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Precipitation of solubilized Na^+/K^+-ATPase by divalent cations

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A method for preparation of membranous fragments of pure and highly active shark rectal gland Na^+/K^+-ATPase by Mn^{2+} precipitation of $C_{12}E_8$-solubilized enzyme is described. The method is rapid and inexpensive, and yields enzyme with a specific Na^+/K^+-ATPase activity of up to 1800 μmol/mg per h at 37°C. The influence of the detergent/protein and lipid/protein ratios on the yield of membrane bound enzyme is described.

Introduction

The Na^+/K^+-ATPase is an integral membrane protein responsible for the active transport of Na^+ and K^+ across the plasma cell membrane. The protein composition of the enzyme is an α-subunit (M_r about 112000, Refs. 1 and 2) and a β-subunit (M_r about 35000, Refs. 3 and 4) in equimolar amounts, and the enzyme requires an annulus of phospholipid at the hydrophobic parts of the protein for activity to be preserved.

Purification of the enzyme has been carried out almost exclusively with the use of detergents such as SDS, DOC, Lubrol or $C_{12}E_8$. There are in principle two approaches for purification, either solubilization of the enzyme followed by a reformation of the membrane structure at a later step [5] or selective extraction of protein impurities [6–8]. Both methods yield preparations which are pure with respect to protein composition, i.e. only α- and β-subunits are present on SDS gels.

The present paper describes a new approach to purification through solubilization of Na^+/K^+-ATPase from shark rectal glands in $C_{12}E_8$. The enzyme is in a fully active state in this detergent, existing as a mixture of αβ-protomers and $(\alpha\beta)_2$-diprotomers [9–11].

It has previously been observed that an increase in ionic strength (for example from 0 to 150 mM KCl) leads to an aggregation of the enzyme [10]. It is here reported that low concentrations of divalent cations such as Ca^{2+} or Mn^{2+} (4–6 mM) also lead to a precipitation of the soluble enzyme in a membranous form.

This observation led us to investigate if the Mn^{2+} precipitation step could be useful in preparation of membrane bound enzyme. The idea is that the starting material of membrane bound shark rectal gland Na^+/K^+-ATPase, which is easily prepared in large quantities [8], is only about 50% pure, and that $C_{12}E_8$ solubilizes the enzyme exclusively, i.e. the solubilized enzyme is 95–100% pure with respect to the α- and β-subunits. If a fully active membrane bound enzyme could be reconstituted from the $C_{12}E_8$-solubilized

Abbreviations: $C_{12}E_8$, octaethyleneglycol dodecyl monoether; α-subunit, the 112 kDa catalytic subunit; β-subunit, the 35 kDa glycoprotein; SDS, sodium dodecyl sulfate; DOC, deoxycholate.

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enzyme by Mn^{2+}-precipitation, a 2-fold increase in purity and specific activity of shark rectal gland Na^+/K^+-ATPase could be obtained by a single centrifugational step.

It was not, however, possible to achieve a specific activity of Na^+/K^+-ATPase of more than 70–80% of the specific activity to be expected from 95–100% pure enzyme isolated from example kidney using the SDS procedure [7]. The activity is, however, comparable to the enzyme activities obtained using other solubilization/precipitation procedures, and the method is much more rapid that previously published procedures.

Methods and Materials

Methods

Preparation of enzyme

Na^+/K^+-ATPase is prepared from rectal glands of *Squalus acanthias* as previously described, omitting saponin from the preparative procedure [8]. 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-subunits (determined by polyacrylamide gel electrophoresis in SDS) and the phosphorylation capacity is about 2 nmol/mg protein. Activity measurements and protein determination of the membrane bound enzyme are performed as previously described [8].

Solubilization of membrane bound enzyme

The membrane bound enzyme in 20 mM histidine (pH 7.0)/25% glycerol is solubilized with $C_{12}E_8$ as follows: To 4 vols. of membrane suspension is added 1 vol. of $C_{12}E_8$ in water at 4°C. Non-solubilized protein is removed by centrifugation for 30 min at 6–8°C in a Beckman Airfuge at $100\,000 \times g$. The supernatant contains the solubilized Na^+/K^+-ATPase and at detergent/protein ratios above 1 (w/w) all the membrane bound enzyme, but almost none of the protein impurities, is solubilized (see Ref. 9). The protein composition of the supernatant is about 65% α-subunit, about 30% β-subunit and 0–5% contaminating protein, as evidenced by scans of SDS gels (cf. Fig. 3 in Ref. 12).

