the enzyme molecules as diprotomers or protomers, predominantly protomers at high detergent concentrations. At a given detergent/protein ratio the protomers are inactivated with the rate constant k_1, whereas the diprotomer is inactivated with the rate constant k_2, which is 15-fold lower (see text).

be done with HPLC, cf. Refs. 12 and 19). Secondly, the $(Na^+ + K^+)$-ATPase activity of each component should be followed with time under conditions where the protomers do not aggregate to form diprotomers. This is the difficult step, since protomers of both the shark and kidney enzyme aggregate with time [8,2]. If the experimental problem of keeping the protomers in the protomeric form could be overcome, a good test of the scheme in Fig. 8 could be made.

An interpretation of the parameters *M* and *D* used for the curve-fitting (cf. Fig. 7) could be that – in the absence of $C_{12}E_8$, i.e., by extrapolation to zero detergent – there is about 25% *M* (and 60% *D*). This interpretation (possibly too far-fetched) leads to the conclusion that about 1/4 of the membrane-bound enzyme is protomeric, with the rest existing as higher oligomers.

In an attempt to further characterize the solubilization step, the following experiment was performed. The enzyme was solubilized at a $C_{12}E_8$/protein ratio of 4, and after 2 min at 37°C (there is no appreciable inactivation within 2 min at this ratio, cf. Fig. 4), $C_{12}E_8$ was added to give a final ratio of 10. The result (not shown) was that the inactivation (after the 2 min 'preincubation') proceeded just as if the enzyme had been solubilized directly at a ratio of 10. This suggests that it is not the $C_{12}E_8$/protein ratio at the solubilization step which 'presets' the amount of protomer and diprotomer, but rather the $C_{12}E_8$/protein ratio in solution (had the rates of inactivation been slower in the above-mentioned case, this would have meant that a solubilized diprotomer could not readily be dissociated to a protomer by addition of $C_{12}E_8$).

The transfer of resonance energy from tryptophan on the enzyme to fluorescent lipid molecules has been used to study the effect of detergent on the lipid/protein interaction [7,11,20], and a biphasic inactivation of energy transfer and $(Na^+ + K^+)$-ATPase activity has been observed with the electroplax enzyme [20]. The earlier experiments suggested a correlation between delipidation and loss of activity [11,20].

Similar experiments with shark enzyme, labelled with dansylphosphatidylethanolamine [7,11], showed that the energy transfer was completely (and immediately, i.e., within 1 min) abolished by solubilization at $C_{12}E_8$/protein ratios of 10–16 at 37°C (data not shown). This suggests that delipidation is not directly related to inactivation, but to a process prior to loss of activity (or – less interesting – that the fluorescent lipid molecules

are not part of the boundary layer of phospholipid molecules thought to be essential for activity).

Note that the curves in Figs. 4 and 6 are identical (same sets of parameters in Eqn. 3). This indicates that the $(Na^+ + K^+)$-ATPase and the K^+-*p*-nitrophenylphosphatase activities are inactivated at about the same rate, at least at the lower $C_{12}E_8$/protein ratios. At higher ratios (10–16), the K^+-*p*-nitrophenylphosphatase activity is lost faster than the $(Na^+ + K^+)$-ATPase activity, in contrast to the common notion that the K^+-*p*-nitrophenylphosphatase activity is more stable (less susceptible to inactivation) than the $(Na^+ + K^+)$-ATPase activity. We have at present no explanation for this – albeit small – effect.

Inactivation of solubilized kidney enzyme at 37°C

The control experiments shown in Figs. 2 and 3 shows that all the $(Na^+ + K^+)$-ATPase activity of the kidney enzyme is solubilized at detergent/protein ratios above 1 and that ATP hydrolysis is linear with time at 23°C at detergent concentrations up to 80 μg/ml in the test solution (and 10 μg protein/ml). It is therefore possible to investigate the inactivating effect of $C_{12}E_8$ on the solubilized kidney enzyme, provided the detergent/protein ratio at the incubation step is above 1 and that the dilution of detergent is sufficiently large to give – at the most – 80 μg $C_{12}E_8$/ml in the test solution.

Fig. 9 shows the results of such an experiment, where the detergent/protein ratio has been varied between 1 and 6 (giving, for the highest ratio, a $C_{12}E_8$ concentration in the test solution of 75 μg/ml; see above). Note that the specific $(Na^+ + K^+)$-ATPase activity corresponding to 100% is the same for all six inactivation curves, i.e., if the enzyme had been solubilized at a low detergent/protein ratio and at 4°C and then transferred to the test solution at 23°C with up to 80 μg $C_{12}E_8$/ml, the activity would have been the same for all six curves. The K^+-*p*-nitrophenylphosphatase was inhibited in parallel with the $(Na^+ + K^+)$-ATPase (not shown).

