Expression, Purification, and Characterization of CTP:Glycerol-3-phosphate Cytidylyltransferase from *Bacillus subtilis* *

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*Bacillus subtilis* contains the gene for CTP:glycerol-3-phosphate cytidylyltransferase, which is involved in biosynthesis of the major teichoic acid of the *B. subtilis* cell wall. When this gene was expressed in *Escherichia coli* under the control of the T7 promoter, the glycerol-3-phosphate cytidylyltransferase accumulated to a level of about 15% of cellular protein. The expressed glycerol-3-phosphate cytidylyltransferase was purified to homogeneity by ion-exchange chromatography, gel filtration, and affinity chromatography on blue Sepharose. Approximately 47 mg of pure enzyme was obtained from a 660-ml culture. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis indicated that the subunit molecular weight of the purified enzyme was about 15,000. The molecular weight of the native enzyme was found to be 30,900 by gel filtration analysis, suggesting that the native enzyme is a homodimer. The pH optimum was very broad, from 6.5 to 9.5, and the enzyme was stable at alkaline conditions. A divalent cation, either Co**, Mg**, Mn**, or Fe**, was required for enzyme activity. *Km* values for CTP and glycerol 3-phosphate were 3.85 and 2.23 mM, respectively, and the *Vmax* was 185 units/mg of protein. Initial rate studies and product inhibition patterns indicated that the enzyme catalyzes the reaction by means of a rapid equilibrium random order mechanism. The availability of large amounts of glycerol-3-phosphate cytidylyltransferase will facilitate enzymological and structural studies on this model cytidylyltransferase.

Teichoic acids are major constituents of the cell walls of most Gram-positive bacteria. Teichoic acids are a chemically diverse group of anionic polymers containing polyols and/or sugar residues linked by phosphodiester bonds (1). Poly(glycerol phosphate) is the major cell wall teichoic acid found in *Bacillus subtilis*, and most of the genes concerned with synthesis of poly(glycerol phosphate) are organized in two divergently transcribed operons denoted *tagABC* and *tagDEF* (2). The first gene of the *tagDEF* operon, *tagD*, encodes glycerol-3-phosphate cytidylyltransferase (GCTase).

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EXPERIMENTAL PROCEDURES

MATERIALS—*B. subtilis* BR151, a derivative of strain 168 and the strain used for the isolation of GCTase genomic DNA, was obtained from Dr. Don B. Clewell, University of Michigan. pbLuescript II KS (+/−) phagemid and *Escherichia coli* XL1-blue cells were obtained from Stratagene. The PET-11a vector and *E. coli* HMS174 (DE3)plac857 were purchased from Novagen. Oligonucleotides were synthesized by the DNA Synthesis Core Facility, University of Michigan. Isopropyl-1-thio-β-D-galactopyranosidase (IPTG), 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), CTP, L-α-glycerophosphate, CDP-choline, DEAE-Sepharose CL-6B, Sephadex G-100, and Reactive Red 120-agarose were supplied from Sigma. Blue Sepharose CL-6B was purchased from Pharmacia LKB Biotechnology Inc. CDP-[methyl-14C]choline and L-[U-14C]glycerol phosphate were from Amersham Corp. NZ broth, restriction endonucleases, and T4 DNA ligase were obtained from Life Technologies, Inc.

Cloning of the GCTase Gene in the PET-11a Vector—The general approach was to obtain the GCTase gene by polymerase chain reaction (PCR) techniques; clone the gene in pbLuescript; transfer the gene to the T7 expression vector, PET-11a; then express the clone in *E. coli*. In the PET-11a vector the expressed protein replaces the coding region of the T7 gene 10 protein. General cloning procedures used for construction of the vectors were as described by Sambrook...
sequences of the yeast contained 22 nucleotides of GCTase plus a PstI site for insertion into the glycerol-3-phosphate cytidylyltransferase plasmid and an
line cytidylyltransferases.
black background. Alignment was done using the program GAP of the
by changing a T to C of 32nd base in the oligonucleotide; this had no
was used for the 5' end of the coding sequence. This oligonucleotide
was used as the primer at
the 3' end. This oligonucleotide contained antisense sequence com-
tained, in a volume of 100
400 base pairs. This fragment was digested with PstI and XbaI and
from the pBluescript vector and inserted into the PET-lla vector
used to transform competent
HMS174(DE3)pLysS cells harboring pETGCT were picked from cells
of chloramphenicol and used to inoculate up to 1-liter cultures (NZ medium containing 50 pg/ml of ampicillin and 25 pg/ml of chloramphenicol). The large cultures were
grown at 37°C until the absorbance at 690 nm reached a value of about 1.0. IPTG was then added to a concentration of 1 mM, and the culture was grown for 5 h at 37°C.

