Regulation by Lipids of Plant Microsomal Enzymes

III. PHOSPHOLIPID DEPENDENCE OF THE CYTIDINE-DIPHOSPHO-CHOLINE PHOSPHOTRANSFERASE OF POTATO MICROSOMES

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ABSTRACT

Cytidine-diphospho-choline diacylglycerol phosphorylcholine phosphotransferase activity was demonstrated in potato (Solanum tuberosum L.) microsomes and the incorporation of cytidine-diphospho[14C]choline into phosphatidylcholine was characterized by the time course of 14C incorporation and the effect of microsomal protein concentration on choline incorporation.

Potato microsomes were progressively depleted by treatments (2 min at 0°C) with increasing amounts of phospholipase C from Bacillus cereus. A decrease in choline phosphotransferase activity was observed in parallel with the progressive hydrolysis of membrane phospholipids. A 70% (or more) phospholipid hydrolysis provoked the total inactivation of the enzyme.

Adding back exogenous phospholipids (in the form of liposomes) to phospholipase C-treated membranes restored the enzymatic activity. Restoration could be obtained with egg yolk phospholipids as well as with potato phospholipids. Restoration was time dependent and completed after 10 minutes; restoration was also dependent on the quantity of liposomes added to lipid-depleted membranes: the best restorations were obtained with 1 to 2.5 milligrams of phospholipid per mg of microsomal protein; higher phospholipid to protein ratios were less efficient or inhibitory.

These results clearly demonstrate the phospholipid dependence of the cytidine-diphospho-choline phosphotransferase from potato microsomes.

CDP1-choline diacylglycerol phosphorylcholine phosphotransferase (EC 2.7.8.2) is the final enzyme in the biosynthetic pathway of phosphatidylcholine (14), one of the major phospholipids of cell membranes (16). The enzyme is bound to ER membranes and catalyzes the following reaction:

CDP-choline + diacylglycerol → phosphatidylcholine + CMP

In plant tissues, this enzyme has been demonstrated and characterized for the first time in spinach leaf microsomes (4). The spinach enzyme was similar to that of animal microsomes with regard to pH optimum, metal requirements (Mg or Mn), sensitivity to SH inhibitors and K_m for CDP-choline (10 μM). The enzyme did not show any diacylglycerol specificity when exogenous diacylglycerols were added to the incubation medium.

CDP-choline phosphotransferase was also found by Donaire and Oursel (5) in the microsomal membranes from Vicia faba roots; high concentrations of calcium (greater than 0.5 mM) in the incubation medium strongly inhibited the phosphotransferase reaction. CDP-choline phosphotransferase was clearly localized in ER (18) and also on both the inner and outer mitochondrial membranes of castor bean endosperm (21). However, the mitochondrial CDP-choline transferase represented about 1% to 2% of the total activity measured in the tissue. Further, Montague and Ray (17) have demonstrated the presence of the enzyme in microsomal and Golgi apparatus membranes from pea stem.

As many membrane-bound enzymes, CDP-choline transferase can be suspected to be regulated in some manner by the lipids surrounding the active protein within microsomal membranes. However, very few authors (9–13) have been successful, up to now, in fully demonstrating the lipid dependence of a plant membrane-bound enzyme. One noticeable exception is a recent work by Coccucci and Ballarin-Denti (3) demonstrating the lipid dependence of an ATPase from plasma membranes of germinating radish seeds: cholate treatment of the membranes removed almost all phospholipids and ATPase activity was barely detectable; addition of polar lipids to lipid-depleted membranes restored the enzymic activity. It is the purpose of this paper to demonstrate the phospholipid dependence of the CDP-choline transferase of potato tuber microsomes.

MATERIALS AND METHODS

Potato tubers (Solanum tuberosum L.) were furnished by 'l'Institut Français de la Pomme de Terre' (Paris) and stored at 4°C. Cytidine 5'-diphospho-[methyl-14C]choline (50 mCi/mmol) was purchased from Amersham (Versailles, France).

