Studies of the Electron Transport Chain of Extremely Halophilic Bacteria

VII. SOLUBILIZATION PROPERTIES OF MENADIONE REDUCTASE

(Received for publication, December 13, 1971)

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SUMMARY

When NaCl concentration was lowered, membrane vesicle-bound menadione reductase of Halobacterium cutirubrum showed 6- to 7-fold increased activity. The enzyme with this increased activity was initially found in a transient, membrane-bound form, followed by release from the membranes. Several detergents caused solubilization and a similar increase in enzyme activity, but sodium dodecyl sulfate, which increased activity, gave only limited solubilization. Unlike mitochondrial DPNH dehydrogenase, the halophilic system showed only minor changes in substrate affinity upon these treatments. Conditions that resulted in highly active, membrane-bound enzyme also released trapped horseradish peroxidase, which had been added as a marker of the interior of these vesicles. These results suggest that menadione reductase is attached to the interior surface of the vesicles and becomes accessible to the substrates upon the breakage of the vesicles during the course of solubilization.

Half of the menadione reductase content of the vesicles was released into the supernatant in 1 M NaCl but was still attached to large membrane fragments. As NaCl concentration was further lowered the enzyme in this fraction was detached and could be eluted from an Agarose column in a position corresponding to 36,000 molecular weight. In addition, at NaCl concentrations lower than 1 m, large membrane fragments containing the second portion of the enzyme were released. Tween 80 solubilized half of the total enzyme in the vesicles; the solubilized enzyme was eluted at 48,000 molecular weight, which probably corresponds to a protein-detergent complex. The sequential action of lowered NaCl concentration and Tween 80 resulted in the recovery of all the enzyme in this low molecular weight fraction.

Menadione reductase of Halobacterium cutirubrum, a membrane-bound, respiratory, chain-linked enzyme, has been shown to require 2 to 3 M NaCl for maximal activity and stability (7, 8). Similarly, the integrity of membranes prepared from the cell envelope of H. cutirubrum is retained only at high concentrations of salt (9, 10). In this report we have examined the relationship between enzyme activity and release from membranes at lower NaCl concentrations. In addition, we tested the effect of various detergents on the solubilization of the enzyme. By doing this, it was our intention to develop a system in which the membrane-dependent properties of DPNH dehydrogenase can be studied.

METHODS

Sources of Chemicals—Menadione, equine cytochrome c (type III), bovine serum albumin, cetyltrimethyl ammonium bromide, 2-hexyl-4-hydroxyquinoline-N-oxide, phenylmercuric acetate, and N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid were from Sigma; human gamma globulins, chymotrypsinogen A (bovine pancreas, six times crystallized), and Tween 80 from Mann; reduced diphosphopyridine nucleotide from CalBiochem; o-dianisidine from Nutritional Biochemicals; Triton X-100 from Rohm & Haas; sodium dodecyl sulfate from Matheson Coleman; Blue Dextran from Pharmacia; and horseradish peroxidase (3100 i.u. per mg, RZ = 2.9) from Worthington.

Buffers—N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid (0.05 M, pH 7.0) was used throughout. The pH was adjusted with NaOH after adding salt, with the aid of a low sodium error Corning electrode.

Preparation of Membrane Vesicles—Growth and harvesting of H. cutirubrum cells has been described elsewhere (11). Cell envelope vesicles were prepared as in a previous publication (10), but vesicle preparations were stored in buffer containing 5.0 M NaCl and basal salts. Membrane protein was determined as described previously (10).

Enzyme Assays—Menadione reductase (1.6.99.2) was assayed as before (7). All manipulations involving this enzyme were carried out in semidarkened rooms because the enzyme was sensitive to light in dilute solution.

Horseradish peroxidases (1.11.1.7) assays contained the following per ml of buffer: 1.3 μmoles of H₂O₂, 0.27 μmoles of o-dianisidine (stock solution 1% in methanol) (12). Absorbance increase at 460 nm was followed in a Cary 14 spectrophotometer (ε = 11.3 × 10⁵ M⁻¹ cm⁻¹).

