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Phosphatidylethanolamine Synthesis by Castor Bean Endosperm[1]

A Base Exchange Reaction

Sungho Shin and Thomas S. Moore, Jr.*

Department of Botany, Louisiana State University, Baton Rouge, Louisiana 70803-1705

ABSTRACT

A base exchange reaction for synthesis of phosphatidylethanolamine by the endoplasmic reticulum of castor bean (*Ricinus comminus* L. var Hale) endosperm has been examined. The calculated Michaelis-Menten constant of the enzyme for ethanolamine was 5 micromolar and the optimal pH was 7.8 in the presence of 2 millimolar $CaCl_2$. L-Serine, *N*-methylethanolamine and *N,N*-dimethylethanolamine all reduced ethanolamine incorporation, while D-serine and *myo*-inositol had little effect. These inhibitions of ethanolamine incorporation were found to be noncompetitive and ethanolamine also noncompetitively inhibited L-serine incorporation by exchange. The activity of the ethanolamine base exchange enzyme was affected by several detergents, with the best activity being obtained with the zwitterionic defjtergent 3-3-cholamidopropyl)dimethylammonio-2-hydroxyl-1-propanesulfonate.

PtdEtn,[2] the second most abundant phospholipid in most eukaryotic cell membranes (6, 8, 21), plays an important role in determining the chemical and physical properties of these membranes and their proteins (26, 31). The three pathways which have been defined for synthesis of this phospholipid are:

CDPethanolamine + 1,2-Diacylglycerol→ PtdEtn + CMP (1)

Phosphatidylserine→ PtdEtn + CO_2 (2)

Phosphatidyl-X + Ethanolamine→ PtdEtn + X (3)

The CDPethanolamine (or nucleotide) pathway (reaction 1) is catalyzed by CDPethanolamine: 1,2 diacylglycerol ethanolaminephosphotransferase (EC 2.7.8.1) and generally predominates. It has been demonstrated in tomato root (32), spinach leaves (15) and castor bean endosperm (26) as well as in animal tissues (13, 28, 31) and bacteria (27). The second reaction, phosphatidylserine decarboxylase, also has been found in plant tissues (16, 32), animals (4) *Escherichia coli* (12), and *Tetrahymena* (7).

The exchange reaction (reaction 3) is distinguished from the other two reactions by utilizing free ethanolamine as a substrate and requiring Ca^{2+} in the mM range as a cofactor (9). The first support for phosphatidylethanolamine synthesis by an exchange reaction in plants was by Miedema and Richardson (17), and Vandor and Richardson (30) later described a Ca^{2+}-stimulated incorporation of ethanolamine, serine, and choline into phospholipids, as well as the decarboxylation of the serine moiety of phosphatidylserine in pea microsomes. This exchange activity has been the object of several studies using mammals (11, 18, 23, 24). Recently, Suzuki and Kanfer (29) purified an ethanolamine exchange enzyme from rat brain microsome approximately 25-fold and characterized it.

In a recent study, Kinney and Moore (14) found an incorporation of [^{14}C]ethanolamine into PtdCho during treatment with diethylethanolamine, a compound which inhibited the incorporation of ethanolamine- and choline-phosphates into phospholipids of the same tissue. One explanation of these results was that the incorporation occurred through an active ethanolamine base exchange reaction, followed by conversion to PtdCho by the phosphoglyceride methylation pathway (14). The purpose of the present work was to confirm the presence of the ethanolamine exchange reaction in castor bean endosperm, determine its capacity, to confirm and extend the results of Vandor and Richardson (30) through additional characterization of this ethanolamine base exchange enzyme, and to provide data necessary for testing various hypotheses concerning the role of this enzyme.

MATERIALS AND METHODS

Materials

[1,2-^{14}C]Ethanolamine hydrochloride (38 mCi/mmol) and [3-^{14}C]L-serine (57 mCi/mmol) were obtained from ICN. The sucrose was from E. M. Science and CHAPSO from Calbiochem. All other organic compounds were purchased from Sigma Chemical Co.