**Purification of Recombinant GCTase**—All purification procedures were carried out at 4°C. The cells from an IPTG-induced 600-ml culture were harvested by centrifugation at 5,000 x g for 40 min and washed once with 66 ml of 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM dithiothreitol (TED). After centrifugation, cells were resuspended in 16 ml of the above buffer and disrupted by passage three times through a French press at 1,000 psi. The lysate was then centrifuged at 15,000 x g for 30 min at 4°C. The supernatant fraction was loaded onto a DRAE-Sepharose CL-6B column (2.5 x 14 cm) equilibrated in TED buffer containing 0.1 M NaCl. The column was washed with 350 ml of the same buffer followed by a linear salt gradient from 0.1 to 0.3 M NaCl (500 ml) at a flow rate of 45 ml/h. Fractions of 5 ml were collected and GCTase activity was assayed. The amount of protein was monitored by measuring the absorbance at 280 nm. The active fractions (47 ml) were pooled and concentrated to 7 ml by dialysis against PEG 20,000.

The concentrated sample was applied to a Sephadex G-100 column (2.5 x 110 cm), equilibrated in TED buffer containing 0.2 M NaCl, and eluted with the same buffer at a flow rate of 5 ml/h. Fractions of 5 ml were collected and assayed for GCTase activity. Active fractions (59 ml) were pooled and desalted by dialysis against TED buffer and then concentrated to 5 ml by dialysis against PEG 20,000. This sample was applied to a blue Sepharose CL-6B column (1.5 x 11 cm) equilibrated with TED buffer at a flow rate of 20 ml/h. After washing with the column volumes of the same buffer, the enzyme was eluted with 1 mM CTP in TED buffer at a flow rate of 20 ml/h. Fractions of 5 ml were collected and the enzyme activity was assayed. Active fractions were collected and dialyzed against TED buffer. The enzyme was concentrated by dialysis against PEG 20,000 and then stored at -80°C. Amino-terminal protein sequencing analysis was performed by the Protein and Carbohydrate Structure Core Facility, University of Michigan.

**Assay of GCTase Activity**—GCTase activity was assayed by a modification of the CTTase assay procedure (16). GCTase activity was measured in a reaction mixture containing 20 mM Tris-HCl, pH 8.0, 1 mM MgCl₂, 5 mM CTP, 4 mM U-[¹⁴C]glycerol 3-phosphate (0.25 µCi/mol) and up to 60 mg of protein in a final volume of 50 ml. After incubation for 10 min at 37°C, the reaction was terminated by the addition of 10% (v/v) trichloroacetic acid. The mixtures were kept on ice for 5 min and 0.5 ml of a freshly prepared suspension of charcoal in water, 10 mg/ml, was added. The suspension was kept on ice at least 30 min and centrifuged in a microcentrifuge for 2 min. The supernatant fluid was removed, and the charcoal pellet was washed twice by adding 0.5 ml of water, vortexing, and centrifuging for 2 min. The washed charcoal was then suspended in 0.5 ml of 10% acetic acid and mixed with 5 ml of scintillation mixture. Scintillation counting was performed in a Beckman LS-1701 liquid scintillation counter. To determine the binding efficiency of the nucleotide-containing product to the charcoal, control tubes containing 0.4 ml CDP-[¹⁴C]choline (0.25 Ci/mol) were carried through the assay. The amount of CDP-glycerol produced during the assay was calculated from the specific radioactivity of the [¹⁴C]glycerol 3-phosphate used, a sample of which was counted with charcoal under identical conditions as the assay mixtures, and from the recovery of the CDP-choline. One unit of enzyme activity was defined as 1 amol of CDP-glycerol produced/min.

**Gel Electrophoresis**—SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 15% slab gels as described by Laemmli (15). Equal volumes of CTTase or GCTase were run on each gel. Gels were stained with Coomassie Brilliant Blue R-250. Phosphorylation b (Mᵦ = 97, 400), bovine serum albumin (Mᵦ = 66, 200), ovalbumin (Mᵦ = 45, 000), carbonic anhydrase (Mᵦ = 31, 000), soybean trypsin inhibitor (Mᵦ = 21, 500), and lysozyme (Mᵦ = 14, 400) were used as molecular weight standards.

**Protein Concentration Determination**—Protein concentration was determined by the Bradford method (18) using reagents purchased from Bio-Rad. Bovine serum albumin was used as a standard.

**DNA Sequencing**—DNA sequencing was performed by the dye-oxynucleotide termination method (19) using T7 DNA polymerase.

