Regulation by Lipids of Plant Microsomal Enzymes

II. LIPID DEPENDENCE OF THE NADH-CYTOCHROME c REDUCTASE OF POTATO TUBERS

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ABSTRACT
Microsomal membranes from potato tubers were treated with a phospholipase C extracted from Bacillus cereus. A positive correlation could be observed between the hydrolysis of membranous phospholipids and the decrease of the NADH-cytochrome c reductase activity. Addition of total lipid or phospholipid micelles to phospholipase C-treated microsomes partially restored the NADH-cytochrome c reductase activity, thus proving the lipid-dependence of this enzyme.

Since 1963, it has been shown by numerous investigators that lipids, and more especially PLPs, may regulate various enzymic activities linked to microsomal membranes prepared from animal tissues (for a review, see ref. 7). Fleischer et al. (6) defined two criteria to prove the lipid-dependence of a membrane-bound enzyme activity: (a) the removal or the alteration of membrane lipids must result in a decrease of enzyme activity; and (b) the addition of extracted lipids (or some definite lipid molecular species) to lipid-depleted membranes must restore the initial enzyme activity. Thus, the lipid-dependence of the microsomal NADH-Cyt c reductase (EC 1.6.2.2) from animal livers had been demonstrated by Dallner et al. (4, 5) and Jones and colleagues (13-15, 20).

To our knowledge, similar experiments have never been performed with plant membranes and it was, therefore, interesting to try to extend the preceding results to plant microsomal enzymes. Following our previous assays on microsomal ATPases (12), the study reported here shows that the microsomal NADH-Cyt c reductase of potato tubers requires PLPs for optimal activity.

MATERIALS AND METHODS
Potato tubers (Solanum tuberosum L., var Bintje) were furnished by the Institut Technique de la Pomme de terre (Saint Rémy l'Honoré, France).

Microsomes were isolated as previously described (10). Two tenths ml of microsomal suspension (representing 1 to 1.5 mg protein) was delipidated at 25 C in 1.8 ml of a medium containing 1 ng phospholipase C extracted from Bacillus cereus (Boehringer, Mannheim), 5 mM CaCl2, 0.2% BSA, and 0.1 M Tris-HCl buffer (pH 7.4). Ten µl of a butylated hydroxytoluene solution (30 mg/ml methanol) must be added to the incubation medium to prevent the decoloration of the microsomal fraction (probably by carotene oxidation). The reaction was initiated by addition of microsomes. The zero time sample was removed immediately after mixing. At fixed time intervals, aliquots were withdrawn; the reaction was stopped by addition of 0.1 ml 25 mM EDTA, 5 mM o-phenanthroline, 0.2% BSA, and 20 mM Tris-HCl (pH 8.0). Enzymic activities were measured on the treated membranes after (or before, when indicated) two centrifugations at 100,000g for 55 min, separated by a resuspension of the pellet in 20 ml 20 mM Tris-HCl (pH 8.0) for washing.

Microsomal lipids were extracted according to the Bligh and Dyer method (3). PLPs, galactolipids, and neutral lipids were separated by TLC using the system of Trémolières and Lepage (22). Fatty acid methyl esters were analyzed by GLC as previously described (2).

Lipids used for reprepidation experiments were extracted from potato tuber parenchyma. PLPs were separated from other molecular species by chromatography on a silicic acid column (23) and were dispersed as liposomes in an aqueous medium by sonication under N2 as previously described (10).

NADH-Cyt c reductase was measured at 25 C by the method of Hackett et al. (9), except that the pH was fixed at 8.0, as indicated by Moreau (16) and Philipp et al. (19). For reprepidation experiments, phospholipase C-treated microsomes and lipid or PLP liposomes were mixed and then incubated at 25 C for 5 min, and the enzymic reaction was measured directly on the mixture.

RESULTS AND DISCUSSION
Potato tuber microsomes presented high NADH-Cyt c reductase activities, ranging from 150 to 280 nmol Cyt c reduced/min/mg protein. These values are in agreement with those found by other authors on plant microsomes (1, 17, 21). The microsomal fraction had no succinate-Cyt c reductase activity nor was the NADH-Cyt c reductase activity inhibited by antimycin A or cyanide; these facts indicated that the microsomal fraction contained no mitocondria.

Phospholipase C Treatments. To study the PLP dependence of NADH-Cyt c reductase, microsomal fractions were incubated with phospholipase C from B. cereus for various times. Figure 1 shows the loss of the reductase activity with the time of incubation. Using 1 ng phospholipase C/mg microsomal proteins, we observed a decrease in activity of about 60% after 10 min and of 75% after 45 min. Twenty ng phospholipase C/mg microsomal proteins gave the same maximal inhibition, but the latter was reached after only 15 min incubation. Standard assays have been carried on with the complete medium except 5 mM CaCl2; in that case, the enzymic activities remained equal to that of untreated microsomes for at least 50 min, proving that neither 5 mM Ca2+ nor concentrated Tris buffer were inhibitory to phospholipase C, as suggested by Ottolenghi (18). When 1 ng phospholipase C/mg protein was used, neutral lipids (probably diacylglycerols) increased 86% in 10 min.