Microsome Preparation. One hundred g of freshly peeled potato parenchyma were ground in a Moulinex apparatus with 100 ml of the following solution: 0.4 m sucrose, BSA (0.75 mg ml−1), 10 mM KCl, 1 mM MgCl2, 4 mM cysteine, and 0.1 M Tris-HCl buffer (pH 8). The homogenate was filtered through one layer of cheese cloth ('Miracloth'; Touzart et Matignon, Paris, France) to eliminate cell debris and centrifuged at 15,000g for 20 min. The crude mitochondrial pellet was discarded and the postmitochondrial supernatant was centrifuged at 100,000g for 60 min to get the microsomal pellet. The microsomes from 100 g of parenchyma (containing about 30 mg of protein) were suspended in 3 ml of 0.05 M Tris-HCl buffer (pH 7.2 or 8).

Measurement of CDP-Choline Transferase Activity. The incubation medium contained, in a final volume of 1.6 ml of 0.05 M Tris-HCl (pH 8): MgCl2, 5 μM; DTT, 20 μM; CDP-[14C]choline, 2 μmol (200,000 dpm); and 0.1 ml of the microsomal suspension (about 1 mg of protein). Unless otherwise indicated, all incubations were run at 30°C in a Warburg apparatus, for 10 min. The reaction was stopped by adding 3.5 ml of boiling methanol, and
the lipids were extracted with chloroform-methanol (see below). Total lipids were analyzed by TLC, and the ^{14}C-radioactivity recovered in the phosphatidylcholine spot was determined in a Kontron-Intertechnique Scintillator SL 3000. Autoradiography of the thin-layer showed that more than 95% of the radioactivity of the lipids was recovered in phosphatidylcholine. Routinely, the total lipid extract in chloroform was counted directly for determining the radioactivity of the reaction product.

**Lipid Extraction and Analysis.** Microsomal lipids were extracted following the Bligh and Dyer method (2). PLP was separated and analyzed by TLC, using the system of Lepage (15). Fatty acid methyl esters were analyzed by GLC, as previously described (1).

**Incubations of Membranes with Phospholipase C.** Phospholipase C (extracted from Bacillus cereus) was obtained from Boehringer (Mannheim, Germany). The activity of the commercial preparation was about 900 enzyme units/mg of protein. Incubations were made by adding 1.5 ml of the microsome suspension (approx. 15 mg of protein) to 4 ml of 0.05 M Tris-HCl buffer (pH 7.2) containing from 7.5 to 12.6 ^{14}C to the phospholipase C solution (2 mg/ml) and 4.5 ml of a 8% solution of CaCl$_2$. Membranes were allowed to stand in this medium for 2 min, at 0°C, and the action of phospholipase C was stopped by adding 5 ml of a solution of 0.15 M EDTA (as tetrasodium salt) in 0.05 M Tris-HCl buffer, containing 10 mg of BSA. The mixture was centrifuged at 100,000g for 35 min. The supernatant was discarded, and the pellet was suspended in 10 ml of 0.05 M Tris-HCl buffer (pH 8) containing 20 mg of BSA to wash the membranes. The washed delipidated membranes were finally obtained by centrifuging the washing medium at 100,000g for 30 min. The final pellet was suspended in 4.5 ml of 0.05 M Tris-HCl buffer (pH 8).

**Liposomes.** Liposomes made from egg yolk phospholipids (20) or from potato tuber phospholipids, prepared by chromatography on a silicic acid column (24), were obtained in the following manner: 20 mg of phospholipids were deposited under 10 ml of 0.05 M Tris-HCl buffer (pH 8) and sonicated for 20 min, under N$_2$, in a M.S.E. apparatus, at the highest speed and at 20°C. The liposome suspension was immediately utilized for relipidation assays.

**Restoration of CDP-Choline Phosphotransferase Activity.** Various quantities of liposomes were added to aliquots of the delipidated microsome suspension (as described in "Results") and the mixture was routinely allowed to stand at 30°C for 10 min. The measurement of CDP-choline transferase activity was made, as described above, in a final volume of 1.6 ml, in the presence of liposomes.

Protein contents were determined by the method of Gornall et al. (8).

