Phosphatidylcholine Synthesis in Castor Bean Endosperm

Occurrence of an S-Adenosyl-L-Methionine:Ethanolamine N-Methyltransferase

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ABSTRACT

Methyl ethanolamine synthesis by S-adenosyl-L-methionine: ethanolamine N-methyltransferase from an extract of castor bean (Ricinus communis L. var Hale) endosperm was characterized. The apparent Michaelis-Menten constants of the enzyme for ethanolamine and S-adenosyl-L-methionine were estimated to be 6.7 and 1.4 μM, respectively, although the Kₘ for ethanolamine is imprecise because of strong substrate inhibition. The pH optimum was 8.0, and a divalent cation was required for activity, with Mg²⁺ giving the greatest stimulation at 5 mM. The enzyme was inhibited by calcium in the micromolar range and relatively high concentrations of ethanolamine (above about 7 μM). The activity was found in the 119,000g supernatant fraction and, therefore, appears to be cytoplasmic. The potential roles of S-adenosyl-L-methionine: ethanolamine N-methyltransferase in choline and phosphatidylcholine synthesis are discussed.

Phospholipids are important constituents of plant seeds. Even in oil-rich species, such as castor bean (Ricinus communis L.), in which triacylglycerols are the predominant lipids, PtdCho and PtdEtN are major components of the cellular membranes that are synthesized during the postgermination period (7). Two pathways are traditionally thought to be involved in biosynthesis of PtdCho: (a) the nucleotide pathway, which derives its headgroup from CDP-Cho, and (b) the methylation pathway, which depends on sequential methylations of PtdEtN. In recent studies, it has been demonstrated that PtdCho synthesis in plants requires methylated intermediates that are both cytosolic and membrane bound. The first steps in synthesis of these intermediates consist of P-EtN synthesis, catalyzed by EtN kinase, followed by its methylation. The product, P-MEtn, was further methylated at either the base phosphate level (Lema), the phospholidip level (soybean), or a combination of both (carrot) to ultimately produce PtdCho (2, 10). Enzyme activities related to these patterns have been assayed (3).

In castor bean endosperm, in which we followed the in vivo kinetics of entry of the radiolabeled methyl group of methionine into the methylated derivatives that lead to PtdCho (13), the first compound to become highly labeled was MEtn rather than P-MEtn. From those results, we suggested that the initial step in PtdCho synthesis in this tissue may be the methylation of free EtN rather than P-EtN. This would require the activity of an AdoMet:Etn N-methyltransferase, which has not been previously described. In this paper, we report success in measuring such an activity in vitro, as well as describe general assay conditions and properties.

MATERIALS AND METHODS

Plant Material

Three-day postgermination endosperms of castor bean (Ricinus communis L. var Hale) were obtained as described previously (5).

Homogenization

Endosperm halves (5 g) were chopped in a Petri dish on ice for 15 min with a single razor blade in 10 mL of grinding medium, which contained 150 mM Tris-HCl (pH 7.6), 10 mM KCl, 0.5 mM sucrose, 3 mM EGTA, and 26 mM reduced GSH (freshly added). The crude homogenate was filtered through two layers of cheesecloth, and this filtrate was used for most of the enzyme assays described here.

Enzyme Assay

AdoMet:Etn N-methyltransferase was routinely assayed for 1 h at 30°C in a final volume of 0.150 mL containing 100 mM Hepes (pH 8.0), 7 μM EtN (42.8 mM Ci/mmol), 0.120 μM AdoMet, and 5 mM MgCl₂. [2-¹⁴C]EtN was obtained from Amersham. In some cases, 100 mM Mes, N-[2-hydroxyethyl]-piperazine-N'-(3-propane-sulfonic acid), or Tris-HCl were substituted for the Hepes. The reaction was started by addition of the enzyme and stopped by precipitation of protein with cold methanol (1.5 mL), followed by acidification with formic acid (10 μL of 2.5 n). After centrifugation for 10 min in a Beckman benchtop centrifuge, the supernatant volume was reduced under a N₂ stream and applied to a paper
Identification of the Product

Descending paper chromatography was carried out on Whatman No. 3 paper at the room temperature for 18 h. The solvent used was: phenol:1-butanol:88% HCOOH:H2O (125:125:6:8:25.7, w/v/v/v); the chromatography paper was predipped in 1 M KCl and dried to avoid tailing. Authentic external standards were used to locate the reaction product. [14C]Etn was methylated with iodomethane to synthesize MEtn and DMEtn standards (10). The chromatograms were cut into 1-cm sections, which were placed into scintillation vials, and their radioactivities were measured directly in CytoScint (ICN) using a Beckman LS-8000 scintillation counter.