Precipitation of supernatant enzyme

Supernatant enzyme is precipitated with cations as follows: To 200 μl of supernatant enzyme is added 30 μl water (or extra phospholipid, see below) and 70 μl of the desired cation as the chloride salt. After 90 min at 4°C the turbid suspension is centrifuged for 30 min in a Beckman Airfuge at $100\,000 \times g$. The supernatant is used for protein determination. The amount of protein in the pellet is determined after resuspension in 20 mM histidine (pH 7.0)/25% glycerol. The specific activity of the enzyme in the pellet is determined after the pellet has been washed twice by centrifugation at $200\,000 \times g$ in 10 ml 20 mM histidine (pH 7.0)/25% glycerol. The pelleted enzyme is termed 'reconstituted enzyme'. The protein composition of the reconstituted enzyme is identical to that of the supernatant enzyme (not shown).

Addition of extra lipid and cholesterol

In some experiments the effect of addition of extra lipid prior to precipitation of the solubilized enzyme was investigated. An appropriate combination of phospholipids and cholesterol was brought into solution by sonication in $C_{12}E_8$ at the lowest possible detergent/lipid ratio. Usually 0.8 g detergent/g lipid was sufficient to make a clear (although slightly turbid) solution of mixed phospholipid/detergent micelles.

Protein determination and activity measurements

Protein determination of solubilized, supernatant and reconstituted enzyme is carried out according to the Peterson modification [13] of the method of Lowry et al. [14] using albumin as reference. Determination of the enzymatic activity of supernatant and solubilized enzyme is carried out as previously described [10].

Materials

$C_{12}E_8$ is obtained from Nikko Chemicals, Tokyo, and $^{14}C_{12}E_8$ from CEA, France. Phosphatidyl-choline, -serine, -ethanolamine and -inositol are obtained from Avanti Polar Lipids and cholesterol from Sigma.

Results

The relative effectiveness of cations in precipitating $C_{12}E_8$-solubilized supernatant enzyme is il-

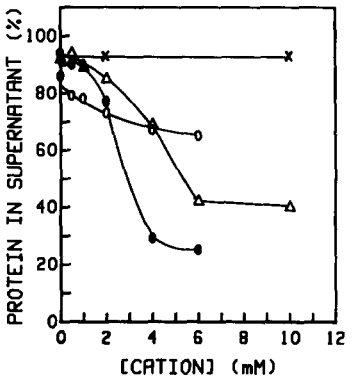

Fig. 1. Precipitation of supernatant enzyme by cations. To 200 µl supernatant enzyme (about 0.2 mg protein/ml in 16 mM histidine (pH 7.0)/20% glycerol and 0.2 mg/$C_{12}E_8$ per ml) is added 100 µl of cation/chloride. After 90 min at 4°C the suspension is centrifuged at 100000 × *g* in a Beckman Airfuge. The amount of protein remaining in the supernatant is shown in percent of the protein content before centrifugation as a function of the total concentration of KCl (crosses), $CaCl_2$ (triangles), $MnCl_2$ (filled circles) or $MnCl_2$ + 150 mM KCl (open circles).

lustrated in Fig. 1. Firstly, it is observed that low concentrations (0–10 mM) of monovalent cation (here K^+) do not lead to precipitation (crosses, see Fig. 1). Secondly, Fig. 1 shows that addition of Ca^{2+} (filled triangles) or Mn^{2+} (filled circles) to supernatant enzyme causes a precipitation of 60–70% of the protein, with Mn^{2+} being more effective than Ca^{2+}.