The properties of respiratory chain-linked DPNH dehydrogenase from mammalian sources depend greatly on the membrane matrix surrounding the catalytic portion of the enzyme. Various degrees of solubilization result in changes of acceptor specificity and enzyme rate (1-4), inhibitor specificity (1, 3, 5, 6), and composition (1, 3, 6).
Gel Exclusion Chromatography—Agarose A-0.5m and A-5m (BioRad), swollen in buffer containing 3.4 M NaCl, were used at flow rates of 18 to 20 ml per hour in a column (2.5 x 85 cm) at 5°. The agarose A-0.5m column was calibrated with Blue Dextran, human gamma globulin, bovine serum albumin, chymotrypsinogen A, and equine cytochrome c as standards. The ratio of the volume of elution, $V_e$, and the void volume, $V_v$, was a linear function of the logarithm of the molecular weight. Enzyme recoveries on overnight column fractionations were 60 to 80%.

RESULTS

Menadione Reductase Activity of Intact and Damaged Vesicles—Membrane vesicles showed considerably less menadione reductase activity than previous preparations, which were obtained by the more drastic treatment of breakage with a French press (7, 8). As shown in Fig. 1, however, where the vesicles were assayed in the presence of Triton X-100, addition of the detergent caused a 6- to 7-fold increase in enzyme activity. This transition occurred at a detergent to protein ratio of 0.15 to 0.25; the amount of detergent required was dependent on the concentration of total protein. As shown in Fig. 1, passing a vesicle preparation through the French press at 25,000 p.s.i resulted in a corresponding increase in enzyme activity and Triton X-100 had no further effect on the vesicle fragments obtained in this way. Menadione reductase was shown previously to exhibit partial activity when NaCl is replaced by lower concentrations of MgCl$_2$ (7). Membrane vesicles in the presence of Triton X-100, however, yielded very little enzyme activity under these conditions. The low activity in 100 mM MgCl$_2$ was at least partly due to rapid inactivation of the enzyme by the detergent during the assays, an effect generally observed in the absence of the stabilizing effect of NaCl.

The appearance of enzyme activity in these experiments was thus related to the fragmentation of the vesicles. Lowering the NaCl concentration had been shown previously to result in the disintegration of H. cutirubrum membranes (9, 11). Menadione reductase activity was therefore assayed at various NaCl concentrations in the presence and absence of excess detergent. In Fig. 2, initial enzyme activity is plotted against NaCl concentration. In the presence of Triton X-100, a typical hyperbolic salt dependence curve is obtained, similar to that reported previously (7) in French press-treated extracts. In the absence of detergent however, enzyme activity shows a maximum at 1.25 M NaCl and decreases at salt concentrations above this value, where the enzyme becomes latent as in Fig. 1. The two curves, obtained in the presence and absence of detergent, approach one another below 1 M NaCl.

Salt-dependent Release of Menadione Reductase—Previously, we reported that at gradually lowered NaCl concentrations various components of the membrane vesicles became detached (10). The solubilization of menadione reductase was tested in similar experiments. Vesicle preparations were added to aliquots of buffer containing a given concentration of NaCl and were incubated for increasing periods of time at room temperature. Equal volumes of buffer containing 5.0 M NaCl were added and the membranes were collected by centrifugation at 35,000 x g for 20 min. The pellets were resuspended in the same volume of buffer as the supernatants, previously adjusted to contain the same concentration of salt (approximately 2.7 to 3.0 M). Both supernatant and pellet fractions were assayed with and without Triton X-100. The detergent had no effect on the enzyme activity of supernatant fractions. Fig. 3 shows the time course of the solubilization of menadione reductase at (a) 0.4 M, (b) 0.6 M, and (c) 1.0 M NaCl. The results are shown as per-

![Fig. 1 (left). Menadione reductase activity of membrane vesicles in the presence of Triton X-100. Membrane protein in assays 0.08 mg per ml. O, assay in buffer containing 3.4 M NaCl; ●, assay in buffer containing 100 mM MgCl$_2$; △, French press-treated vesicles (25,000 p.s.i.) assayed in buffer containing 3.4 M NaCl.](http://www.jbc.org/)

![Fig. 2 (right). Salt dependence of menadione reductase activity in the presence and absence of Triton X-100. Membrane protein in the assay mixture and initial rates were determined within the first 20 sec. O, assay in the presence of 0.004% Triton X-100; ●, assay without detergent.](http://www.jbc.org/)