Protein Determination

Protein concentrations were measured by the method of Bradford (1).

RESULTS

A protein- and Etn-dependent activity was detected that formed a 14C-labeled product that comigrated with MEtn (Fig. 1). None of the product migrated with the other methylated free bases, DMEtn and Cho, or with the phosphorylated bases. Under the assay conditions, product formation was linear between 15 and 60 min and proportional to enzyme up to at least 150 μg of protein (Fig. 2). Maximal activity occurred at 30°C (Fig. 2). The enzyme had a narrow pH optimum with maximal activity observed at pH 8.0 in Hepes buffer (Fig. 3). Ca2+ inhibited the reaction, whereas Mg2+ strongly promoted it, reaching a peak at 5 mM (Fig. 3); Mn2+ stimulated the reaction slightly, also reaching a peak at 5 mM (Fig. 3). The effects of increasing AdoMet and Etn concentrations are shown in Figure 4. From these data, the apparent K_m values were calculated to be 1.4 and 6.7 μM for AdoMet and Etn, respectively. The enzyme affinity for its substrates was, therefore, very high. As the concentration of Etn was raised above approximately 7 μM, there was a progressive decrease in the reaction rate, and therefore, the true K_m for Etn remains uncertain; however, strong affinity for this substrate is clear.

The effects of various components of the medium were tested by preparing breis in media with one or more components omitted; removal of GSH and EGTA led to losses of 60 and 40% of the activity, respectively. In jack bean extracts, PEG greatly enhanced AdoMet:methionine S-methyltransferase activity (4), but in castor bean extracts, addition of PEG had no effect on the rate of the AdoMet-dependent Etn N-methylation.

To provide an estimate of the location of the enzyme activity within the cell, the filtrate was centrifuged at 119,000g for 60 min, followed by resuspension of the precipitate in 5 mL of grinding medium using a glass homogenizer. Activity could be found only in the supernatant fraction (Table I). The addition of 200 mM KCl to the filtrate for 10 min before centrifugation did not lead to an increase in supernatant activity (Table I). We conclude, therefore, that AdoMet:Etn N-methyltransferase is located entirely in the cytoplasm of endosperm cells.

DISCUSSION

The in vivo kinetic studies of incorporation of the methyl group of AdoMet into PtdCho by castor bean have suggested the presence of an enzyme capable of direct N-methylation of Etn (13). In this paper, we confirm the existence of such

![Figure 1. A representative chromatogram of free bases. Radioactivity of the standards is indicated by hatched bars, and the radioactivity due to the precursors and products under study is indicated by open bars. Separations were on paper as described in "Materials and Methods."](image)
an enzyme by more direct in vitro measurement of the activity. The combined results of this and our previous (13) investigations provide evidence that MEtn may be produced by direct methylation in plants. The resulting MEtn may directly enter the phospholipid fractions by one or both of two pathways, either by the CDP derivative pathway for incorporation into phospholipids or by direct exchange for other headgroups. If the nucleotide pathway were followed, MEtn would be converted to P-MEtn (13). It is interesting that this product is utilized as well as P-Cho by a purified cytidylyltransferase (15), thereby allowing formation of CDP-MEtn by the enzyme normally thought to produce CDP-Cho; neither P-Etn nor P-DMEtn served as substrates for that enzyme (15). The alternative means of P-MEtn incorporation by exchange of free MEtn into a phospholipid fraction, by substituting the MEtn for an existing headgroup in a Ca2+-requiring reaction, has been found in this tissue, but the activity is low and difficult to measure (9). The final phospholipid products of both of these pathways would be PtdMEtn, which would be expected to undergo additional methylations to form PtdCho. Thus, the AdoMet:Ettn N-methyltransferase could play a central role in providing MEtn in either scenario.