Fig. 1. Alignment of the predicted amino acid sequence of the glycerol-3-phosphate cytidylyltransferase (Bsu) with the sequences of the yeast (Sce) and rat liver (Rat) phosphocholine cytidylyltransferases. Identical sequences are shown with a black background. Alignment was done using the program GAP of the Genetics Computer Group Software Package.

et al. (13). All plasmids were propagated in E. coli XL1-blue cells. The primer TTTCTGCCAGCATGAAAAGTTTAGCATACAG was used for the 5' end of the coding sequence. This oligonucleotide contained 25 nucleotides of GCTase plus a PstI site for insertion into the pBluescript vector and an NdeI site at the initiation methionine codon. In addition, an NdeI site in the coding region was eliminated by changing a T to C of 32nd base in the oligonucleotide; this had no effect on the amino acid sequence. The oligonucleotide TTTTCTAAGGATCTTTAAAAATTTAACGCAA was used as the primer at the 3' end. This oligonucleotide contained antisense sequence complementary to 24 bases of the 3' end of the GCTase coding region plus an XbaI site and a BamHI site for insertion into the pBluescript vector and the pET-11a vector, respectively. The PCR reaction mixture contained, in a volume of 100 µl, 50 mM KCl, 10 mM Tris-HCl, pH 8.4, 0.1 mg/ml gelatin, 4.5 mM MgCl₂, 0.2 mM of each deoxynucleoside triphosphates, 5 µM of each oligonucleotide, 272 µg of template DNA, and 2.5 units of Taq DNA polymerase. The PCR conditions used were 94°C, 45 s; 48°C, 45 s; 72°C, 2 min for 30 cycles. DNA amplification was done using a Perkin-Elmer Cetus DNA Thermal Cycler. The PCR product was a single band of about 400 base pairs. This fragment was digested with PstI and XbaI and then inserted into the pBluescript KS phagemid vector which had been digested with the same enzymes. The fidelity of the inserted sequence was confirmed by DNA sequence analysis of the recombinant pBluescript vector (pBGCT). The GCTase gene was then excised from the pBluescript vector and inserted into the pET-11a vector using the NdeI site at the initiator methionine codon for the T7 gene 10 protein (14, 15). The construct (pETGCT) was then used to transform competent E. coli HMS174(DE3)pLysS cells. Expression of GCTase from pETGCT—Single colonies from E. coli HMS174(DE3)pLysS cells harboring pETGCT were picked from cells plated on NZ plates containing 50 µg/ml of ampicillin and 25 µg/ml of chloramphenicol. The 5-ml cultures were grown overnight at 37°C and then used to inoculate up to 1-liter cultures (NZ medium containing 50 µg/ml of ampicillin and 25 µg/ml of chloramphenicol). The large cultures were
**RESULTS AND DISCUSSION**

**Expression of Recombinant GCTase**—GCTase was cloned by PCR from the genomic DNA of *B. subtilis* BR151. The entire nucleotide sequence of the GCTase gene was the same as the sequence previously reported. For producing GCTase in *E. coli*, we used the T7 expression system. The entire coding region of the GCTase gene was put under the control of the T7 promoter and translation signals. The gene for T7 RNA polymerase, engineered into the bacterial chromosome under lacUV5 control, was induced by the addition of IPTG. The time course of induction of GCTase is shown in Fig. 2. Expression of GCTase was induced rapidly after the addition of IPTG and did not increase further after 3 h.

**Purification of Recombinant GCTase**—GCTase from an IPTG-induced culture was purified from the cleared lysate through ion-exchange chromatography on DEAE-Sepharose, gel filtration on Sephadex G-100, and affinity chromatography on blue Sepharose. The activity bound to DEAE-Sepharose in TED buffer, pH 8.0, and was eluted as a single peak between 150 and 200 mM NaCl (Fig. 3A). The yield in this step was 91%. The GCTase was then chromatographed on Sephadex G-100 (Fig. 3B). After this step, the enzyme was almost pure as determined by SDS-PAGE; only one minor contaminant, the molecular weight of which was about 31,000, appeared when 20 µg of sample was analyzed (Fig. 4). This contaminant was removed by affinity chromatography on blue

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**Fig. 2. Expression of the recombinant GCTase in the pET vector.** A, time course of induction of GCTase in a culture of *E. coli* harboring the pETGCT plasmid. Samples (1.5 ml) from a 250-ml culture, grown as described under “Experimental Procedures,” were taken at various time intervals. The absorbance of the culture at 600 nm reached about 1.0 and further grown with (+) or without (−) 0.4 mM of IPTG. The cells were harvested at the indicated time, sonicated, and centrifuged. The cleared lysate was then subjected to SDS-PAGE and stained with Coomassie Blue. The position of molecular weight standards is shown on the right of the figure.