![Fig. 3. Time course of solubilization of menadione reductase at lower NaCl concentrations. Procedure: 0.05 ml of vesicle preparation was added to 5 ml of buffer containing (a) 0.4 M, (b) 0.6 M, and (c) 1.0 M NaCl. Incubation for the indicated time at room temperature was terminated by adding 5 ml of 5 M NaCl. The suspension was centrifuged at 35,000 x g for 20 min and the pellet was suspended in 10 ml of buffer previously adjusted to contain the same NaCl concentration as the supernatant. Enzyme activity in the pellet fractions is shown as assayed with and without Triton X-100 (0.004%); the supernatant activity was not affected by the detergent. On the top of the graphs the percentage of recovery of total activity (supernatant plus pellet assayed with detergent) is plotted. Symbols in the lower graphs: O, pellet assayed with detergent; ●, supernatant; △, pellet assayed without detergent.](http://www.jbc.org/)
centage of total enzyme activity in various fractions versus the length of exposure to the salt buffer. The total enzyme activities (sum of supernatant and pellet with detergent activities) are plotted at the top of Fig. 3 and show relatively slow inactivation, with half-lives of 5 to 10 min, during the incubation. In contrast, the appearance of enzyme activity in the supernatants is rapid, with a half-life of a few seconds even at 1 M NaCl. Lower NaCl concentration resulted in a greater proportion of the enzyme released. Adding detergent to the pellet fractions caused an increase in enzyme activity similar to the results in Figs. 1 and 2. However, the pellet-bound enzyme activity, even when determined in the absence of detergent, showed increases in the first minute of incubation at all NaCl concentrations tested. The subsequent disappearance of this transitory membrane-bound activity was not due to preferential inactivation during further incubation since pellets obtained by incubating with 0.25 M NaCl for 30 sec exhibited the same rate of inactivation at 1.0 M NaCl as supernatant fractions. Rather, it is likely that the rapid decrease in this pellet-bound activity after the initial part of the incubation is due to detachment of the enzyme from the membranes.

The above experiments were also carried out at NaCl concentrations from 0.03 to 2.6 M, with 5-min incubation at the given salt concentration, followed by addition of an equal volume of 5 M NaCl and centrifugation. The enzyme activities in various fractions are shown in Fig. 4a as functions of salt concentration. The release of menadione reductase into the supernatant is nearly complete in 5 min at less than 0.5 M NaCl; above this concentration, the release of activity decreases with increasing NaCl concentration. Increased pellet-bound enzyme activity appearing at the same salt concentrations as solubilization, is maximal at 1.25 M NaCl, and declines below this concentration. The increased enzyme activity at intermediate salt concentrations in Fig. 4a is thus seen to be due to both the increased vesicle-bound activity and the higher activity of the solubilized enzyme. Similar experiments were carried out by incubating the vesicles in the absence of NaCl but with MgCl₂. As before, the 5-min incubation period was terminated by adding an equal volume of buffer containing 5 M NaCl. The results are shown in Fig. 4b, plotted in an analogous manner to Fig. 4a. The results are similar to those obtained with NaCl; solubilization occurs between 4 and 20 mM MgCl₂, maximal pellet activity is seen at 14 mM MgCl₂.

Kinetic parameters for substrates and inhibitors were obtained for menadione reductase in untreated membranes, membranes plus detergent, membranes in MgCl₂, low salt-treated pellets, and low salt supernatants. Table I contains Kₐ values for menadione and DPNH, maximal enzyme activity, and Kᵢ values for 2-heptyl-4-hydroxyquinoline-N-oxide (13) and phenylmercuric acetate, determined under the above conditions. These data indicate that despite the great variety of treatments, substrate affinities assume only a few discrete values. The menadione-binding site was thus found to exist in two possible forms: (a) with Kₐ = 40 to 50 μM in untreated vesicles, in low salt disrupted membranes, and in French press-prepared preparations (7), and (b) with Kₐ = 125 μM in low salt and detergent-solubilized fractions, as well as in membrane-bound form when assayed in MgCl₂ buffer (Table I). Although this change in the affinity of the enzyme for menadione is not yet understood, it may be significant that detachment from the membranes brings about the same increase in Kₐ as is found to result from the

