Phosphatidylinositol Synthesis by a Mn²⁺-Dependent Exchange Enzyme in Castor Bean Endosperm

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ABSTRACT
myo-Inositol is incorporated into phosphatidylinositol by an exchange reaction associated with the endoplasmic reticulum fraction isolated from post-germination castor bean endosperm. The reaction requires Mn²⁺, has a pH optimum of 8.0, an apparent K_m for myo-inositol of 26 micromolar, and is stimulated about 15-fold by certain cytidine derivatives. The cytidine derivatives appear to be converted to CMP, which may be the only active stimulator. These optimal exchange reaction conditions, both with and without CMP, differ from those for cytidine-5'-diphosphoglyceride: myo-inositol transferase (EC 2.7.8), so the exchange does not appear to be a reversal of the transferase. This conclusion is augmented by the low rates of CDP-diglyceride formation from cytidine derivatives when compared to the high rate of myo-inositol incorporation into phosphatidylinositol in the presence of the same cytidine derivatives and identical reaction conditions.

Two pathways are known for the incorporation of myo-inositol into phosphatidylinositol. The de novo pathway via CDP-diglyceride: myo-inositol transferase (EC 2.7.8) has been characterized in both animal (1, 3, 13, 16, 20, 21) and plant (5, 17, 19) tissues. The alternate pathway is the phosphatidylinositol-myo-inositol exchange reaction, which appears to proceed by the enzymic exchange of free myo-inositol for the inositol moiety of PI². This reaction requires Mn²⁺, does not utilize CDP-diglyceride, and is affected by cytidine nucleotides in animal tissues (2, 9, 13, 21). It has been proposed that the exchange reaction in animal tissues is a reversal of the transferase reaction (13), which could result in maintaining a low level of CDP-diglyceride in microsomal membranes (9, 14). On the other hand, evidence of differences between the transferase and exchange reaction enzymes was obtained by Takenawa et al. (21) using solubilized membrane preparations. In addition, Holub (6, 7) has shown that the acyl components of PI synthesized via the exchange reaction are not typical of the acyl components of endogenous PI or CDP-diglyceride.

Our purpose in these experiments was to establish the presence of CDP-diglyceride-independent PI formation (exchange enzyme) in a plant system, to characterize this activity, to compare it to the previously described (5, 17, 19) transferase enzyme, and to assess its role in plant phospholipid metabolism.

MATERIALS AND METHODS
Lipids were purchased from Serdary Research Laboratories, Inc., London, Ontario. Triton X-100, sodium deoxycholate

1 This investigation was supported by National Science Foundation Grants PCM 76-11933 and PCM 78-06817.
2 Abbreviations: PI, phosphatidylinositol; CDP-diglyceride, cytidine-5'-diphosphoglyceride.
isobutyric acid: 1 M ammonium hydroxide (5:3, v/v) containing 0.1 M EDTA.

**Protein Determination.** Protein quantities were determined after the method of Lowry et al. (11).

**RESULTS**

**Intracellular Localization.** The CDP-diglyceride-independent incorporation of [2-3H]myo-inositol into chloroform-soluble material by various castor bean endosperm organelles was estimated using sucrose density gradient fractions (Fig. 1). The peak of incorporation of label corresponded to the ER peak identified with NADH:Cyt c reductase (12). No significant activity occurred in the remainder of the gradient.

**Reaction Characteristics.** Incorporation of inositol into PI utilizing the standard assay conditions was linear for 90 min (Fig. 2A), in contrast to the transferase activity which was linear for less than 30 min (17). Increasing quantities of protein resulted in an exponential increase in the rate of the reaction (Fig. 2B).

A metal ion was absolutely required for the exchange reaction (Fig. 3A). Mn²⁺, the preferred cation, saturated the reaction at approximately 15 mM. Incorporation of substrate was reduced 80 to 90% if equimolar MgCl₂ was substituted for MnCl₂. CaCl₂ was totally ineffective in promoting the reaction, but PI formation was reduced (about 50%) if CaCl₂ equimolar to the MnCl₂ was added to the normal reaction mixture.

The pH optimum occurred at 8.0 (Fig. 3B). Substantial enzyme activity was present from pH 7.5 to 8.5 in Tris-Mes buffer. The rate of incorporation of substrate was higher using Hepes buffer but the range of high activity was slightly narrower than with Tris-Mes.

Triton X-100 and sodium deoxycholate affected the exchange activity differently. Triton X-100 stimulated activity at concentrations below 0.05% (w/w) while deoxycholate decreased activity at all concentrations tested (lowest concentration was 0.015%, w/w).