Plant Materials

Seeds of castor bean (*Ricinus communis* L. var Hale) endosperm were removed from their seed coats, surface-steri-

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[2] Abbreviations: PtdEtn, phosphatidylethanolamine; PtdCho, phosphatidylcholine; OGP, octyl-β-D-glucopyranoside, CHAPSO 3-(3-cholamidopropyl)dimethylammonio-2-hydroxyl-1-propanesulfonate.

lized in 10% bleach (5.25% hypochlorite) for 90 s, and planted in moist vermiculite contained in preparation dishes. They were germinated and grown at 30°C for 3 d.

Homogenization of Tissue

This procedure was similar to the methods described previously (20). Thirty endosperm halves were removed from 3-d-old seedlings, rinsed in distilled water and placed in a Petri dish on ice. They were chopped (2 strokes/s) for 15 min with a single razor blade in 11 mL of homogenization medium containing 150 mM Tricine buffer (pH 7.5), 10 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, and 0.5 mM sucrose. The crude homogenate was filtered through two layers of Dacron cloth.

Fractionation of Cellular Components

Five mL of the filtered homogenate were layered onto the top of a gradient contained in a 37.5 mL centrifuge tube. The gradient was composed of: (a) a 2.0 mL cushion of 2.25 M sucrose, (b) 20 mL of sucrose solution decreasing linearly in concentration from 2.25 to 1.0 M, and (c) a 10 mL layer of 0.6 M sucrose. All gradient solutions were prepared in 3 mM EDTA (pH 7.5). The gradients were centrifuged at 20,000 rpm in a Sorvall AH-629 motor with a Sorvall OTD-65B ultracentrifuge for 3 hours.

Enzyme Assays

The ethanolamine and serine exchange assays were based on the methods of Raghavan *et al.* (23) and Moore (19). The standard incubation medium for the incorporation of ethanolamine into a chloroform-soluble fraction consisted of 200 mM Hepes buffer (pH 7.8), 2 mM $CaCl_2$, and 0.01 mM [2-^{14}C] ethanolamine hydrochloride (2.4 mCi/mmol) in a final volume of 0.5 mL. The incubation medium for the incorporation of serine consisted of 40 mM Hepes buffer (pH 7.8), 2 mM $CaCl_2$, and 0.4 mM [^{14}C-3]L-serine (5 mCi/mmol) in a final volume of 0.5 mL (24). The reactions were started by the addition of 150 to 200 μg of endoplasmic reticulum fraction, usually incubated for 1 h at 30°C, and stopped by adding 3.3 mL chloroform:methanol:water (1:2:0.3 v/v). The lipids were extracted by the method of Bligh and Dyer (3). The radioactivity of the chloroform fraction was determined by drying the chloroform extract in a 3 mL scintillation vial, followed by the addition of 2.5 mL of Betacount scintillation solution (J. T. Baker) and measuring radioactivity in a Beckman LS-8000 scintillation counter.

Chromatography

The products of the reactions were identified by TLC with known standards. The chloroform-soluble products were applied to 250 μm thick Silica Gel G TLC plates (Analtech, Inc.) and chromatographed in two dimensions (10) with: (a) chloroform-methanol-7 N ammonium hydroxide (70:30:5, v/v), and (b) chloroform-methanol-acetic acid-water (25:15:4:2, v/v).

Protein Determination

Protein was determined by the method of Bradford (5).

RESULTS

General Requirement for Ethanolamine Incorporation

The exchange activity under the standard assay conditions exhibited a linear increased in PtdEtn formed with increasing amounts of added endoplasmic reticulum protein up to 150 μg or with time up to 75 min. The general requirements for synthesis of PtdEtn by the exchange reaction are given in Table I. Boiled endoplasmic reticulum lost all activity. In most previously described exchange reactions Ca^{2+} was an absolute requirement (9), and the same appears true for this activity. Ethanolamine incorporation was inhibited about 30% by 1 mM L-serine, but the same concentration of D-serine was stimulatory. In the presence of 1 mM *N, N*-dimethylethanolamine, incorporation of ethanolamine was inhibited by 30%, while *N*-monomethylethanolamine reduced incorporation of ethanolamine by only 20%. Addition of unlabeled 1 mM ethanolamine yielded an 86% inhibition of isotope incorporation, while choline at the same concentration inhibited incorporation of ^{14}C-ethanolamine into the products by only 11%. Inositol increased incorporation of ^{14}C-ethanolamine to the products by 8%. Greater than 91% of the radiolabel was found in phosphatidylethanolamine.

