A spectrophotometric method for the assay of cytidine 5′-diphospho-1,2-diacyl-*sn*-glycerol-dependent enzymes of phospholipid metabolism

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Summary Cytidine 5′-diphospho-1,2-diacyl-*sn*-glycerol (CDP-diglyceride) hydrolase, CDP-diglyceride:L-serine *O*-phosphatidyltransferase, and CDP-diglyceride:*sn*-glycero-3-phosphate phosphatidyltransferase all release CMP from their liponucleotide substrate, CDP-diglyceride. We have developed a spectrophotometric assay for these enzymes using CMP kinase, pyruvate kinase, and lactate dehydrogenase to couple the release of CMP with the oxidation of NADH. The assay for each of the phospholipid-dependent enzymes was found to be linear both with time and with enzyme concentration. The assay should prove useful for continuous monitoring of enzymatic activity, determination of initial rates of reaction, and detailed kinetic analysis of these enzymes. Since several enzymes and substrates are used in the coupled assay system, the method is limited to analysis of partially purified preparations lacking competing activities.

Supplementary key words CMP · phosphatidylserine synthase · phosphatidylglycerophosphate synthase · CDP-diglyceride hydrolase

Metabolic enzymes involving lipid substrates and products are routinely assayed by single-point determinations using radioactive substrates. Such assays are unsuitable for the detailed kinetic analysis of these enzymes because initial rates of reaction are not necessarily measured. Therefore the development of assays that continuously monitor the formation of product and that are generally applicable to several enzymes of lipid metabolism would be desirable.

In bacterial systems there are at least three enzymes (**Fig. 1**) that are dependent on cytidine 5′-diphospho-1,2-diacyl-*sn*-glycerol (CDP-diglyceride) and release CMP as one of the products, i.e., CDP-diglyceride hydrolase, CDP-diglyceride:L-serine *O*-phosphatidyltransferase (PS synthase, EC 2.7.8.8) and CDP-diglyceride:*sn*-glycero-3-phosphate phosphatidyltransferase (PGP synthase, EC. 2.7.8.5). From *Escherichia coli* the first enzyme has been purified about 1000-fold (1), while the other two enzymes have been obtained in near homogeneous form (2, 3). The PGP synthase of *Bacillus licheniformis* has been purified approximately 500-fold (4).

We have developed a recording spectrophotometric assay applicable to the quantitation of the above enzymatic activities. The assay is dependent on the release of CMP which is then coupled via reactions 5–7 (below) using CMP kinase, pyruvate kinase, and lactate dehydrogenase, respectively, to the ultimate formation of NAD^+ which is measured by the decrease in absorbance at 340 nm. Such a coupled assay system allows the continuous monitoring of product formation and the determination of initial reaction rates. The use of this assay for enzymes involved in eukaryotic phospholipid metabolism is also discussed.

5) $\text{CMP} + \text{ATP} \xrightarrow[Mg^{2+}]{\text{CMPK}} \text{CDP} + \text{ADP}$

6) $\text{ADP} + \text{phosphoenolpyruvate} \xrightarrow[Mg^{2+},K^+]{\text{PK}} \text{ATP} + \text{pyruvate}$

7) $\text{pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{LDH}} \text{lactate} + \text{NAD}^+$

Materials and methods

All chemicals were reagent grade or better. Phosphoenolpyruvate, L-serine, ATP, CMP, NADH, pyruvate kinase, and lactate dehydrogenase were purchased from Sigma Chemical Co., St. Louis, MO. *sn*-Glycero-3-phosphate was purchased from Calbiochem, San Diego, CA. Triton X-100 was a product of

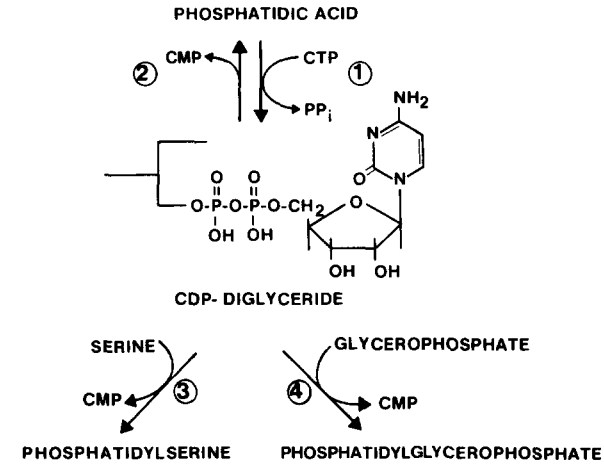

Fig. 1. Metabolism of phospholipids in bacteria as catalyzed by the following enzymes: *1*, CTP:phosphatidic acid cytidylyltransferase; *2*, CDP-diglyceride hydrolase; *3*, PS synthase; *4*, PGP synthase.