The data given in Fig. 9 can, at least qualitatively, be interpreted as showing a rapid drop in $(Na^+ + K^+)$-ATPase activity, followed by a reasonably monoexponential decay. The quality of the data does not allow for a more refined analysis.

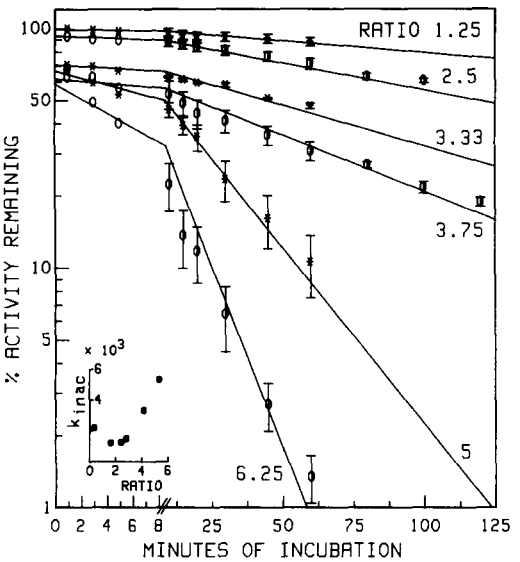

Fig. 9. Inactivation of kidney $(Na^+ + K^+)$-ATPase activity by $C_{12}E_8$ at 37°C. Kidney enzyme (0.6 mg protein/ml) was incubated at 37°C with the detergent/protein weight ratios indicated and the residual $(Na^+ + K^+)$-ATPase activity is shown as function of the time spent at 37°C. The solid lines represent a single exponential with the second-order rate constants (k_{inac}) for inactivation shown in the inset. The dimension of k_{inac} is $(min \cdot mg\ C_{12}E_8/mg\ protein)^{-1}$. About 0.8 mg $C_{12}E_8$ is bound per mg protein in the solubilized state [2]. For the purpose of showing the relationship between the rate of inactivation and the free $C_{12}E_8$ concentration, the bound $C_{12}E_8$ has been subtracted from the total $C_{12}E_8$ concentrations, giving the abcissa values in the inset. The standard deviations of 3–5 experiments are shown.

The observed rate constant for the slow phase inactivation is not proportional to the $C_{12}E_8$ concentration, in contrast to what is found with the shark enzyme. The inset in Fig. 9 shows the calculated rate constant (k_{inac}) in $(min \cdot mg\ C_{12}E_8/mg\ protein)^{-1}$ at the $C_{12}E_8$/protein ratios tested. Had the inactivation followed the same kinetics as the shark enzyme, this constant would have been independent of the $C_{12}E_8$/protein ratio, cf. Eqn. 2. Clearly, the value for k_{inac} is not constant, but tends to increase with the ratio.

Another point of discrepancy is that the amplitude of the rapid phase (the rate of which is measurable with the shark enzyme, but too rapid for the kidney enzyme) seems to level off at about 30–40% at detergent/protein ratios of 2–4, whereas there is a linear relationship between the amplitude of the rapid phase and the detergent/protein ratio for the shark enzyme, cf. Fig. 7.

There seems to be no straightforward explanation for the kinetics of the detergent inactivation of kidney enzyme, but if the data are to be interpreted in terms of the model shown in Fig. 8, the following points can be made: (a) the maximal amount of protomer formed is limited to 40% of the enzyme molecules, the activity of which is lost rapidly at 37°C; and (b) the residual activity – the diprotomer – is inactivated with an observed rate constant which is not proportional to the $C_{12}E_8$ concentration (i.e., k_{obs} is not equal to k_{inac} times the $C_{12}E_8$/protein ratio, cf. Eqn. 2). A qualitative explanation for this could be that the diprotomer of the kidney enzyme, at high $C_{12}E_8$/protein ratios, is dissociated into the protomer with time, leading to an extra rapid decrease in activity.

Another explanation, which could be in line with the observations of a larger protomeric content of the kidney enzyme than the shark enzyme [1–8,10–12,19], is the following. Up to 40% of the enzyme is – for unknown reasons – denatured immediately upon solubilization and the remaining 60% of active protomer looses activity with the rate constant (k_{inac}) of 0.002–0.005 (min · mg $C_{12}E_8$/mg protein)$^{-1}$. However, this rate constant is, interestingly, comparable to the inactivation rate constant for the shark enzyme diprotomer.