Cation and pH Specificity

The results summarized in Figure 1 show the requirement for divalent cations in the ethanolamine base exchange reaction. Calcium was strongly preferred over other cations tested, with an optimal concentration of about 2 mM (Fig. 1A). A low rate of ethanolamine incorporation was observed in the presence of Mg^{2+} and Mn^{2+} (Fig. 1B) but was reduced by 75 to 85% when compared to Ca^{2+}. The optimal pH for the enzyme was 7.8 in 200 mM Hepes buffer (Fig. 2).

Table I. *General Requirements for the Synthesis of PdtEtn by the Exchange Reaction*

The control activity was 0.32 nmol h^{-1} (mg protein)$^{-1}$. Reaction mixtures and assay were as described in the "Materials and Methods" except for the variations indicated. The data represent the averages of four experiments.

Treatment	Activity
	%
Complete	100
Boiled enzyme	0
$-Ca^{2+}$	10
+D-Serine, 1 mM	114
+L-Serine, 1 mM	69
+ Choline, 1 mM	89
+ Ethanolamine, 1 mM	14
+ Inositol, 1 mM	108
+ *N*-Monomethylethanolamine, 1 mM	79
+ *N,N*-Dimethylethanolamine, 1 mM	69

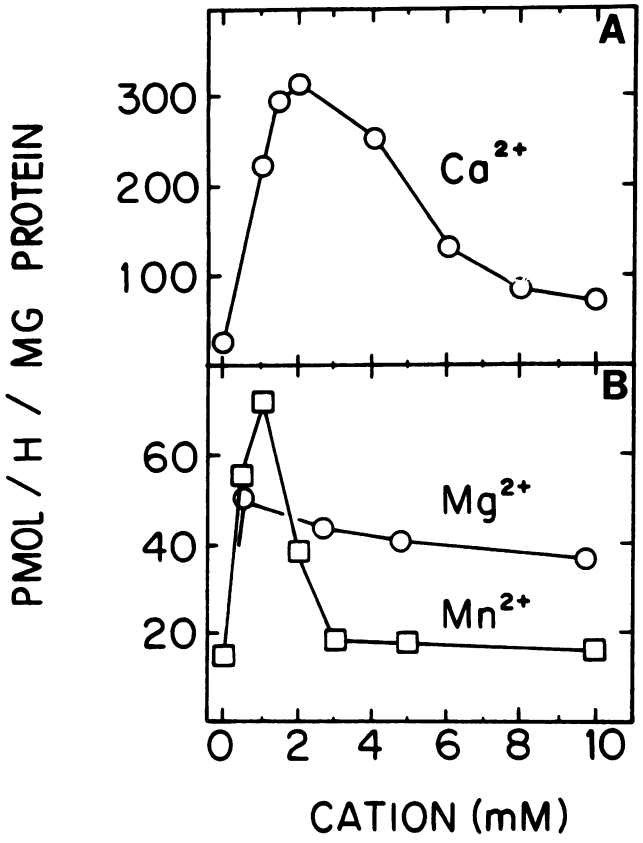

Figure 1. Effects of divalent cations on the incorporation of ethanolamine to form phosphatidylethanolamine. The results are expressed as pmol PtdEtn produced in 1 h by 1 mg of protein and represent the averages of three experiments.