Abbreviations: CDP-diglyceride, cytidine 5′-diphospho-1,2-diacyl-*sn*-glycerol; PGP, 3-*sn*-phosphatidyl-1′-*sn*-glycero-3′-phosphate; PS, 3-*sn*-phosphatidyl-L-serine; CMPK, CMP kinase; PK, pyruvate kinase; LDH, lactate dehydrogenase.

Rohm and Haas, Philadelphia, PA. Bovine serum albumin was purchased from Miles Laboratories, Kankakee, IL. Radiochemicals were purchased from Amersham/Searle, Arlington Heights, IL. CDP-diglyceride was prepared as previously described (3) from egg lecithin. [5-^{3}H]CDP-diglyceride and *sn*-[2-^{3}H]-glycero-3-phosphate were prepared as described by Larson, Hirabayashi, and Dowhan (4). *Escherichia coli* B (¾ log), grown on rich medium, was purchased as the frozen cell paste from Grain Processing, Muscatine, IA. Protein was determined as described by Lowry et al. (5).

Enzymes. CDP-diglyceride hydrolase was partially purified from *E. coli* B as described previously (1) through the DEAE–cellulose step. The enzyme had a sp act of 790 U/mg. PS synthase was purified to homogeneity as described by Larson and Dowhan (2) from *E. coli* strain RA324 isolated by Raetz, Larson, and Dowhan (6). The enzyme had a sp act of 33,000 U/mg. PGP synthase was partially purified from *B. licheniformis* as described by Larson et al. (4) and had a sp act of 360 U/mg.

Spectrophotometric assays. CDP-diglyceride hydrolase activity was determined by measuring the release of CMP from CDP-diglyceride at 30°C by following the decrease in absorbance at 340 nm on a recording Gilford 240 spectrophotometer in the presence of excess NADH, coupling enzymes, and their substrates. The reaction mixture contained 50 mM Tris-HCl (pH 7.5), 0.1 M KCl, 10 mM $MgCl_2$, 1 mg/ml bovine serum albumin, 0.4 mM CDP-diglyceride, 1.6 mM Triton X-100, 1 mM ATP, 1 mM phosphoenolpyruvate, 0.3 mM NADH, 50 units of partially purified CMP kinase (see Results), 100 units of pyruvate kinase, 100 units of lactate dehydrogenase, and an appropriate dilution of CDP-diglyceride hydrolase in a total volume of 0.25 ml. The coupled system was assembled with the exception of CDP-diglyceride hydrolase. A short incubation was required to eliminate trace amounts of CMP or ADP in the reaction mixture. After absorbancy at 340 nm was stabilized, the reaction was initiated by the addition of hydrolase.

PS synthase activity was measured in a similar assay mixture. The CDP-diglyceride concentration was reduced to 0.2 mM and 1 mM L-serine was included. PGP synthase activity was measured in an assay mixture similar to that for the PS synthase reaction with the exception of replacing L-serine with 0.5 mM *sn*-glycero-3-phosphate and increasing the concentrations of Triton X-100 and $MgCl_2$ to 6 mM and 50 mM, respectively. CMP kinase activity was measured spectrophotometrically using pyruvate kinase and lactate dehydrogenase as coupling enzymes in an assay mixture similar to that described above for the CDP-diglyceride hydrolase that included 0.5 mM CMP and excluded CDP-diglyceride. A unit of enzymatic activity is defined as the amount of enzyme that catalyzes the formation of 1 nmol of product per min under the assay conditions described above. The specific activity is defined as units per mg of protein.

Radioactive assays. CDP-diglyceride hydrolase activity was measured as previously described (1) using 0.4 mM [5-^{3}H]CDP-diglyceride (120 cpm/nmol). PS synthase activity was measured as previously described (2) using 1 mM L-[3-^{3}H]serine (190 cpm/nmol). PGP synthase activity was measured as previously described (4) using 0.5 mM *sn*-[2-^{3}H]glycero-3-phosphate (130 cpm/nmol).