1) This is Paper No. 2 in the series "Regulation by lipids of plant microsomal enzymes." The preceding paper in the series is reference 12.

2) Abbreviation: PLP, phospholipid.

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We have not found in the literature similar experiments with NADH-Cyt c reductase from animal microsomes but, for the UDP-glucuronyltransferase (8) or the glucose-6-phosphatase (24), animal physiologists generally used 1 to 1.5 \times 10^9 ng phospholipase C (from Clostridium welchii)/mg microsomal protein, i.e. 5,000 to 150,000 times more enzyme to obtain similar inhibitions. We conclude that plant microsomes are very sensitive to the action of exogenous phospholipase C. In agreement with this fact, we can also note that butylated hydroxytoluene must be added during the delipidation treatment to prevent a decoloration of the microsomal fraction.

After a 10 min delipidation of the microsomes, which results in a decrease of NADH-Cyt c reductase activity of about 55 to 60% (Table I), the membranes are centrifuged as indicated under "Materials and Methods" to eliminate the hydrolysis products; the remnant reductase activity was about 10 to 30% of the initial activity of the nondelipidated fraction. The new loss of activity caused by the two last centrifugations and the washing was another proof of the great fragility of plant microsomal membranes.

Table I shows phosphatidyleholine and phosphatidylinositol are the most and the least hydrolyzed species, respectively, as compared to the mean of all PLPs. Figure 1 and Table I show a good correlation between the decreases in both reductase activity and phosphatidyleholine content, the major PLP of the microsomes.

To demonstrate fully the PLP dependence of the reductase, it was necessary to restore enzyme activity after addition of exogenous PLPs to lipid-depleted membranes.

Relipidation Treatments. Numerous assays have been attempted to restore the NADH-Cyt c reductase activity of microsomes treated for 10 min by phospholipase C. Figure 2 shows the results of a sample experiment. Ten to 20 \( \mu \)L PLPs extracted from potato tuber parenchyma provoked a 2-fold stimulation of the NADH-Cyt c reductase activity of lipid-depleted microsomes. However, our restoration assays with total lipids or potato PLPs did not allow us to reach more than 22% of the initial activity of standard untreated microsomes. At low lipid concentrations, some correlation could be observed between the quantity of added lipids and the percentages of restoration. Above a given lipid concentration (20 \( \mu \)L PLPs/mg proteins), an excess of PLPs produced smaller reactivations. PLPs were slightly more effective than total lipids. These percentages of restoration could not be enhanced by increasing the incubation time (from 5 to 60 min), thus indicating that the relipidation phenomenon was rather rapid. Addition of 0.020% sodium deoxycholate (a concentration that did not modify the reductase activity [11]) could not produce better restoration. Sonication of delipidated microsomes with exogenous lipids, a procedure that was found necessary to obtain the reactivation of some plant mitochondrial enzymes [11], could not be used for microsomal NADH-Cyt c reductase because this activity was strongly lowered by sonication.

In contrast to animal membranes, plant microsomes apparently are perturbed by delipidation treatments since it appears impossible, under our experimental conditions, to restore completely the lowered NADH-Cyt c reductase activity of lipid-depleted membranes. In a preceding paper (10), we showed that no restoration of the enzyme activity could be obtained after delipidation of the microsomes by a 90% acetone solution. Some denaturation could explain this result. In the study here, phospholipase C treatment could also cause a partial inactivation of the reductase activity. Suitable relipidation conditions remain to be found for total restoration, but these preliminary results prove, for the first time, the PLP dependence of potato microsomal NADH-Cyt c reductase.

![Graph showing the effect of phospholipase C treatments on the NADH-Cyt c reductase activity and PLP content of potato microsomes.](image-url)

**Table I. Effects of Phospholipase C Treatment on NADH-Cyt c Reductase Activity and PLP Content of Potato Microsomes**

<table>
<thead>
<tr>
<th>Hydrolysis Times (min)</th>
<th>NADH-Cyt c reductase</th>
<th>Lipids*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without washing &amp; centrifugations</td>
<td>After washing + centrifugations</td>
</tr>
<tr>
<td></td>
<td>nmol Cyt c reduced/min-mg protein</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>259 (100)*</td>
<td>225 (100)</td>
</tr>
<tr>
<td>5</td>
<td>148 (57)</td>
<td>131 (58)</td>
</tr>
<tr>
<td>10</td>
<td>119 (46)</td>
<td>65 (29)</td>
</tr>
<tr>
<td>30</td>
<td>86 (33)</td>
<td>32 (14)</td>
</tr>
</tbody>
</table>

* Abbreviations: PC, phosphatidyleholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol.

* Values in parentheses were adjusted to 100 for the 0-min time sample.
FIG. 2. Reactivation by total lipids (-----) or PLPs (●○●) of the NADH-Cyt c reductase of potato microsomes treated for 10 min by phospholipase C (phospholipase C-treated fraction activity: 36 nanomol Cyt c reduced/min-mg protein). Delipidated microsomes (32 μg protein) were incubated for 5 min at 25°C with increasing quantities of lipids extracted from potato parenchyma before the measure of the enzymic activity.

LITERATURE CITED