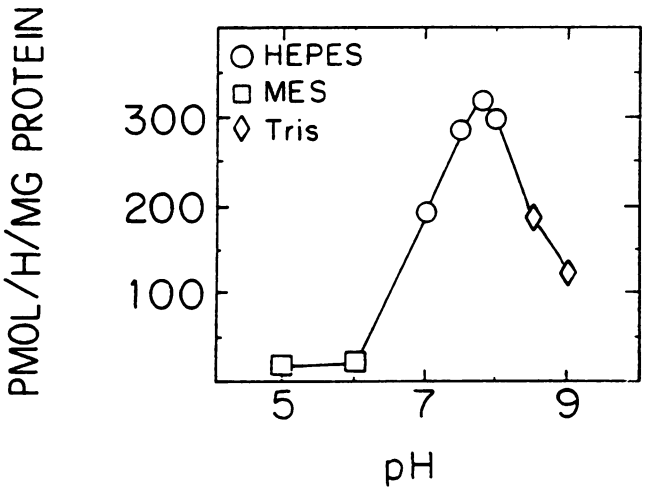

Figure 2. Ethanolamine base exchange enzyme activity as a function of pH in Mes, Hepes, and Tris. The results are expressed as pmole PtdEtn produced in 1 h by 1 mg protein and represent the averages of three experiments.

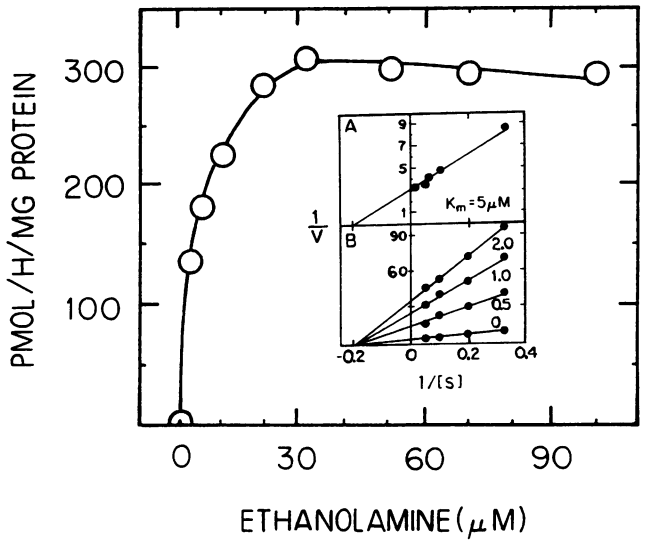

Figure 3. Effect of increasing ethanolamine concentrations on the formation of PdtEtn, determination of the Michaelis constant (inset A), and the effect of nonradioactive serine on the incorporation of ethanolamine by the base exchange reaction (inset B). The results are expressed as the pmol of PtdEtn produced by 1 mg protein in 1 h and are the averages of four experiments.

Substrate Requirement

The ethanolamine base exchange enzyme achieved a maximum velocity of approximately 0.3 nmol h^{-1} (mg protein)$^{-1}$, which was attained at an ethanolamine concentration of about 30 μM (Fig. 3). The K_m for ethanolamine, calculated from a double-reciprocal plot, was 5.0 μM (Fig. 3,A).

Serine decreased the V_{mac} for ethanolamine with no effect on the K_m (Fig. 3,B). Both methylethanolamine and dimethylethanolamine also were found to behave as noncompetitive inhibitors (Fig. 4).

Detergent Effects

The effects on the ethanolamine exchange of various detergents at their critical micelle concentrations (CMCs) are summarized in Table II. The enzyme exhibited a 20% increase in activity in the presence of the zwitterionic detergent CHAPSO. By contrast, Triton X-100 reduced the activity almost 35% while digitonin, OGP and sodium cholate had no significant effect.

Serine Base Exchange Reaction

A maximum velocity of approximately 1.7 nmol h^{-1} (mg protein)$^{-1}$ was obtained with L-serine at about 400 μM and exhibited a K_m of about 116 μM (Fig. 5). Ethanolamine decreased the maximum velocity for serine with no effect on the K_m (Fig. 5,B).