The MEtn might also be further methylated and phosphorylated to produce P-Cho, with subsequent conversion to CDP-Cho and finally incorporation into PtdCho. Relatively little is known about the regulation of the methylation and nucleotide pathways with respect to each other. Available data show that when Cho is present the nucleotide pathway is operative and the methylation pathway is reduced, whereas the methylation pathway is active when Cho concentrations are reduced (14). For these and other reasons, the committing step for PtdCho synthesis in these tissues has been placed at P-Etn methylation (2, 10). For example, the presence of Cho in the growth medium partially suppressed the operation of P-Etn methylation by decreasing the activity of the enzyme.

Figure 2. Effects of varying time (top), protein concentration (middle), and temperature (bottom) on the rate of AdoMet-dependent Etn N-methylation. The product was quantified after purification by paper chromatography as in Figure 1. Each result is the mean of two separate measurements.

Figure 3. Effects of varying pH (A) and divalent cations (B) on the rate of AdoMet-dependent Etn N-methylation. Other details are as described in the legend to Figure 2.
involved, AdoMet:P-Etn N-methyltransferase, in * Lemma* and cultured cell suspensions of both carrot and soybean (11, 12). We cannot state at this time whether an analogous situation exists with the castor bean endosperm AdoMet:Etn N-methyltransferase. Indeed, Cho synthesized in vivo from exogenously added [14C]AdoMet and [2-14C]P-Ser did not appear to be utilized for the nucleotide pathway but accumulated in a major sequestered pool (13). Cho kinase has been found to be highly reversible and thus may not have a normal role in phosphorylating Cho but rather as a phosphatase-producing Cho from P-Cho for other purposes (6). One of them could be the regulation of PtdCho synthesis through the regulation of AdoMet:Etn N-methyltransferase activity.

It is not clear whether the activity of the AdoMet:Etn N-methyltransferase reported here is adequate for the ultimate production of PtdCho in these tissues. In particular, Cho kinase, P-Cho cytidylyltransferase, and P-Cho transferase have measured activities in this tissue of 4.7 (6), 0.45 (15), and 4.5 (7) nmol min⁻¹ mg⁻¹ of protein, respectively, as compared with only 1.3 pmol min⁻¹ mg⁻¹ of protein for the in vitro N-methyltransferase activity measured here. On the other hand, the extent of recycling of Cho (13) is not known, and the strong affinities of the two substrates, both in the low micromolar range, indicate a capacity to work efficiently with small, highly regulated pools. Although Etn clearly inhibited activity at concentrations greater than 7 μM, the enzyme appears to demonstrate a stronger affinity for Etn than the enzyme that utilizes P-MEtn for methylation in * Lemma* has for that phospho-base (3). Etn also may serve as a substrate for Etn kinase in this tissue, as demonstrated by P-Etn, CDP-Etn, and PtdEtn becoming rapidly labeled when endosperm halves were supplied with [2-14C]Etn (13). Etn kinase has been purified from both spinach leaf (8) and soybean seeds (16), and we have found it in the soluble fraction of castor bean endosperm extracts (our unpublished results). It is noteworthy that MEtn, but not Cho (5 mm), inhibited Etn kinase in spinach and soybean tissues. Therefore, to get a better understanding of PtdCho and PtdEtn biosynthesis in castor bean endosperm, it seems of particular interest to study further the regulation of both AdoMet:Etn N-methyltransferase and Etn kinase. However, these studies should await purification of the two enzymes mentioned to reduce ambiguities in the results.

**Table I. Intracellular Localization of AdoMet-Dependent Etn N-Methylation**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Activity (pmol h⁻¹ mg⁻¹ protein)</th>
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</thead>
<tbody>
<tr>
<td>Supernatant</td>
<td>92.5</td>
</tr>
<tr>
<td>Pellet</td>
<td>0.0</td>
</tr>
<tr>
<td>Supernatant (KCl)</td>
<td>90.5</td>
</tr>
<tr>
<td>Pellet (KCl)</td>
<td>0.0</td>
</tr>
</tbody>
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**Figure 4.** Effects of increasing AdoMet (A) and Etn (B) concentrations on the rate of AdoMet-dependent Etn N-methylation. Other details are as in the legend to Figure 2.

**LITERATURE CITED**


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