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**Table I**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>K₀, M</th>
<th>V₀, a</th>
<th>Kᵢ¹</th>
<th>Kᵢ²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated membranes in 3.4 M NaCl</td>
<td>125</td>
<td>746</td>
<td>0.30</td>
<td></td>
</tr>
<tr>
<td>Untreated membranes in 3.4 M NaCl + 0.0004 M MgCl₂</td>
<td>128</td>
<td>626</td>
<td>0.53</td>
<td></td>
</tr>
<tr>
<td>Pellet fraction in 3.0 M NaCl</td>
<td>53</td>
<td>50</td>
<td>0.30</td>
<td>1.0</td>
</tr>
<tr>
<td>Supernatant fraction in 3.0 M NaCl</td>
<td>143</td>
<td>20</td>
<td>0.61</td>
<td>1.6</td>
</tr>
</tbody>
</table>

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*Measured in micromoles per min per mg of protein DPNH oxidized.

1 2-Heptyl-4-hydroxyquinoline-N-oxide.

2 Phenylmercuric acetate. The enzyme was incubated 2 min with the inhibitor before addition of DPNH.

3 Vesicles were added to buffer containing no NaCl (20-fold dilution), incubation was terminated at 30 sec by adding 5 M NaCl. The pellet was collected by centrifugation at 35,000 × g for 20 min and was resuspended in 2.5 M NaCl of the same volume as the supernatant. In the assays, both fractions were diluted 21 times with 3.4 M NaCl.

4 Removal of hydrophobic stabilization, on replacing NaCl with low concentrations of MgCl₂ in the medium (7, 8).

Inhibition by 2-Heptyl-4-hydroxyquinoline-N-oxide and phenylmercuric acetate was observed under all conditions tested.
In general, any treatment, particularly solubilization, decreased the effectiveness of the former inhibitor.

In other respiratory chain-linked DPNH dehydrogenase systems, denaturing conditions bring about solubilization, large increases in affinity to nonspecific electron acceptors (1-4), and changes in inhibitor specificity (1, 3, 5, 6). In contrast with the behavior of mitochondrial DPNH dehydrogenase, the increase of enzyme activity in the halophilic system is accompanied by either no change in $K_m$ (for the pellet-bound fraction) or an increase in the $K_m$ (supernatant fraction). While the mitochondrial enzyme acquires sensitivity to mercurials only on denaturation (1, 3, 5), the halophilic enzyme is inhibited by phenylmercuric acetate in all its forms. In *H. cutirubrum* membranes, therefore, the appearance of highly active menadione reductase seems to be related mostly to the topological integrity of the vesicles.

**Detergent Solubilization of Menadione Reductase**—As shown in Figs. 1 and 2, solubilization of menadione reductase either by detergent treatment or by exposure to lowered NaCl concentrations caused a large increase in enzyme activity. For low salt treatment this increase was due to both the increase in membrane-bound activity and the higher activity of the supernatant fraction. This point was investigated in the case of detergent solubilization as well. In these experiments, vesicle preparations were added to buffer containing 3.4 M NaCl and the given concentration of detergent. After centrifugation at 35,000 $\times$ $g$ for 20 min the pellet was resuspended in the same volume of salt buffer and both pellet and supernatant fractions were assayed. Menadione reductase activities in these fractions are plotted against detergent to protein ratios in Fig. 5 for Triton X-100, cetyl trimethylammonium bromide, sodium dodecyl sulfate, and Tween 80. As Fig. 5 indicates, in all cases except sodium dodecyl sulfate, highly active enzyme was liberated from the membranes while pellet activity declined. The detergent to protein ratios required for solubilization varied for the different detergents; with Tween 80 only half of the total membrane-bound enzyme could be solubilized. The results with sodium dodecyl sulfate show little solubilization until 0.3 to 0.4 detergent to protein ratios are reached. Pellet-bound enzyme activity is seen to increase, however, at ratios of 0.1 to 0.3. The limited solubility of this detergent at the high concentration of NaCl present in the buffer made it impossible to carry out the experiment at higher detergent concentrations. The data indicate that the anionic detergent is very ineffective in solubilizing the enzyme. Conversely, the cationic detergent is most active in solubilization.