DISCUSSION

This work confirms our previous indirect evidence for the presence in castor bean endosperm of an ethanolamine base exchange (14) enzyme, which appeared to catalyze the incor-

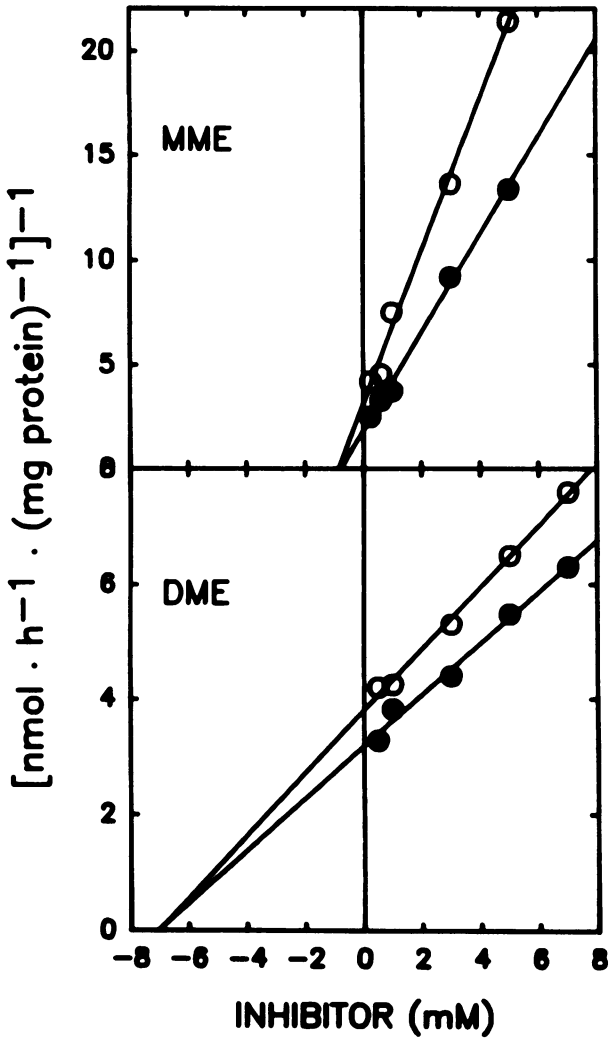

Figure 4. Dixon plots of the effects of varying concentrations of monomethylethanolamine (MME, panel A) and dimethylethanolamine (DME, panel B) on the incorporation of 15 μM (●) and 30 μM (○) ethanolamine by the base exchange reaction. The results are expressed as the reciprocals of the nmoles of PtdEtn produced by 1 mg of protein in 1 h and are the averages of four experiments.

poration of ethanolamine into phospholipid in apparent exchange for an existing headgroup. In a previous study (19), we showed that this activity cannot be attributed to the presence of phospholipase D, and we have been unable to directly measure phospholipase D activity in this tissue (data not shown). Vandor and Richardson (30) provided the first evidence for the occurrence of ethanolamine exchange in plants. They utilized pea seedlings and found that both choline and L-serine incorporation also were stimulated by Ca^{2+} (30). L-serine base exchange also has been reported from castor bean endosperm, but choline exchange has not (19). There is considerable evidence for such an ethanolamine-requiring exchange reactions in animal cells. For example, Gaiti *et al.* (9) established the Ca^{2+} requirement for that reaction, while Bjerve (2) studied kinetic properties in rat microsome. Recently, Suzuki and Kanfer (29) purified this base exchange enzyme 25-fold from rat brain microsomes.

Table II. *Effects of Detergents on the Ethanolamine Base Exchange Enzyme Activity*

Concentrations are the approximate critical micelle concentrations for each detergent (33). The control activity was 0.32 nmol h^{-1} (mg protein)$^{-1}$. The experiment was performed under the standard assay condition given in "Materials and Methods" except that detergent was varied as shown. All data represent the averages of three experiments.

Detergent	Concentration	Activity
	mM	%
Control		100.0
Sodium cholate	8.0	101.4
Triton X-100	3.3	64.8
OGP	25.0	93.6
Digitonin	3.0	94.6
CHAPSO	8.0	121.0