**RESULTS**

Characterization of the CDP-Choline Phosphotransferase of Potato Microsomes. As potato microsomes represented a new source of enzyme, preliminary experiments were done to characterize the choline phosphotransferase activity of these microsomes. Autoradiograms of the lipids, extracted from various incubation media containing CDP-[^{14}C]choline and separated by TLC, clearly showed one single major radioactive compound corresponding to [^{14}C]PC. Labeled PC contained alone more than 95% of the radioactivity incorporated into the lipids. Traces of lyso-[^{14}C]PC were apparent on the autoradiograms under the major radioactive spot. PC synthesis did not proceed linearly with time; after 15 min of incubation, a decrease in the rate of synthesis was apparent and a plateau was reached at 30 min. A 10-min incubation time was chosen for routine experiments. Incorporation of [^{14}C]choline into PC was related to protein concentration in the range 0 to 1 mg per assay; a plateau was reached at 1.8 mg of protein per ml. For subsequent assays, 0.8 to 0.9 mg of protein were used for one incubation (final volume, 1.6 ml).

**Effects of Phospholipase C on Microsomal Membranes.** Phospholipase C was chosen as a specific agent of delipidation of microsomal membranes because the products of the phospholipid hydrolysates achieved by this particular enzyme are diacylglycerols and phosphorylalcohols. Those products are not harmful for microsomal enzymes: indeed, endogenous DG are substrates for the CDP-choline transferase.

Potato microsome phospholipids were degraded by phospholipase C; among the different phospholipids, PC and PE were the most severely hydrolyzed (Fig. 1), while PI appeared slightly less susceptible to degradation. In the same time, DG accumulated within the treated membranes. However, the susceptibility to degradation of microsomal phospholipids varied from experiment to experiment; also, the length of the cold storage of potatoes influenced greatly this susceptibility of membranes to phospholipase C. Membranes from freshly stored potatoes (in October) were far less susceptible to degradation than membranes from 8 month-stored potatoes (in June). For example, 250 ng of phospholipase per mg of protein were sufficient in June to degrade half the content of PC and PE of microsomal membranes; to obtain the same extent of degradation, 5,000 ng of phospholipase were necessary in October.

A positive correlation can be observed between CDP-choline phosphotransferase activity and the phospholipid content of microsomal membranes (Fig. 2). Mild treatment with phospholipase C does not stimulate the enzymic reaction, although DG are generated as a consequence of phospholipid hydrolysis (Fig. 1). This result confirms that endogenous DG, present in untreated microsomal membrane, is not rate-limiting for CDP-choline transferase.

**CDP-Choline Transferase Activity Restoration.** In order to fully demonstrate the phospholipid dependence of the transferase, it remained to obtain the restoration of the enzyme activity after addition of exogenous phospholipids to lipid-depleted membranes. Delipidated membranes were incubated in the presence of liposomes (as described in "Materials and Methods"). Egg yolk phospholipids or potato tuber phospholipids were used to prepare the liposomes.

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**Fig. 1.** Effects of increasing quantities of phospholipase C on lipid content of microsomes from potato tubers stored for 8 months.
Membrane Reconstitution with Egg Yolk Phospholipids. Figure 3 shows that the enzymic activity of delipidated membranes (treated by phospholipase C and washed) could be fully restored by incubating those membranes with egg yolk phospholipids dispersed in the form of liposomes. Full restoration was completed after 10 min of incubation when a plateau was reached.

Figure 4 shows the effects of increasing quantities of phospholipids on the enzymic activity of delipidated membranes. Restoration was directly dependent on the quantity of added phospholipids in the range 0 to 6 mg of phospholipid per mg of microsomal protein. Higher ratios of lipid to protein provoked a noticeable inhibition of the CDP-choline transferase activity of the standard untreated membranes (up to 30% for 37.5 mg of phospholipids per mg of microsomal proteins). Consequently, the efficiency of higher ratios of phospholipids to protein for enzyme restoration was also diminished.

Membrane Reconstitution with Potato Phospholipids. Similar results can be obtained with potato phospholipids, but very noticeable is the marked inhibition exerted by potato phospholipids upon the enzymic activity of standard microsomes: the addition of 5 mg of potato phospholipid per mg of microsomal protein provoked a 40% inhibition on standard untreated membranes. Nevertheless, the enzymic activity of delipidated membranes could be partially restored (from 30–50% of the standard) by low and increasing quantities of potato phospholipids (from 0.25–2 mg of phospholipid per mg of microsomal protein). Higher quantities of potato phospholipids appeared less efficient or without any efficiency for enzyme activity restoration; this could be easily explained by the marked inhibitory effect of added exogenous potato phospholipids.