Since the bulk of membrane proteins in extreme halophiles is hydrophobic, the cationic detergent is most active in solubilization. In extremely halophilic bacteria the lipids are almost entirely ether-type phospholipids (18) lacking both carbonyl and amino groups and the membrane proteins of extreme halophiles are strongly acidic (19, 20). Thus, the possibility that interactions between polar groups is involved in the structure of these membranes seems to be limited. Indeed, sodium dodecyl sulfate, an anionic species, interacts very poorly with the acidic halophilic membranes (Fig. 5). The ease of removal of menadione reductase from the membrane matrix by a weak detergent, Tween 80, suggests that this enzyme is bound mostly, if not exclusively, by hydrophobic forces. Such a conclusion was suggested previously for membrane-bound flavoproteins of *H. cutirubrum* (10).

**Release of Horseradish Peroxidase (Marker for Interior of Vesicles)**—The disintegration of the membrane vesicles probably involves, at an early stage, the opening of the vesicles exposing the interior surface of the membrane. This process was investigated by following the release of an enzyme trapped during the preparation of the vesicles. For this purpose, horseradish peroxidase was added, at approximately 0.1 mg per ml, to the cell paste before breaking. During isolation the membrane vesicles retained a small fraction of this enzyme. Table II shows experiments designed to test the assumption that the retained enzyme is contained inside the vesicles. The assays were conducted in buffer containing 3.4 M NaCl, under conditions of controlled damage to vesicles. The peroxidase activity of intact vesicles in Table II is probably overestimated because handling during resuspension of the pellet very likely caused some re-equilibration of the intravesicle space with the medium. Nevertheless, as Table II shows, over 2-fold enhancement of enzyme activity is observed in the presence of detergent and substantial increases are seen for sonicated and freeze-thawed preparations. Endogenous peroxidase activity was not measurable in membranes. When peroxidase was added to an unmarked vesicle preparation, the pellet recovered after centrifugation contained negligible enzyme activity. These results indicate that the peroxidase is not adsorbed on the outside of the vesicles but rather appears to be in the intravesicle space.

The release of trapped peroxidase was investigated under two experimental conditions. First, the vesicles were incubated at various concentrations of NaCl, followed by addition of 5 M NaCl and centrifugation. The pellets were resuspended in buffer containing no NaCl to disintegrate the vesicles and thus make the total retained peroxidase content of the pellet available for assay. The results are plotted in Fig. 6a as percentage peroxidase released from the vesicles, using the peroxidase content of a pellet derived from intact vesicles for total peroxidase. Fig. 6a shows that 50% of the trapped enzyme is released at
exposed to increasing amounts of sodium dodecyl sulfate (as in Fig. 5) and the pellet-bound peroxidase activity was determined under "Methods."

...peroxidase activity as described under "Methods." b, 0.2 ml of vesicles was added to 9 ml of buffer containing 3.4 m NaCl; the pellets obtained by centrifugation at 35,000 X g for 20 min were resuspended in 5 ml of buffer containing 0, 0.4, and 1.0 m NaCl, respectively. Incubation for 60 sec at room temperature was followed by adding solid NaCl to saturation and the supernatants from these solutions were applied to the column. a, a pellet fraction, obtained at 1% Tween was added to 1% Tween 80 and the excess detergent was removed by dialysis as before. Experiments with added peroxidase as internal controls indicated that the detergent did not inhibit the enzyme. As shown in Fig. 6b, the release of peroxidase begins at a detergent to protein ratio of 0.1; half of the enzyme is released at a ratio of about 0.25.

Fig. 6. Release of trapped horseradish peroxidase from membrane vesicles by (a) incubation at lowered NaCl concentrations and (b) addition of sodium dodecyl sulfate. Procedure: a, 0.1 ml of vesicles was added to 5 ml of buffer containing the indicated concentration of NaCl. After 5 min of incubation at room temperature equal volumes of 5 m NaCl were added and the pellets were recovered by centrifugation at 35,000 X g for 20 min. Each pellet was resuspended in 1 ml of buffer containing no salt. Peroxidase assay is described under "Methods." b, 0.2 ml of vesicles was added to 10 ml of buffer containing 3.4 m NaCl and sodium dodecyl sulfate (final protein concentration 0.24 mg per ml). Pellets were removed and assayed as under a.