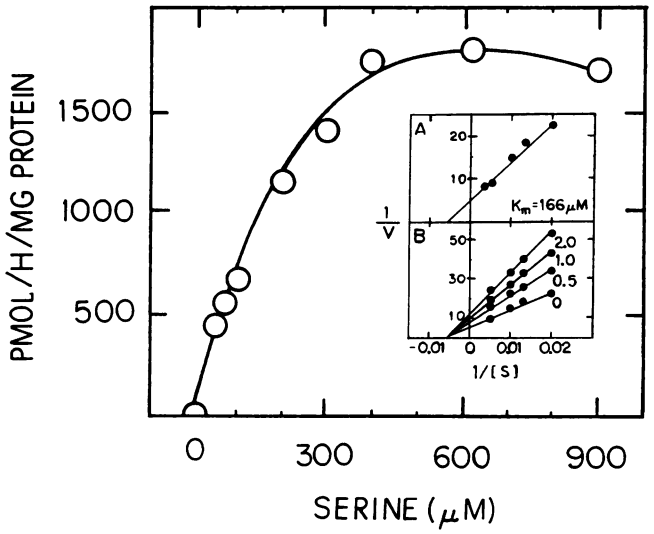

Figure 5. Effect of increasing L-serine concentrations on the formation of phosphatidylserine, determination of the Michaelis constant (inset A), and the effect of nonradioactive ethanolamine on the incorporation of serine by a serine base exchange reaction (inset B). The results are expressed as the pmol of phosphatidylserine produced in 1 h by 1 mg protein and are the averages of four experiments.

The optimal conditions for the ethanolamine and serine base exchange reactions described here are similar to those previously reported for serine base exchange in this tissue (19). The strong requirement for Ca^{2+} is similar to the enzyme from animal cells (2, 9). Suzuki and Kanfer (29) found optimal Ca^{2+} concentrations for ethanolamine and serine incorporation of 8 and 10 mM, respectively, in rat brain microsomes. Bjerve (2) reported that at pH 7.5, 4 mM Ca^{2+} was optimal for the incorporation of both ethanolamine and serine in rat liver microsomes, but the optimal Ca^{2+} concentration was found to increase with decreasing pH and vice versa. While details were not presented, the maximum incorporation of ^{14}C-ethanolamine in pea seedling microsomes also occurred with Ca^{2+} in the mM range. The physiological significance of a requirement for these relatively high Ca^{2+}

concentrations is not clear, but the possibility exists that the enzyme occurs in a site with elevated ion concentrations. This possibility is supported by its apparent exposure to the lumen side of the ER (25). The sarcoplasmic reticulum is well known for its capacity to serve as a Ca^{2+} reservoir (1), and a similar situation might exist here.

It is not clear whether the incorporations of ethanolamine and serine into phospholipid are catalyzed by the same or separate enzymes. The similarities in requirements and characteristics, as well as topology (25), might suggest that a single enzyme is involved and that ethanolamine is the strongly preferred substrate. On the other hand, L-serine is not a competitive inhibitor of ethanolamine incorporation, and indeed is noncompetitive, which indicates that the substrate and inhibitor bind to different sites. This might indicate two enzymes. Vandor and Richardson (30) reported that both the D- and L-isoforms of serine inhibited the pea microsomal enzyme, but only L-serine was effective in our tests. In addition, they, as well as Miedema and Richardson (17), mentioned that these compounds were competitive inhibitors, but insufficient data were presented for comparison and that was not the case here. Each substrate was a competitive inhibitor of the base exchange enzymes from rat brain microsomes (29) while in rat liver ethanolamine was a competitive inhibitor of serine incorporation and serine a noncompetitive inhibitor of ethanolamine (2). Thus, the situation may be complex and no direct comparisons are possible at this time. Unfortunately, our attempts to purify the ethanolamine exchange enzyme have been unsuccessful due to a rapid loss of activity following solubilization.

The effects of methylethanolamine and dimethylethanolamine were of particular interest in light of the recent data of Mudd and Datko (22) indicating that PtdCho may arise by methylation of phosphatidylmethylethanolamine in some plant tissues, and that PtdEtn is not itself methylated. However, the possibility that this enzyme utilizes these substrates for incorporating these potential substrates to form the substrates for methylation seems unlikely in light of the absence of competitive inhibition.

The physiological role of the ethanolamine and serine base exchange reactions is not clear. One possibility may be that they serve as a mechanism for a rapid change of membrane phospholipid composition as a response to different chemical and physical influences on a cell. Alternatively, the enzyme might also provide products to a specific side the membrane and allow independent control of the PtdEtn composition of that leaflet of the membrane. This latter possibility is explored in the accompanying paper (25).

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