**Fig. 3. Purification of GCTase.** A, DEAE-Sepharose CL-6B column chromatography. The *E. coli* lysate from 660 ml of culture was chromatographed on a column of DEAE-Sepharose CL-6B (2.5 × 14 cm) and eluted with a linear gradient of NaCl (−−−) at a flow rate of 45 ml/h as described under “Experimental Procedures.” Fractions of 5 ml were collected and assayed for GCTase activity (■) and absorbance at 280 nm (▲). B, Sephadex G-100 column chromatography. The active fractions from the DEAE-Sepharose CL-6B chromatography step were pooled and chromatographed on a Sephadex G-100 column (2.5 × 110 cm) and eluted at a flow rate of 9 ml/h as described under “Experimental Procedure.” Fractions of 5 ml were collected and assayed for GCTase activity (■) and absorbance at 280 nm (▲).
was 50 °C, although the enzyme was not stable for longer than the presence of MgCl₂. Whereas the addition of monovalent cations (K⁺, Na⁺, or Li⁺) to the reaction mixture had no effect, the divalent cations Ca²⁺, Cu²⁺, and Zn²⁺ strongly inhibited enzyme activity (92, 100, 97, 100, and 98%, respectively).

Substrate Specificity—When dCTP, ATP, and GTP were examined as possible substrates, only dCTP was active as a substrate. The activity with 5 mM dCTP was 95% of the activity with 5 mM CTP. The $K_m$ and $V_{max}$ values for dCTP were 4.00 mM and 250 units/mg of protein, respectively, which were very similar to those for CTP.

Kinetic Properties of the GCtase—Initial velocity measurements were made with five concentrations of CTP ranging from 0.125 to 2 mM, and six concentrations of glycerol 3-phosphate ranging from 0.125 to 4 mM. The double-reciprocal plots are shown in Figs. 7 and 8. The kinetic constants were determined from secondary plots of the slopes and intercepts versus the reciprocal of the fixed substrate concentration (Fig. 7 and 8). The $K_m$ values for CTP and glycerol 3-phosphate were 3.85 and 3.23 mM, respectively. The secondary plots for both substrates extrapolated to the same $V_{max}$ value of 185 units/mg protein.

Although the families of double-reciprocal lines appeared nearly parallel, and parallel lines would be consistent with a ping-pong mechanism, product inhibition studies were indicative of a rapid equilibrium random order mechanism. Inhibition of the GCtase-catalyzed reaction was observed when either PPi, or CDP-glycerol was present. Inhibition by PPi produced competitive patterns with CTP or glycerol 3-phosphate as the varied substrates (Fig. 9). The apparent inhibition constants for PPi were determined by a fit of the results of Fig. 9 to following equation (20, 21),

$$v = \frac{V[A]}{K(1 + [I]/K_m) + [A]}$$

where $K$ is the Michaelis constant for substrate, $[I]$ is the concentration of inhibitor, and $K_m$ is the dissociation constant for inhibitor.

When CTP was used as the varied substrate (Fig. 9A), the replot of slope versus inhibitor showed a parabola (data not presented).

### Table 1

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Protein Activity</th>
<th>Specific activity</th>
<th>Purification Yield</th>
</tr>
</thead>
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<td></td>
<td>Protein [μg/min]</td>
<td>Activity [μg/min/mg]</td>
<td>Fold</td>
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<tr>
<td>Cleared lysate</td>
<td>336.0</td>
<td>4,579</td>
<td>13.6</td>
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<tr>
<td>DEAE-Sepharose</td>
<td>84.6</td>
<td>4,170</td>
<td>49.3</td>
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<tr>
<td>Sephadex G-100</td>
<td>47.2</td>
<td>4,027</td>
<td>85.4</td>
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<tr>
<td>Blue-Sepharose</td>
<td>46.4</td>
<td>3,962</td>
<td>85.7</td>
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</tbody>
</table>

The molecular weight of denatured GCtase, determined by 15% SDS-PAGE, was 30,900 (Fig. 4). This is close to the molecular weight, calculated from the predicted protein sequence, of 15,271. The molecular weight of native GCtase, determined by gel filtration, was 30,900 (Fig. 5). Thus it appears that GCtase is a homodimer. GCtase in crude extracts also appears to be a dimer (3).

