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ARE PLANT MICROSOMAL ATPases LIPID-DEPENDENT ENZYMES?*

ALAIN JOLLIOT, CHANTAL DEMANDRE and PAUL MAZLIAK
Laboratoire de Physiologie Cellulaire, ERA 323, Université Pierre et Marie Curie, Tour 53, 4 place Jussieu, 75230 Paris Cédex 05, France

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Key Word Index—*Solanum tuberosum*; Solanaceae; potato; lipid-dependence; ATPases; microsomes.

Abstract—Potato microsomes were delipidated by aqueous acetone solutions of increasing concentrations. Lipid extraction did not change the basal ATPase activity of these membranes (measured in the absence of added mineral ions), but affected the Mg^{2+}-dependent ATPase activity. Low acetone concentrations (5–15%) moderately stimulated the Mg^{2+}-ATPase; more concentrated solutions (20–50% acetone) dramatically decreased the activity of this enzyme, but 70 and 90% acetone solutions enhanced it again, as compared to the activity of the 50% acetone-treated fraction. This last stimulation could be explained by the selective extraction of an inhibitor of Mg^{2+}-ATPase by concentrated acetone solutions. After lipid extraction with 50–90% acetone solutions, the initial Mg^{2+}-dependent ATPase activity could not be restored by adding lipids to delipidated microsomes. These results strongly suggest that, in potato microsomes, the Mg^{2+}-dependent ATPase was a lipid-dependent enzyme, but suitable relipidation conditions remain to be found to definitely prove this lipid dependence.

INTRODUCTION

Ca^{2+}- and $(Na^+ + K^+)$-ATPases from animal tissues are intrinsic membrane proteins known to be phospholipid-dependent for their enzymatic function (for reviews, see [1–5]). During the last decade, numerous reports have shown that most animal ATPase activities are decreased after delipidation of their supporting membranes by organic solvents, phospholipases or detergents. Adding phospholipids to delipidated membranes can restore the lowered enzymatic activities. $(Na^+ + K^+)$-ATPase, for instance, appears to require phosphatidylserine or other negatively charged phospholipids to function, but this requirement is not absolute and enzymatic restorations have been observed with phosphatidylcholine. Some discrepancies on the nature of the phospholipid dependence could be explained by (a) the different sources of membranes, (b) the various methods of delipidation employed and (c) the impurities present in the added lipids. In a recent paper, Dean and Tanford [6] found that a microsomal Ca^{2+}-activated ATPase could be obtained in a stable, inactive and soluble form, virtually free of phospholipid, and that the activity of this enzyme could be fully restored by adding detergents in place of phospholipids.

In a plant membrane fraction (2500 or 4000 *g*–20000 *g*) isolated from sugar beet roots, the $(Na^+ + K^+)$-ATPase activity could be enhanced by treatments with deoxycholate, dithiothreitol or cystein [7, 8]; this stimulation could be correlated with the balance between the long-chain (*ca* C_{20}) fatty acid conjugates of zwitterionic phosphatidylcholine and anionic sulfolipids. Only one report [9] on a plant material indicated that the activity of a *soluble* and unspecific ATPase from bean roots, lowered after a 90% acetone treatment, could be partially restored by adding either phosphatidylcholine or sulfolipid. To the best of our knowledge, no similar experiments have ever been performed with ATPase of plant *membranes*; it was therefore interesting to extend the preceding results to ATPase from plant microsomes.

* Part 1 in the series "Regulation by lipids of plant microsomal enzymes".

RESULTS

Potato tuber microsomes have high ATPase activities. The basal ATPase activity measured with these membranes was *ca* 1.5–2.4 μmol of Pi liberated per hr per mg protein and the Mg^{2+}-dependent ATPase activity was 3.4–5.5 μmol of Pi liberated per hr per mg protein. The microsomal fraction had no succinate–cytochrome *c* reductase activity and NADH–cytochrome *c* reductase was not inhibited by antimycin A or cyanide, thus indicating that the microsomes were not contaminated by mitochondria.

Delipidation by acetone solutions

To extract increasing quantities of membrane lipids, aliquots of a microsomal suspension were treated with a range of different aqueous acetone solutions (5, 10, 15, 20, 30, 50, 70 and 90% of acetone). A standard microsomal aliquot was treated under the same conditions by Tris–HCl buffer (0% acetone). Fig. 1 shows the effects of acetone treatments upon the phospholipid content and the ATPase activities of potato microsomes. No significant loss of membrane phospholipids occurred for low acetone solutions treatments (5 and 10%), but for higher concentrations phospholipids were progressively extracted.