TABLE II

Assay of vesicle-trapped horseradish peroxidase

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Peroxidase activity (molecules/min H2O)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None...</td>
<td>9.5</td>
</tr>
<tr>
<td>0.004% Triton X-100 (detergent to protein ratio, 0.1)</td>
<td>18.4</td>
</tr>
<tr>
<td>0.008% Triton X-100 (detergent to protein ratio, 0.2)</td>
<td>19.9</td>
</tr>
<tr>
<td>30-sec sonicationa</td>
<td>12.0</td>
</tr>
<tr>
<td>120-sec sonicationa</td>
<td>14.2</td>
</tr>
<tr>
<td>Freeze-thaw</td>
<td>11.7</td>
</tr>
<tr>
<td>Unmarked membranes</td>
<td>0</td>
</tr>
<tr>
<td>Unmarked membranes plus peroxidase, pellet fractionb</td>
<td>1.4</td>
</tr>
</tbody>
</table>

* Sonication in a model 8845-3 Cole-Palmer ultrasonic tank.

b Vesicles not marked with horseradish peroxidase were resuspended (0.5 mg per ml membrane protein) in buffer containing 3.4 m NaCl and peroxidase to give an enzyme activity of 20 mules per min. The pellet recovered after centrifugation at 35,000 X g for 20 min was resuspended in the same volume of buffer and was assayed for peroxidase.

Electrophorographs have shown before that the vesicle membrane is continuous above 1 m NaCl (9, 10). In contrast, results with trapped horseradish peroxidase (reported here) indicate that the interior of the vesicles begins to become accessible to the environment as NaCl concentration is lowered to 2 to 2.5 m. Comparison of the conditions that cause release of the trapped enzyme (Fig. 6) with those conditions that result in increased membrane-bound menadione reductase activity (Figs. 4a and 5) shows that the increase in enzyme activity occurs concurrently with the opening of the vesicles. On the basis of these observations we suggest the following hypothesis. Menadione reductase is attached to the interior surface of the vesicle membrane. The low enzyme activity of untreated vesicles is due to the inability of the substrates to reach the enzyme; the enzyme activity detected in the preparations may be due to damaged vesicles. Membrane-bound enzyme activity increases when the vesicles break open and the enzyme becomes accessible to the medium. The data do not reveal whether full activity can be attained while the enzyme is still attached to the membrane since both low salt and detergent treatments also cause the...
The high enzyme activity of French press-fragmented vesicles release of the enzyme, following closely the opening of the vesicles. (Fig. 1) suggests that the enzyme need not be solubilized to exhibit full activity.

**Gel Exclusion Chromatography of Released Menadione Reductase—Agarose gels of 0.5 and 5.0 million exclusion molecular weights, A-0.5m and A-5m, were used to test supernatant fractions obtained from vesicles by various means. Elution profiles of enzyme activity and absorption at 280 nm, obtained with Agarose A-0.5m columns, are given in Fig. 7, where a, b, c, and d show results from experiments with low salt released menadione reductase. Membrane vesicles were resuspended in buffer containing 3.4 M NaCl but no “basal salts” (10) were centrifuged at 35,000 × g for 20 min, and the pellets were resuspended in buffers containing no salt, 0.4, and 1.0 M NaCl (5 ml total volume). The final NaCl concentrations, determined by atomic absorption, were 0.14, 0.53, and 1.12 M, respectively, while MgCl₂ concentrations were estimated at 0.2 M. After a 60-s incubation solid NaCl was added to saturation, the solutions were centrifuged, and the supernatants were introduced onto the top of the Agarose column. Fig. 7, a, b, and c, shows the elution profiles obtained for the supernatants at the above three salt concentrations. In all three graphs, two peaks of enzyme activity are seen, one eluted at the void volume (both on Agarose A-0.5m and A-5m columns) and the other at a volume corresponding to 36,000 molecular weight. The proportion of enzyme activity under the two peaks varies; it approaches a ratio of 1:1 at low NaCl concentrations while the amount of enzyme found in the second peak diminishes at higher NaCl concentrations. The equal ratio of enzyme activity in the two peaks at low NaCl concentrations was not altered by the following changes: decreasing the amount of vesicles in the same volume by 10-fold throughout the experiment, varying the duration of low salt exposure between 10 and 120 sec, or varying MgCl₂ concentration during the low salt exposure between 0.2 and 4 M. The enzyme in both peaks exhibited DPNH-cytochrome c reductase activity (13).