Increasing myo-inositol concentrations (Fig. 4) led to estimates of saturation by about 0.3 mM and an apparent \( K_m \) of 26 \( \mu M \).

**Phospholipid Effects.** The addition of phospholipid vesicles to the reaction resulted in varied effects. A 2-fold increase in activity was obtained with 0.4 mM PI from castor bean and 0.6 mM PI from yeast. Soybean PI had no effect. The addition of other phospholipid vesicles either had no effect (phosphatidylethanolamine, phosphatidic acid) or stimulated the reaction up to only 50% (phosphatidylserine, phosphatidylcholine, phosphatidylglycerol). These variable and limited effects elicited by phospholipids suggest the possibility that this is largely a detergent effect, rather than utilization of the phospholipids as substrates by the enzyme. The results with PI from varying sources suggest acyl-dependent detergent effects instead of acyl-dependent substrate effects as has been shown in rat liver (6).

**Substrate Specificity.** scyllo-Inositol, choline, ethanolamine, and glycerol had little effect beyond a slight promotion of the reaction rate by scyllo-inositol (23% at 25 \( \mu M \)).

**Effects of Cytidine Derivatives.** A variety of cytidine derivatives stimulated the exchange activity (Table 1). Cytidine and cCMP were exceptions. CTP, CDP-choline, and CDP-ethanolamine maximally stimulated inositol incorporation into PI above 40, 60,
and 80 μM, respectively. CDP-diglyceride stimulated the exchange reaction at low concentrations (<40 μM), but activity was reduced at higher concentrations (Fig. 5). Several other nucleotides (see Table I), as well as Pi and PPI, did not affect activity at the concentrations tested.

This apparent lack of specificity for the cytidine nucleotides appears to be superficial. [5-3H]CDP is metabolized to CMP during the course of the reaction (Fig. 6), even in the absence of inositol, and thus CMP may be the only active compound. After

![Fig. 3. Cation requirements (A) and optimum pH (B) for PI-inositol exchange activity in the absence of cytidine derivatives (178 μg ER protein used).](image)

![Fig. 4. Effect of increasing myo-inositol concentrations on PI-inositol exchange activity and determination of the Michaelis constant in the absence of cytidine derivatives (175 μg ER protein used).](image)

![Fig. 5. Effects of CDP-diglyceride on PI-inositol exchange activity under the standard assay conditions (171 μg ER protein used).](image)

Table 1. Nucleotide Effects on Inositol Exchange Activity

<table>
<thead>
<tr>
<th>Addition</th>
<th>Concentration (μM)</th>
<th>Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CDP</td>
<td>40</td>
<td>1,500</td>
</tr>
<tr>
<td>CTP</td>
<td>40</td>
<td>1,500</td>
</tr>
<tr>
<td>CMP</td>
<td>40</td>
<td>1,500</td>
</tr>
<tr>
<td>ATP</td>
<td>40</td>
<td>102</td>
</tr>
<tr>
<td>ADP</td>
<td>40</td>
<td>104</td>
</tr>
<tr>
<td>TTP</td>
<td>40</td>
<td>100</td>
</tr>
<tr>
<td>UTP</td>
<td>40</td>
<td>110</td>
</tr>
<tr>
<td>IDP</td>
<td>40</td>
<td>112</td>
</tr>
<tr>
<td>3' → 5' cCMP</td>
<td>40</td>
<td>113</td>
</tr>
<tr>
<td>Cytidine</td>
<td>40</td>
<td>114</td>
</tr>
<tr>
<td>CDP-choline</td>
<td>40</td>
<td>875</td>
</tr>
<tr>
<td>CDP-ethanolamine</td>
<td>40</td>
<td>755</td>
</tr>
<tr>
<td>CDP-diglyceride</td>
<td>40</td>
<td>601</td>
</tr>
<tr>
<td>Pi</td>
<td>40</td>
<td>100</td>
</tr>
<tr>
<td>PPI</td>
<td>40</td>
<td>106</td>
</tr>
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</table>

The reaction mixture was as described under "Materials and Methods." Control activity was 1.42 nmol PI formed/h/mg protein.

The addition of castor bean PI did not stimulate the reaction as it did in the absence of CMP. CMP addition did not change the apparent \( K_m \) for myo-inositol. Unlike both the exchange reaction without CMP and the CDP-diglyceride:inositol transferase reaction (17), both Triton X-100 and the sodium deoxycholate inhibited the reaction at all concentrations tested.