The basal ATPase activity was not markedly decreased by all the acetone treatments. A decrease which paralleled the slight decrease in protein content provoked by the delipidation treatment was noted. In contrast, the delipidation of microsomal membranes had 3 different effects on Mg^{2+}-dependent ATPase activity. First, treatments by acetone solutions of low concentrations (5, 10 and

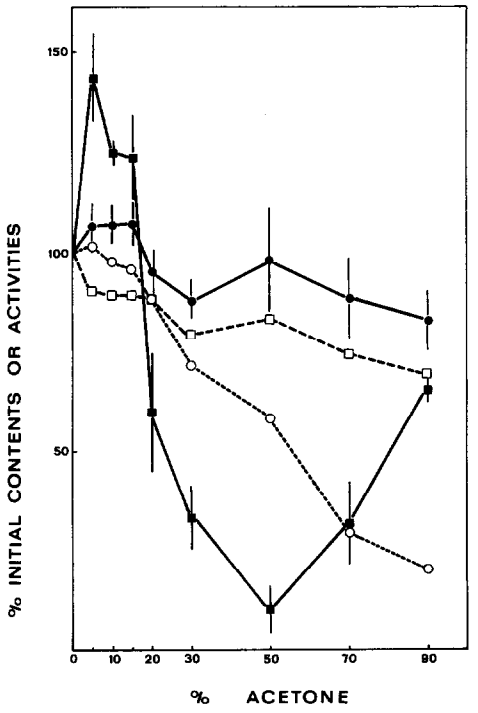

Fig. 1. Effects of acetone treatments on the protein and phospholipid contents and the ATPase activities of potato microsomes. All measured values were adjusted to 100 for 0% acetone (Tris–HCl buffer) treatment. Means and standard deviations are calculated from two or three different experiments. □---□, Protein content; ○---○, phospholipid content (per mg of protein); ●——●, basal ATPase activity (per mg of protein); ■——■, Mg^{2+}-dependent ATPase activity (per mg of protein).

15%) caused a marked stimulation (20–45%) of the Mg^{2+}-dependent ATPase activity. Secondly, when membrane phospholipids began to be extracted, the activity decreased to 10% of the reference sample activity. Finally, when 70 and 90% acetone solutions were used, a 50% enhancement of the Mg^{2+}-ATPase activity could be observed (as compared to the activity of the 50% acetone-treated fraction).

Relipidation treatments

All attempts to restore the lowered Mg^{2+}-dependent ATPase activity of 50 or 90% acetone-treated microsomal membranes by adding lipids to delipidated membranes failed. Varying the quantities of added lipids and/or the times of relipidation (5–60 min) gave no response in any of our assays.

DISCUSSION

To check the lipid dependence of plant microsomal ATPases, rather than extracting abruptly the bulk of membrane lipids, we decided to extract these substances progressively, in order to follow the behaviour of the enzymes in various lipid environments. The basal ATPase activity of potato microsomes was not significantly affected by all delipidation treatments. This fact does not allow us to conclude that this enzyme is not regulated by lipids because a 90% acetone solution did not extract all membrane lipids. About 20% of total phospholipids cannot be extracted by this method and they could perhaps be sufficient to preserve the basal ATPase activity. Hesketh *et al.* [10] have shown, for instance, that the activity of a Ca^{2+}-dependent ATPase from rat microsomes depended to a great extent on an annulus of linked lipids representing a small percentage of the bulk of membrane lipids (30 lipid molecules per ATPase).