Effect of protein concentration and conformational state of the inactivation

Protein concentration. If there is a protomer-diprotomer equilibrium in $C_{12}E_8$ solution, then a variation in the protein concentration – at a fixed detergent/protein ratio – could give information on the equilibrium between the two oligomers, since the protomer is inactivated 15-fold more rapidly than the diprotomer.

However, a variation in the protein concentration of shark enzyme between 0.25 and 2 mg/ml protein and a $C_{12}E_8$/protein ratio of 4 gave, within experimental error, the same inactivation curves. This indicates that the ratio between the diprotomer and protomer is independent of the protein concentration, in contrast to what has been found with, for example, Triton X-100-solubilized Ca^{2+}-ATPase (see Ref. 21 for a discussion).

Conformational state. Kidney enzyme, purified from pig kidney, has been reported to be several orders of magnitude more labile at 37°C in the $E_1 \cdot Na$ conformation than in the $E_2(K)$ conformation (see Ref. 10). This prompted us to see (a) whether there is a large difference in lability between $E_2(K)$ and $E_1 \cdot Na$ in the shark enzyme and (b) to repeat the above-mentioned studies on $(Na^+ + K^+)$-ATPase from ox kidney under our experimental conditions.

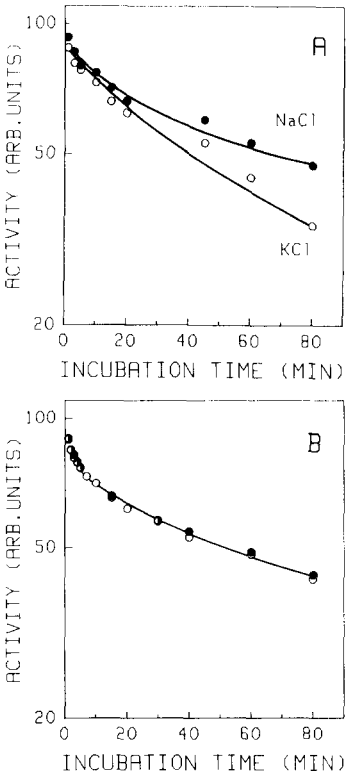

Fig. 10. Inactivation of $(Na^+ + K^+)$-ATPase in the $E_2(K)$ and $E_1 \cdot Na$ states. Kidney enzyme (A) or shark enzyme (B) was treated with $C_{12}E_8$ at a detergent/protein ratio of 4 in the presence of 150 mM NaCl (filled symbols) or 150 mM KCl (open symbols). The remaining activities were determined as described in the legend to Fig. 4 and in Materials and Methods.

Fig. 10 shows the inactivation at a $C_{12}E_8$/protein ratio of 4 of the $(Na^+ + K^+)$-ATPase activity at 37°C in the presence of 150 mM NaCl or KCl for shark enzyme (panel A) and ox kidney enzyme (panel B). For the kidney enzyme, a slightly more rapid inactivation is seen in the presence of NaCl than in KCl, but the difference between rate of inactivation in the E_1 form and $E_2(K)$ form is less than a factor of 2. The rates of

inactivation of the shark enzyme is the same in KCl and NaCl (cf. Fig. 10B). Qualitatively, the inactivation follows the previously observed bi-exponential pattern (cf. Figs. 4 and 9).

Both the rates of inactivation and the biphasic pattern reported here for the kidney enzyme are in contrast to the findings reported in the literature (e.g., Refs. 10, 11). At present we have no explanation for this apart from (trivial?) differences in temperatures [10], and time of preincubation, or methods of measurement of $(Na^+ + K^+)$-ATPase activity [10,11].

Conclusion

The kinetics of inactivation of shark rectal gland enzyme by $C_{12}E_8$ at 37°C can be interpreted in terms of a relatively simple model: The enzyme is solubilized at $(\alpha\beta)_2$ diprotomers at low $C_{12}E_8$/protein weight ratios and as $\alpha\beta$ protomers at high ratios (e.g., 12–16). The protomer is inactivated with a rate constant about 15-fold larger than that of the diprotomer.

The kidney enzyme exhibits a more complex inactivation kinetics than the shark enzyme. Firstly there is a rapid loss of up to 30–40% of the $(Na^+ + K^+)$-ATPase activity (but not more than 40%, even at high $C_{12}E_8$/protein ratios), and secondly the observed rate constant for inactivation in the 'slow' phase is not proportional to the $C_{12}E_8$ concentration over the range tested. There is only a small (for kidney) or no (for shark) difference between the rates of inactivation in the $E_1 \cdot Na$ and $E_2(K)$ forms.

The results are in agreement with earlier observations [1–13] of a higher protomeric content in – and greater lability of – the $C_{12}E_8$-solubilized kidney enzyme than for the shark enzyme.

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