The reaction rate increased linearly with increasing protein concentrations up to at least 0.4 mg (Fig. 8A) and the product of the reaction increased linearly with increasing time for up to 20 min (Fig. 8B). The optimal Mn\(^{2+}\) concentration was about one-half that for the same reaction minus CMP. The addition of castor bean PI did not stimulate the reaction as it did in the absence of CMP. CMP addition did not change the apparent \( K_m \) for myo-inositol. Unlike both the exchange reaction without CMP and the CDP-diglyceride:inositol transferase reaction (17), both Triton X-100 and the sodium deoxycholate inhibited the reaction at all concentrations tested.
DISCUSSION

This, to our knowledge, is the first report of the inositol exchange reaction in plant tissue. Relatively few descriptions of this activity exist for other systems as well (6, 13, 21). In general, the characteristics of the castor bean exchange activity are similar to those of the other reported enzymes.

A crucial question concerning this activity is whether it is catalyzed by a specific enzyme or by reversal of CDP-diglyceride: myo-inositol transferase, followed immediately by the forward reaction (9, 13, 14, 22). The final answer must await purification of both enzymes. The general characteristics of the exchange reaction, both with and without added CMP, are different from those of the transferase in the same tissue (17). The exchange enzyme is maximally active at a slightly lower pH and a higher Mn²⁺ concentration and has a significantly lower Kₘ for myo-inositol, although both reactions are highly specific for this substrate. There is also a dissimilarity in their susceptibilities to detergents. These differences are similar to those reported for the respective reactions in animal tissues (1, 6, 13, 21). Such data do not absolutely dictate that the reactions are catalyzed by different enzymes.

Stimulation of the exchange reaction by cytidine derivatives has been reported for certain animal tissues (2, 13, 21), but the concentrations were higher than used here (mm) and no attempt was made to determine effects on enzyme characteristics. The active cytidine nucleotide in the castor bean most likely is CMP, since the castor bean ER fraction is capable of hydrolyzing other cytidine nucleotides to CMP, and CMP appears to stimulate maximally at a lower concentration than the other nucleotide derivatives. This may account for certain ambiguous results in animal tissues (6, 9, 13, 21), since the microsomal fractions from chicken brain, guinea pig liver, and beef liver all have been shown to contain a nucleotide phosphohydrolase (14, 22). The mechanism of the potential regulation by CMP is unknown (15), but the effect of CMP in converting the activity response to increasing protein concentration from an exponential to a linear increase in activity may be relevant. Such behavior is normally attributed to

Fig. 6. Elution from anion exchange resins of CMP and CDP following addition of [5-3H]CDP to the standard assay mixture with (●) and without (x) myo-inositol. The (O–O) had chloroform-methanol-water (1:2:0, v/v) added prior to addition of the enzyme.

CDP-Diglyceride Formation. To examine further the possibility that the apparent exchange reaction was due to a reversal of the transferase reaction, CDP-diglyceride formation was examined under typical exchange reaction conditions (Table II). Some CDP-diglyceride was synthesized in the presence of CTP or CDP. The rate of CDP-diglyceride formation in the presence of CDP was considerably less than the measured rate of PI synthesis, and so seems incapable of supporting the reaction. The addition of inositol decreased the accumulation of CDP-diglyceride as in cauliflower (18) and chicken brain (3), indicating a small portion may be used for PI synthesis. It is also possible that it increases the synthesis as well as utilization of CDP-diglyceride by forcing the formation of PI. The CDP-diglyceride that remained may represent the minimum endogenous concentration in the ER of castor bean endosperm.
the regulation of the association-disassociation of enzyme subunits in partially purified allosteric proteins (10). Direct evidence for this is lacking for the exchange enzyme.

One plausible explanation for the CMP stimulation of exchange activity which must be dealt with is the enhancement of the reversal of the transferase reaction by the addition of a product (CMP) of that reaction. This seems unlikely because (a) an inadequate accumulation of CDP-diglyceride occurred in the absence of myo-inositol, (b) the quantity of CMP required for stimulation was quite low, (c) there was an absence of stimulation by the other transferase product (PI) when CMP was present, and (d) the exchange reaction could be distinguished from the transferase by its higher affinity for myo-inositol.

The role of the exchange reaction in phospholipid metabolism is obscure. Purification of the enzyme in order to allow a precise definition of substrate preference and regulation of activity will be required to elucidate this function.

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