**Release of menadione reductase from the membrane vesicles** occurs below 2 M NaCl and is nearly complete at 0.5 M NaCl (Fig. 4a). Near 1 M NaCl the solubilized enzyme is seen to be associated with large fragments (Fig. 7c) while at lower salt concentrations more enzyme is released and another species is detected which elutes at 36,000 molecular weight on Agarose (Fig. 7a). It was possible to test whether the low molecular weight form of the enzyme, recovered below 0.2 M NaCl, originates from the membrane fragments released at 1 M NaCl or directly from the vesicles. The vesicles were first exposed to 1 M NaCl, the solubilized enzyme removed, and the residual pellet was treated at low NaCl concentration. As seen in Fig. 7d, the enzyme released in the second step eluted at the void volume. Thus, the sequence of events occurring on lowering the NaCl concentration may be reconstructed as follows. At 1 M NaCl the released menadione reductase, about half of the total enzyme present, is still attached to large membrane fragments. As NaCl concentration is lowered, these fragments further disintegrate to give low molecular weight enzyme. In addition, the second half of the menadione reductase content of the membranes is released, this fraction also being attached to large fragments.

The data in Fig. 7, e and f were obtained with detergent-solubilized samples. Vesicle preparations (1 ml) were suspended in 5 ml total volume of buffer containing 3.4 M NaCl and 1% Tween 80, giving a detergent to protein ratio of approximately 4. The solution was centrifuged at 35,000 × g for 20 min; the supernatant was dialyzed against 100 volumes of the same buffer at 0° for 6 hours to remove excess detergent, and was applied to the Agarose column. As shown in Fig. 5, Tween 80 solubilizes about half of the total amount of vesicle-bound menadione reductase. In Fig. 7f, this solubilized fraction is seen to elute at a volume corresponding to 48,000 molecular weight. This molecular weight is consistent with a 3:1 protein-detergent complex (21) containing the low salt released species. Very little ultraviolet-absorbing material is found in the supernatants obtained with Tween 80, indicating that the enzyme behaves unlike the bulk of the membrane protein. Fig. 7f shows the elution pattern of a supernatant fraction obtained by the sequential action of low salt and detergent exposure. The supernatant enzyme fraction was collected after incubation at 0.14 M NaCl as in Fig. 7a. Tween 80 was then added to a detergent to protein ratio of 4 and the excess detergent was removed by dialysis as above. In Fig. 7f the enzyme activity (which in Fig. 7a appears in two peaks) eluted in one peak at the position of 48,000 molecular weight. The elution pattern of other proteins, as measured by absorbance at 280 nm, showed virtually no change. In a similar experiment, Tween 80 was added to a solubilized fraction obtained at 1.12 M NaCl (as in Fig. 7c) and after dialysis the elution pattern on Agarose A-0.5m was determined. As in Fig. 7f, all enzyme activity was recovered in the peak corresponding to 48,000 molecular weight. It was not possible to carry out the experiment in the reverse sequence, that is, to solubilize first with the detergent, followed by low salt exposure, since at low NaCl concentrations even traces of detergent inactivated the enzyme.

Enzyme fractions solubilized with Triton X-100 and cetaryl trimethylammonium bromide eluted in two peaks also, one at the void volume and one at a volume corresponding to several hundred thousand molecular weight. However, the latter peak appeared consistently at the expected position for this molecular weight range on both Agarose A-0.5m and A-8m columns, the ratios of enzyme activity found in the two peaks varied greatly. It is likely that these detergents form complexes with membrane proteins in which menadione reductase is included in a nonspecific manner.

The heterogeneity of menadione reductase in the above solubilization experiments almost certainly reflects the existence of two kinds of binding of the enzyme in the membrane matrix. Although kinetic data for enzymes do not reveal a 2-fold distribution, menadione reductase activity may correspond to two distinct enzymes, perhaps to the two DPNH dehydrogenases suggested in a previous publication (13).

**REFERENCES**

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