The effect of divalent cations is diminished if 150 mM KCl is included, as shown with Mn^{2+} + K^+ in Fig. 1 (open circles). It is known that an increase in the ionic strength with monovalent cations leads to a precipitation of solubilized enzyme if the detergent/protein ratio is low (see Ref. 10), but at the present detergent/protein ratio (about 2) only 10–15% is precipitated by addition of 150 mM KCl (open circles in Fig. 1). Addition of 6 mM $MnCl_2$ leads only to a small increase in precipitation when 150 mM KCl is present, whereas 4 mM $MnCl_2$ precipitates about 60% of the supernatant enzyme in the absence of other cations (filled circles in Fig. 1). In the presence of 150 mM KCl there should thus only be a small effect of divalent cations on the aggregational state of the enzyme. In fact, no change in the aggregational state as determined by sedimentation equilibrium ultracentrifugation was observed when 1 mM $MgCl_2$ was added to gel filtered enzyme in the presence of 150 mM KCl (see Ref. 15).

Fig. 2 shows an experiment designed to see whether the protein concentration plays a role in the percentage of protein precipitated by Mn^{2+}. There is a fairly linear relationship between the amount of protein remaining in the supernatant as a function of the total amount of protein (circles in Fig. 2). Under these conditions (a detergent/protein ratio of 2, and 6 mM $MnCl_2$) about 50% of the protein is precipitated, independent of the protein concentration as Fig. 2 shows.

In order to see whether Mn^{2+} selectively precipitated active (or inactive) enzyme the activity of the enzyme remaining in the supernatant was measured. Fig. 2 (crosses) shows that the enzyme activity in the supernatant increases roughly in proportion to the amount of protein. The activity (in µmol/ml per h) is shown as the percentage of the activity at the highest protein concentration. The protein precipitated by Mn^{2+} (i.e., enzyme not appearing in the supernatant after centrifugation) can thus be expected to be active and representative of the solubilized enzyme. In agreement with this the specific activity of the pelleted enzyme was independent of the protein recovery (see

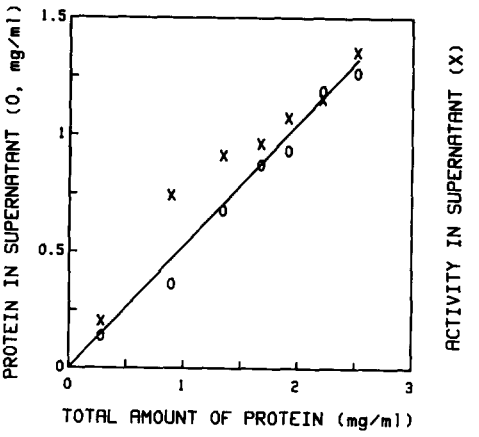

Fig. 2. Protein recovery as a function of protein concentration. This figure shows the amount of protein remaining in the supernatant (mg/ml) after precipitation with 6 mM $MnCl_2$ at protein concentrations from 0.3 to 2.5 mg per ml (open circles). The Na^+/K^+-ATPase activity remaining in the supernatant is also shown. The activity (in µmol/ml per h) is given in arbitrary units relative to the activity of the value for the highest protein concentration (crosses).

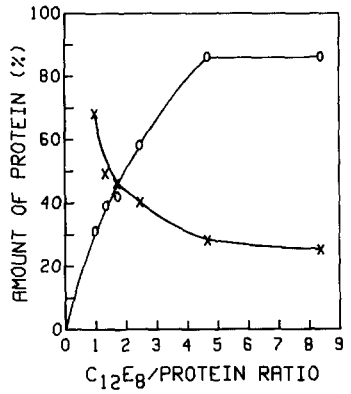

Fig. 3. Detergent protects against precipitation. This figure shows the amount of protein precipitated by 6 mM $MnCl_2$ at detergent protein ratios from 1 to about 9 (crosses). The amount of protein remaining in the supernatant is also shown, indicating a full recovery of protein (open circles). Other conditions as in Fig. 1.

below). There is thus no preferential precipitation of active or inactive enzyme.