The changes observed in Mg^{2+}-dependent ATPase activity with delipidation treatments are more difficult to explain. The stimulation caused by low acetone concentrations could be due to (a) changes in the permeability barriers of membranes and/or (b) release of peripheral proteins that would cause a better accessibility of ATP molecules to the enzyme protein. Similar stimulations have been already observed under the same conditions with microsomal NADH–cytochrome *c* reductase [11]. Treatment of potato microsomes with 15–50% acetone solutions results in a marked decrease of the Mg^{2+}-dependent ATPase activity that can be correlated with the lipid depletion. The most surprising result of this work is the increase of the ATPase activity after 70 and 90% acetone solution treatments. Three explanations can be offered to account for this enhancement (a) new enzymatic active sites could be uncovered by the lipid depletion, (b) loss of other proteins (*ca* 25–30% for these treatments) and (c) an ATPase-inhibitor could be selectively extracted by concentrated acetone solutions. In potato mitochondria, Jung and Laties [12] have demonstrated the tight association of a mitochondrial Mg^{2+}-dependent ATPase with an inhibitor protein (that could be destroyed by trypsin, incubation at alkaline pH or heating at 60° for 2 min). We postulate the existence of a similar inhibitor in potato microsomes. In a preceding paper [11], we have shown that treatment of the microsomal fraction by low acetone solutions (5–20%) did not change the proportions of the predominant phospholipids (phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol). Then phosphatidylethanolamine is extracted in preference to phosphatidylinositol by concentrated acetone solutions (30–90%). Since it has been previously observed that the activation of ATPase was dependent on the nature of the added lipids [7, 8], then perhaps the loss and the subsequent increase in activity could be due to the nature of the lipids remaining in the membranes. This would provide a fourth explanation.

The decrease of the Mg^{2+}-dependent ATPase activity after 15–50% acetone treatments can be correlated with the membrane lipid depletion. But to definitely prove the lipid dependence of this enzyme, a restoration of the ATPase activity must occur when lipids are added to delipidated microsomes. We failed to restore the Mg^{2+}-dependent ATPase activity. Two reasons can be advanced to explain this failure (a) concentrated acetone solutions could cause some denaturation of the Mg^{2+}-ATPase, (b) plant microsomes would be more sensitive than animal membranes to delipidation treatments (possibly because they are richer in polyunsaturated components or in pigments). Such important alterations following the treatments would prevent any restoration of activity.

Finally this work strongly suggests, but does not definitely prove that, as in animal membranes, the Mg^{2+}-dependent ATPase of plant microsomes is a lipid-dependent enzyme.

EXPERIMENTAL

Potato tubers (*Solanum tuberosum* L. cv Bintje) were furnished by C.E.R.D.I.A. (Massy, France). Microsomes were isolated as previously described [13].

Delipidation treatments. To 1 ml of a microsomal suspension (containing 20–30 mg protein) were added 31 ml Me_2CO solns in 20 mM Tris–HCl buffer (pH 7) such that final concns were equivalent to 5, 10, 15, 20, 30, 50, 70 and 90% or 31 ml of Tris–HCl buffer (non-delipidated microsomes). The mixtures were gently stirred during 10 min, then centrifuged at 100000 *g* for 50 min, which allowed us to obtain 5, 10, 15, 20, 30, 50, 70 and 90% Me_2CO-treated and Tris–HCl-treated (0% Me_2CO) microsomal fractions.

Relipidation treatments. Lipids used for relipidation experiments were extracted from potato tubers microsomes and solubilized in an aq. medium by sonication following the methods previously described [11]. 50 or 90% Me_2CO-treated microsomes were mixed with various quantities of microsomal lipid micelles, then incubated for 5 min at 30° and the ATPases activities measured directly on the mixture.

ATPase activities were measured at 30° by determination of Pi liberated from ATP. The assay medium (final vol. 0.5 ml) contained 4 mM ATP, 4 mM $MgCl_2$ (for Mg^{2+}-ATPase) in 10 mM Tris–HCl buffer (pH 7); these values gave maximal activity as described previously [13]. Reaction was initiated by addition of 20–40 μg of microsomal proteins and after 10 min of incubation at 30°, stopped by 0.2 ml 10% TCA. Pi was measured by the procedure of ref. [14]. Two blanks (substrate and membranes) were subtracted from observed values to calculate enzyme activities. When ATPase activities were measured on a mixture of delipidated microsomes + lipid micelles, 0.3 ml pentane must be added before phosphate determination to dissolve the lipids (which otherwise would prevent the *A* measurement). We have previously checked that addition of 0.3 ml pentane did not change the phosphate measurement. In this paper, we have called *basal ATPase activity* the activity measured in the absence of added ions, and Mg^{2+}*-dependent ATPase activity* the activity measured in presence of Mg^{2+} ions minus the basal activity. Succinate–cytochrome *c* and NADH–cytochrome *c* reductases were measured as previously described [15]. Proteins were estimated by the procedure of ref. [16] and phospholipidic phosphorus by the method of ref. [17].

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