Short Communication

Phosphatidylethanolamine *N*-Methyltransferase from *Zymomonas mobilis*: Purification and Characterization

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The conversion of phosphatidylethanolamine (PE) to phosphatidylcholine (PC) occurs through three-step methylation *via* the two *N*-methylated intermediates of phosphatidyl-*N*-monomethylethanolamine (PME) and phosphatidyl-*N,N*-dimethylethanolamine (PDE), and in mammalian cells the PE-*N*-methylation pathway has been shown to involve two distinct enzymes.¹⁾ One is the enzyme that catalyzes the first methylation of PE to PME, and the other catalyzes the two-step methylation from the latter to PC. The participation of two enzymes in the pathway was also demonstrated by experiments using mutant strains of *Neurospora crassa*²⁾ and *Saccharomyces cerevisiae*.³⁾ However, little has been reported as to the enzyme catalyzing the PE-*N*-methylation pathway of bacterial cells except for in the case of *Agrobacterium tumefaciens*,⁴⁾ probably because PC is a rare component of bacterial phospholipids.⁵⁾ In this communication, we describe the purification of PE *N*-methyltransferase that catalyzes the three-step methylation pathway from *Zymomonas mobilis* to homogeneity, indicating that the three-step methylation is catalyzed by a single enzyme.

Zymomonas mobilis IFO 13756 was grown statically at 30°C in a medium containing 2% glucose, 0.5% peptone and 0.3% yeast extract, pH 5.5. Cells were harvested at the late-logarithmic phase, washed twice with 80 mM

Tris-HCl buffer (pH 8.5) containing 10% glycerol and 10 mM L-cysteine (Buffer A) and then disrupted by sonic oscillation for 3 min at 180 watts. The disrupted cells were centrifuged at $9000 \times g$ for 10 min and then the supernatant was centrifuged again at $105,000 \times g$ for 1 h. The resulting precipitate, the membrane fraction, was suspended in a small volume of Buffer A containing 1% Triton X-100 and 1 M KCl, and then solubilized in a Teflon-pestle homogenizer at 0°C for 1 hr. The mixture was then centrifuged at $105,000 \times g$ for 1 hr and the supernatant, the soluble fraction, was diluted to lower the concentration of Triton X-100 to 0.04% with 10 mM phosphate buffer (pH 7.0) containing 10% glycerol. The diluted soluble fraction was immediately applied to a hydroxylapatite column (1.3 $\times$ 7.5 cm) which had been equilibrated with 10 mM phosphate buffer (pH 7.0) containing 0.04% Triton X-100 and 10% glycerol and washed thoroughly with the equilibration buffer. The bulk of the enzyme showing the *N*-methyltransferase activities dependent on PE, PME and PDE was adsorbed to the column and eluted with a linear gradient of 10~200 mM phosphate in the equilibration buffer. The active fractions were pooled, dialyzed against Buffer A containing 0.04% Triton X-100 and then applied to a DEAE-Sephacrose CL-6B column (1.3 $\times$ 7.0 cm) which had been equilibrated with the dialysis buffer. Almost all of the three activities passed through the column with the equilibration buffer, whereas only a very small amount of the activities and the majority of the protein were adsorbed and eluted with increasing NaCl concentration. Finally, the active fractions were pooled and applied to a 110 ml-LKB electrofocusing column with linear gradients of 0~50% sucrose and 0.5~1.5% Ampholine (pH 3.5~10). After electrophoresis at 800 volts for 42 hr, the enzyme showing the three *N*-methyltransferase activities was eluted as a single peak with an isoelectric point of 8.5. The purification procedure is summarized in Table I. Throughout the purification from the hydroxylapatite column chromatography to the final step of isoelec-

TABLE I. PURIFICATION OF PHOSPHATIDYLETHANOLAMINE *N*-METHYLTRANSFERASE FROM *Zymomonas mobilis*

N-Methyltransferase activities dependent on PE, PME and PDE were assayed by measuring the incorporation of the methyl groups from *S*-adenosyl-L-[CH₃-¹⁴C]methionine (AdoMet) into PME, PDE and PC, respectively. However, since the PE and PME-dependent activities accompanied further methylation to PDE and PC, and to PC, the two enzyme activities were calculated as the total radioactivities of PME + 1/2 PDE + 1/3 PC, and PDE + 1/2 PC, respectively, according to the method of Schneider and Vance.⁶⁾ The assay mixture, in a 10 ml-stoppered glass tube, contained 1.36 μ M [CH₃-¹⁴C]AdoMet (New England Nuclear, U.S.A., 58.9 μ Ci/ μ mol), 58 μ M PE, PME or PDE, 100 mM Tris-HCl buffer (pH 8.5), 10 mM L-cysteine, 10% glycerol, 0.04% Triton X-100 and the enzyme source, in a total volume of 1.0 ml. The reaction was carried out at 30°C for 30, 10 and 20 min for the PE, PME and PDE-dependent activities, respectively, and terminated by the addition of 0.25 ml of 1 N HCl. The lipids in the mixtures were extracted by the method of Bligh and Dyer,⁷⁾ and separated by thin-layer chromatography on Silica gel G with a solvent system of CHCl₃/CH₃OH/H₂O (65:25:4, by vol). The radioactive lipids corresponding to the PME, PDE and PC standards were scraped into vials and their radioactivities were counted in a toluene scintillation solution. One unit of enzyme activity was expressed as pmol of methyl-¹⁴C-transferred phospholipids, formed per min under the conditions described above. Protein was determined by the method of Lowry *et al.*⁸⁾ with bovine serum albumin as a standard.