In an attempt to obtain a larger fraction of precipitated membrane enzyme the detergent/protein ratio was varied in the supernatant enzyme and precipitation was induced at a given $MnCl_2$ concentration (6 mM). Fig. 3 (crosses) shows that the lower ratio, the higher the percentage of precipitated enzyme, i.e. a $MnCl_2$/detergent 'antagonism'. This is in line with the results obtained with monovalent cations (see Ref. 10), the difference being a much lower concentration of divalent cation required for precipitation.

It is clear from Fig. 3 that a low detergent/protein ratio will maximize the yield of reconstituted enzyme. It was observed, however, that the specific activity of the reconstituted enzyme (1600–1800 μmol/mg protein per h) was less than what could be expected from an enzyme which is 95–100% pure as judged from SDS gel scans. A pure enzyme should have an activity of about 2200–2400 μmol/mg protein per hour (if the Lowry procedure is used without corrections for the different staining efficiencies of albumin and the Na^+/K^+-ATPase proteins, see Refs. 16 and 17 for a discussion) whereas the reconstituted enzyme here has only 70–80% of this activity. Since the method for preparation of reconstituted enzyme is both rapid and inexpensive it seemed worthwhile to see if the specific activity could be increased to 100%. For this purpose three courses were followed.

First the residual amount of $C_{12}E_8$ in the membranes was determined and its effect on the activity of the membrane-bound enzyme was determined. Fig. 4 shows that the amount of $C_{12}E_8$ in the Mn^{2+}-precipitated enzyme is about 0.22 mg/mg protein. This can be reduced to less than 0.07 mg/mg protein by three centrifugations. These concentrations of $C_{12}E_8$ do not, however, influence the overall Na^+/K^+-ATPase activity, since addition of up to 0.25 mg $C_{12}E_8$ to 1 mg of a 'native' membrane enzyme does not inhibit Na^+/K^+-ATPase activity (not shown)). The lower specific Na^+/K^+-ATPase activity of the reconstituted enzyme can thus not be due to an inactivating effect of the residual detergent present. In agreement with this the specific activity of the reconstituted enzyme remained constant irrespective of the number of centrifugations used for removal of $C_{12}E_8$.

A second possibility is that closed vesicles are formed upon precipitation with $MnCl_2$, and the lower than optimal specific activity is due to the lack of access of substrates to both sides of the membrane. However, this seems not to be the case since addition of saponin, which is known to make the Na^+/K^+-ATPase-containing membranes permeable [8], did not increase the specific activity (data not shown).

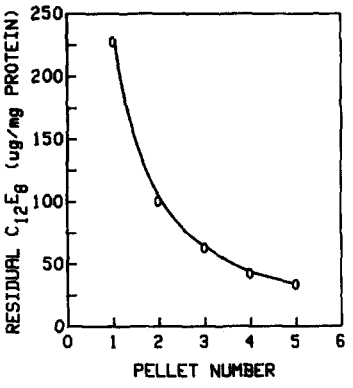

Fig. 4. Removal of $C_{12}E_8$ by centrifugation. This figure shows the amount of $C_{12}E_8$ remaining in the pellet (μg $C_{12}E_8$/mg protein) as a function of the number of centrifugation steps used. The first pellet is the one obtained directly from precipitation with $MnCl_2$. The following pellets are obtained after resuspension of the first pellet in 10 ml histidine (pH 7.0)/25% glycerol and centrifugation for 60 min at 200000 × g.

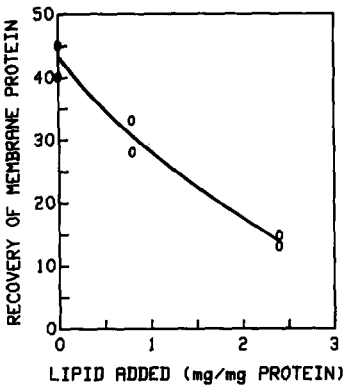

Fig. 5. Effect of added lipid on recovery (in %) of reconstituted enzyme. In these experiments extra lipid was added to the solubilized enzyme as given in Methods. One mg lipid is made up from 0.67 mg phosphatidylcholine and 0.33 mg cholesterol. Two separate experiments are shown for each lipid/protein ratio.