Results

Purification of CMP kinase. CMP kinase is the only coupling enzyme that is not commercially available and requires a partial purification. The enzyme was partially purified from *E. coli* B as described by Brynolf (7). Cells were broken using a French pressure cell and the crude enzyme preparation was fractionated by treatments with streptomycin sulfate and ammonium sulfate as previously described (7). The preparation was then subjected to column chromatography on DEAE–cellulose and phosphocellulose. Brynolf's procedure was modified in that 0.2 mg/ml of bovine serum albumin was added to the equilibration and elution buffers for the phosphocellulose step. After phosphocellulose chromatography the enzyme was concentrated using an Amicon ultrafiltration device equipped with a PM 10 filter. The use of bovine serum albumin for the phosphocellulose column resulted in a 36% recovery from the previous DEAE–cellulose step as compared to an 8% recovery without bovine serum albumin. Approximately an 8-fold increase in yield is obtained by using the above modification of Brynolf's procedure. About 220,000 units of CMP kinase activity may be obtained from 450 g of *E. coli* cell paste (sufficient for 4,400 assays).

The CMP kinase preparation, although not homogeneous, was judged free of competing kinase and phosphatase activities and suitable for use in the coupled assay. The presence of a CDP kinase or phosphatase activity in the CMP kinase preparation should result in the formation of ADP in the presence of ATP and CDP while an ATP phosphatase activity should result in ADP formation in the presence of only ATP. The lack of ADP formation was verified by coupling the above reactions to NAD^+ formation via reactions *6* and *7*. The lack of competing CMP phosphatase and ADP phosphatase activities in the CMP kinase preparation was verified by the stoi-

chiometric relationship between CMP added and NAD^+ produced (via reactions 5–7) or ADP added and NAD^+ produced (via reactions 6 and 7), respectively.

Spectrophotometric assay. The reaction rates of CDP-diglyceride hydrolase, PS synthase, and PGP synthase using the coupled spectrophotometric assay are shown in **Fig. 2.** An initial lag period (not shown) of about 40–60 sec was followed by a constant rate of NAD^+ formation which was found to be linear for at least 10 min. The reaction was also found to be linear with enzyme concentration, indicating that initial rates of reaction were being measured. As little as 0.1 unit of enzymatic activity may be determined using this coupled assay mixture. Radioactive assays are potentially more sensitive since they are usually limited only by the specific activity of the substrates. The activities of CDP-diglyceride hydrolase, PS synthase, and PGP synthase were in good agreement with the activities as determined by the radioactive assays (**Table 1**). The variability, as indicated by the standard deviation, was consistently less using the spectrophotometric assay.

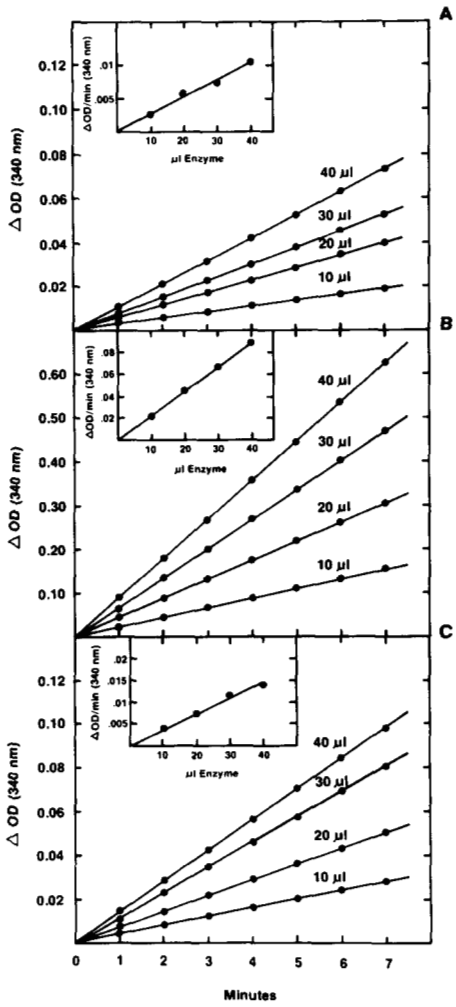

Fig. 2. Time course and enzyme concentration dependence of the spectrophotometric assay. Enzymatic activity in each preparation was determined by the radioactive assays. *A*, CDP-diglyceride hydrolase containing 10 U/ml of activity; *B*, PS synthase containing 88 U/ml of activity; *C*, PGP synthase containing 16 U/ml of activity. Each point was calculated from a continuous recorder tracing monitoring the disappearance of NADH. Inserts are replots of the slopes for each time course plot.