DISCUSSION

One can find, in the literature, few papers concerning the regulation of CDP-choline transferase, and studies upon the regulation of this enzyme have been realized only on animal tissues. Particularly, the regulation of the microsomal enzyme by its lipid environment has been suggested twice. Freysz et al. (7) have studied the effects of deoxycholate and phospholipase A₂ on the CDP-choline transferase of chicken brain microsomes; however, their demonstration was not total since restoration experiments were unsuccessful. Morimoto and Kanoh (19) treated rat liver microsomes with phospholipase A₂. The activity of CDP-choline transferase was rapidly lost following the accumulation of lysophospholipids and free fatty acids. Microsomal total phospholipids partially reactivated the treated enzyme and among the lipids, PC alone was found to be effective. Thus, some phospholipid dependence was proven for the enzyme of rat liver microsomes; as pointed out by the authors, the stimulatory effect of phosphatidylcholine on choline phosphotransferase is difficult to explain, in view of the reversibility of the action of the transferase. Two recent papers are also related to the question of the lipid dependence of the phospholipid synthesizing enzymes. Tanaka et al. (23) have studied the biosynthesis of PC by the pathway of the successive methylations of PE in mouse liver microsomes. These authors have shown that methyl incorporation into phospholipids was activated by PE and its methylated intermediates. Finally, Stuhne-Sekalec and Stanacev (22) have degraded guinea pig liver microsomes with phospholipase C and D and observed that the activity of PI synthetase was almost completely lost after the treatment. A nearly complete restoration of the original activity was achieved after endogenous biosynthesis of PC in degraded microsomes, the substrate CDP-choline being furnished to the treated membranes.

As far as we know, the problem of the lipid-dependence of the CDP-choline transferase had never been studied previously on an enzyme from plant microsomes. In the present paper, we demonstrate that the CDP-choline transferase of potato microsomes is indeed a phospholipid-dependent enzyme. After partial hydrolysis of microsomal phospholipids by phospholipase C, the enzymic activity of delipidated membranes can be fully restored by reconstituting the membranes with exogenously added phospholipids.

As egg yolk phospholipids were far less inhibitory on the enzymic activity of standard untreated membranes than potato phospholipids, the efficiency of restoration was always greater with the former than with the latter. The reason for the great inhibitory action of potato phospholipids on CDP-choline trans-
membranous enzymes interesting, since a of independence addition of ase, (6) substrate experiments. exerted by that of ferase activity are stored 2. BLIGH BEN ABDELKADER 1.

Table 1. Molecular Compositions of Egg Yolk and Potato Phospholipids Used for Membrane Reconstitutions

<table>
<thead>
<tr>
<th>Total Phospholipids</th>
<th>Egg yolk phospholipids</th>
<th>Potato phospholipids</th>
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<tr>
<td>%</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>PC</td>
<td>82.8</td>
<td>50</td>
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<tr>
<td>PE</td>
<td>14.2</td>
<td>35</td>
</tr>
<tr>
<td>PI</td>
<td>2.9</td>
<td>7</td>
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<tr>
<td>PG</td>
<td>3</td>
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<td>DPG</td>
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<td>PS</td>
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ferase activity is unknown: when comparing the molecular compositions of egg yolk and potato phospholipids (Table I), one can see that more PE, PI, and acidic phospholipids (PG, DPG, PS) are found in potato phospholipids. This difference in molecular compositions might explain the differences in inhibitory effects exerted by egg or potato phospholipids during reconstitution experiments.

In conclusion, the two conditions usually required to demonstrate (6) the phospholipid-dependence of CDP-choline transferase, decrease of enzyme activity following phospholipid hydrolysis by phospholipase C and restoration of enzyme activity following addition of exogenous phospholipids to lipid-depleted membranes, have been fulfilled by our experiments. This phospholipid dependence of a membranous enzyme, involved in the biosynthesis of a major lipid component of membranes, seems particularly interesting, since it suggests that phospholipid dependency of some membranous enzymes might control membrane biogenesis.

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