A third possibility is that the enzyme is precipitated with an unsatisfactory type or amount of lipid. Fig. 5 shows that an increase in the amount of lipid (phosphatidylcholine + cholesterol) in the supernatant (i.e. before addition of Mn^{2+}) decreases the amount of protein precipitated by Mn^{2+}. The specific activity was, however, about the same for all the types of reconstituted membranes, i.e. 1400–1800 μmol/mg protein per h. Addition of phosphatidyl-serine, -ethanolamine or -inositol (or combinations of these) did not increase the specific activity (not shown).

It is not clear why the higher lipid/protein ratio leads to a decrease in the yield of reconstituted enzyme, but the effect seems to parallel the lower yield of reconstituted enzyme when the detergent/protein ratio is increased. Sucrose density gradient centrifugation of reconstituted membranes formed at different lipid/protein ratios showed that the average density of the membrane decreased in parallel with an increase in the lipid/protein ratio in the supernatant, i.e. membranes with a higher lipid/protein ratio were formed. The lower yield could thus be due to a lower density of the membranes, i.e. the centrifugation steps after Mn^{2+} precipitation (200 000 $\times$ g for 60 min in 25% glycerol with a density of about 1.06 g/ml) do not recover all the formed membranes.

Discussion

The results presented here show that a pure membranous enzyme preparation can be easily obtained by solubilization of the Na^+/K^+-ATPase from shark rectal gland in $C_{12}E_8$ and precipitation of the solubilized enzyme by 4–6 mM $MnCl_2$. The method is rapid and inexpensive, i.e. requires only two centrifugations. The precipitation with Mn^{2+} and formation of reconstituted, active membranes is also seen with enzyme prepared from ox and rabbit kidney (data not shown). Mn^{2+} is preferred as divalent cation instead of Ca^{2+} since Ca^{2+} inhibits the enzyme. It should be noted that Mn^{2+} also has been used to induce 2-dimensional crystals of the Na^+/K^+-ATPase [18].

The major problem associated with this method is that full enzymatic activity is not retained, i.e. the reconstituted enzyme has only 70–80% of the specific activity to be expected from the protein purity, which is 95–100% (see Refs. 16 and 17). This lower specific activity of reconstituted enzyme is also observed with Na^+/K^+-ATPase solubilized with deoxycholate [19] or Lubrol [5], both precipitated by addition of either glycerol [19] or ammonium sulfate [5].

There are several possible reasons for the lower specific activity of the reconstituted enzymes. The most common is to assume that it is due to an irreversible denaturation by the detergent used, either through a change in protein structure or possibly as a result of peroxides in the detergent [20]. However, the enzyme can be obtained in a fully active and stable form in the solubilized state [12], so the denaturation is probably related to the reconstitution process, i.e. the re-formation of the membrane. A plausible explanation could then be that the protein–lipid interaction is disturbed, with a lower specific activity as a result.

It is, however, tempting to speculate whether there is another reason than denaturation for the 20–50% lower specific activity of reconstituted enzymes (Refs. 5, 8 and 19, and this paper). The major difference between enzymes purified by the solubilization/precipitation procedures and the selective extraction procedure is that the former method leads to a random insertion of protein molecules in the membrane. About half will be oriented inside out relative to the rest, i.e. half of

the molecules will have for example the ATP-site on one side of the membrane, the other half on the opposite side. If we assume that there is a protein–protein interaction both between aligned $\alpha\beta$-protomers and nonaligned protomers, and that Na^+/K^+-ATPase activity requires two identically oriented (aligned) protomers, then, as is observed, a lower than optimal specific activity should be obtained, since the solubilized $\alpha\beta$-protomers, but not $(\alpha\beta)_2$-diprotomers, could give rise to incorrectly aligned protomers. The decrease in specific activity should be proportional to the amount of $\alpha\beta$-protomer in the solubilized state.

Clearly this proposal needs further testing, especially since it is implicit that the functional unit in the membrane is an $(\alpha\beta)_2$-diprotomer or a higher oligomer of the $\alpha\beta$-protomer, whereas the $C_{12}E_8$-solubilized enzyme can function in the solubilized state as an $\alpha\beta$-protomer [9,11,21,22].

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