TABLE 1. Comparison of coupled spectrophotometric assay with radioactive assay[a]

Enzyme	Spectrophotometric	Radioactive
	U/ml	
CDP-diglyceride hydrolase	11 ± 0.2	10 ± 1.5
PS synthase	88 ± 0.7	88 ± 5.4
PGP synthase	15 ± 0.7	16 ± 1.5

[a] Enzymatic activity was measured as described in Materials and Methods. Each value is an average of two determinations ± SD.

Discussion

The kinetic analysis of the CDP-diglyceride-dependent enzymes of bacterial phospholipid metabolism using single-point radioactive assays requires a great deal of effort to ensure that initial velocities are being measured. Such assays are most useful when dealing with crude enzyme preparations where a more detailed kinetic analysis would be of little interest due to an inability to control the reaction components. With the availability in recent years of highly purified preparations of enzymes involved in phospholipid metabolism, a need for a rapid, continuous assay method has arisen in order to carry out a detailed kinetic analysis of these enzymes. We have developed a spectrophotometric assay for monitoring the zero order kinetics of these enzymes utilizing CMP kinase, pyruvate kinase, and lactate dehydrogenase as coupling enzymes. The CMP kinase used in this assay shows equal activity with CMP and dCMP, slight activity with UMP and dUMP, and no activity with other nucleoside monophosphates (7).

Crude preparations of the CDP-diglyceride-dependent enzymes used in this study were unsuitable for assay by this method and required at least a partial purification. The presence of competing activities was usually evidenced by lack of good agreement between the spectrophotometric assay and an assay that measured product formation directly (such as the formation of a characterizable radioactive product). The presence of competing phosphatase and kinase activities was assessed in the same manner as for the CMP kinase preparation. It was necessary to purify CMP kinase through the phosphocellulose step to eliminate the interfering nucleoside diphosphate

kinase and phosphatase activities. The CDP-diglyceride hydrolase required approximately a 100-fold purification before assay using the spectrophotometric method was possible. However, small background rates of activity due to contaminating activities may be corrected for by using a zero substrate blank. In addition the coupled assay system for measuring CMP kinase activity is convenient and an improvement over the cumbersome radioactive assay involving high voltage paper electrophoresis used by Brynolf (7).

CDP-diglyceride hydrolase (1), PS synthase (2), and PGP synthase (3, 4) all require the nonionic surfactant Triton X-100 for solubilization and purification. Triton X-100 also functions as a solubilizing agent for the phospholipid substrate, CDP-diglyceride, and has a stimulatory effect on enzymatic activity. The presence of Triton X-100 in the coupled assay mixture does not interfere with the measurement of activity. Presumably other nonionic detergents, and even ionic detergents, would also be compatible with this coupled assay system, making possible the extension of this assay to other membrane-associated CMP-releasing enzymes.

CMP kinase is equally active with both dCMP and CMP (7); therefore, the coupled assay should be equally effective in the measurement of dCDP-diglyceride-dependent activities. We have been able to use dCDP-diglyceride as substrate for the PS synthase in a coupled assay system identical to that reported here. In addition to the three bacterial enzymes discussed in this report, there are several mammalian enzymes of phospholipid metabolism that release CMP as a product (8), i.e., CDP-choline: 1,2-diacyl-*sn*-glycerol cholinephosphotransferase, CDP-ethanolamine:1,2-diacyl-*sn*-glycerol ethanolaminephosphotransferase, CDP-diglyceride:inositol phosphatidyltransferase, and cardiolipin synthase. Providing the partially purified preparations of these enzymes contain only small amounts of interfering activities, the coupled spectrophotometric assay should prove applicable in these cases. Finally, the CTP:phosphatidic acid cytidylyltransferase (8) could be assayed by coupling CDP-diglyceride formation to the CMP kinase using partially purified CDP-diglyceride hydrolase. Therefore, this assay system should be useful in kinetic analysis of a large number of enzymes involved in phospholipid metabolism.

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