Purification step	Total volume (ml)	Total protein (mg)	Total activity (units) dependent on			Specific activity (units/mg protein) dependent on		
			PE	PME	PDE	PE	PME	PDE
Membrane fraction	13.0	509	11.3	2332	981	0.02	4.6	1.9
Soluble fraction	8.0	212	41.5	3837	1722	0.20	18.1	8.1
Hydroxylapatite	46.0	21.8	70.5	2888	1329	3.2	133	61.0
DEAE-Sephadex CL-6B	19.8	2.7	25.9	1437	619	9.6	532	229
Isoelectric focusing	4.0	0.54	14.3	587	262	26.5	1087	485

tric focusing, the proportions of the three activities dependent on PE, PME and PDE remained almost unchanged, in the ratio of 1:41~55:18~23, and the specific activities were equally elevated about 8-fold.

SDS-Polyacrylamide gel electrophoresis of the purified enzyme gave a single protein band, as shown in Fig. 1. By comparison with the migration of marker proteins, the molecular weight of the enzyme was estimated to be 42,000. The molecular weight of the enzyme was found to be about 120,000 on gel filtration on a Sephadex G-200 column. These results indicate that the native enzyme is composed of three identical subunits and that the three-step methylation pathway is catalyzed by a single enzyme.

The conversion of PE to PME accompanied further methylation to PDE and PC, and the ratio of the three ¹⁴C-methylated products was 100:17:15, respectively. The formation of the three products was linear with time for 30 min with 6.5 μ g of the enzyme under the optimal

conditions given in Table I. The conversion of PME to PDE was also accompanied by the formation of PC in the ratio of 100:7. The three *N*-methyltransferase activities showed similar pH profiles with an optimum at pH 8.5, and were stimulated in the same manner by Triton X-100 and L-cysteine at the optimal concentrations of 0.04% and 10 mM, respectively. No requirement of divalent metal ions was observed for the activities.

The apparent Michaelis constants and maximal velocities of the three reactions were determined from Lineweaver-Burk plots. The *K_m* values of the PE, PME and PDE-dependent reactions for *S*-adenosyl-L-methionine (AdoMet) were calculated to be 3.6, 1.9 and 3.9 μ M with *V_{max}* values of 0.04, 1.36 and 0.69 nmol/mg enzyme/min, respectively. The *K_m* values for PE, PME and PDE were 33.6, 17.5 and 40.5 μ M, respectively, and the *V_{max}* values were almost the same as those for AdoMet. These results indicate that the first-step methylation is rate-limiting in the

FIG. 1. SDS-Polyacrylamide Gel Electrophoresis of the Purified Enzyme.

Ten μ g of the purified enzyme was subjected to SDS-polyacrylamide gel electrophoresis using 7.5% acrylamide by the method of Weber and Osborn.⁹⁾ The marker proteins used were bovine serum albumin (66.0 K), egg albumin (45.0 K), pepsin (34.7 K), trypsinogen (24.0 K), bovine milk β -lactoglobulin (18.4 K) and Lysozyme (14.3 K).

pathway. *S*-Adenosyl-L-homocysteine was a competitive inhibitor with respect to AdoMet, the K_i values toward the PE, PME and PDE-dependent reactions being 11.8, 8.7 and 4.8 μ M, respectively.

Several investigators have purified enzymes catalyzing the PE-*N*-methylation pathway, i.e. from *Agrobacterium tumefaciens*,⁴⁾ *Neurospora crassa*¹⁰⁾ and rat liver microsomes,^{6,11)} but these enzymes were only solubilized or partially purified preparations. Therefore, this is the first report of an enzyme involved in the PE-*N*-methylation pathway being purified to a homogeneous state. One of our interests is

how many enzymes participate in the PE-*N*-methylation pathway in cells of *Zymomonas mobilis*. The PE *N*-methyltransferase described here catalyzes all of the three-step methylation from PE to PC, which is quite different from the finding that two enzymes take part in the PE-*N*-methylation pathways of *Neurospora crassa*,²⁾ *Saccharomyces cerevisiae*³⁾ and bovine adrenal medulla.¹²⁾

A detailed description of this work will be published elsewhere.

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