GCtase activity had a very broad optimum pH range from 6.5 to 9.5, and the enzyme was stable at alkaline pH (Fig. 6). When the enzyme was assayed at temperatures ranging between 20 and 70 °C, the temperature optimum for activity was 50 °C, although the enzyme was not stable for longer than 30 min at temperatures over 40 °C (data not shown). The pure enzyme could be stored at −80 °C for at least 7 months without loss of activity. About 20% of the activity was lost after a month at −20 °C.

We examined whether GCtase is completely dependent on metal ions for its activity (Table II). A divalent cation was required for activity, and either Co₂⁺, Mg²⁺, Mn²⁺, or Fe²⁺ was suitable. The effect of cations was also examined in the presence of MgCl₂. Whereas the addition of monovalent cations (K⁺, Na⁺, or Li⁺) to the reaction mixture had no effect, the divalent cations Cd²⁺, Hg²⁺, Sn²⁺, Cu²⁺, and Zn²⁺ strongly inhibited enzyme activity (92, 100, 97, 100, and 98%, respectively).
**Fig. 6. Effect of pH on the activity (A) and stability (B) of GCTase.**
The buffers used were 100 mM sodium phosphate buffer, pH 6-7, 100 mM Tris-HCl buffer, pH 7-9, and glycine-NaOH buffer, pH 9-10. In B, the enzyme was preincubated at the given pH values at 37 °C for 4 h and then assayed in the assay condition described under "Experimental Procedures."

**TABLE II**

<table>
<thead>
<tr>
<th>Metal ions</th>
<th>Relative activity</th>
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<tr>
<td></td>
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<tr>
<td>None</td>
<td>0 100</td>
</tr>
<tr>
<td>NaCl</td>
<td>0 103</td>
</tr>
<tr>
<td>LiCl</td>
<td>0 99</td>
</tr>
<tr>
<td>KCl</td>
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<td>CaCl₂</td>
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</tr>
<tr>
<td>MgCl₂</td>
<td>9 8</td>
</tr>
<tr>
<td>CoCl₂</td>
<td>100 107</td>
</tr>
<tr>
<td>HgCl₂</td>
<td>0 0</td>
</tr>
<tr>
<td>CdCl₂</td>
<td>92 104</td>
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<td>72 102</td>
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<tr>
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<td>CuSO₄</td>
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</tr>
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<td>MgSO₄</td>
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<tr>
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<td>2 2</td>
</tr>
<tr>
<td>AgNO₃</td>
<td>0 97</td>
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</table>

shown), and an inhibition constant was not calculated. When glycerol 3-phosphate was used as the varied substrate (Fig. 9B) the apparent inhibition constant for PP₁ was calculated to be 0.51 mM. CDP-glycerol also behaved as a competitive inhibitor when either CTP or glycerol 3-phosphate was used as the varied substrate (Fig. 10). The apparent inhibition constants for CDP-glycerol with respect to CTP and glycerol 3-phosphate were calculated to be 1.63 and 2.38 mM, respectively. Each product therefore, displayed competitive inhibition with respect to each substrate. This behavior is indicative of a rapid equilibrium random order mechanism (22). The apparently parallel double-reciprocal plots are consistent with such a mechanism if the binding of one substrate decreases the affinity of the enzyme for the other substrate, as has been observed previously for a Neurospora protein kinase (23).

**Comparison of Properties of GCTase and CCTase—**In addition to the apparent homologies in protein sequence between GCTase and the CCTase, there are some similarities in the properties of the enzymes. For example, the pH optimum of purified rat CCTase is quite broad, from pH 6.5 to 8 (12), although not extending into the basic range as far as GCTase. In addition, like GCTase, CCTase from rat liver is a homodimer (24, 25). The Kᵥ values of rat CCTase for CTP and phosphocholine are rather high, 0.29 and 0.14 mM, respectively (26), although these are only about 5-10% of the values of GCTase for CTP and glycerol 3-phosphate. CCTase from yeast has not been purified, and true Kᵥ values have not been reported. Rat CCTase uses both CTP and dCTP as substrate (27), similar to GCTase. Further comparisons of the properties of the these cytidylyltransferases await further analyses of the kinetic and mechanistic properties of the enzymes. The ability to express large amounts of GCTase as a model cyti-
FIG. 8. Initial rate analysis of the GCTase with CTP as the variable substrate. A, double-reciprocal plot for the GCTase-catalyzed reaction with CTP as the variable substrate and a fixed concentration of glycerol 3-phosphate of: 0.125 mM (△), 0.25 mM (▲), 0.5 mM (◆), 1 mM (◇), 2 mM (□), and 4 mM (■). B, secondary plots of slopes (■) and intercepts (△) from the primary plots versus the reciprocal substrate, glycerol 3-phosphate, concentrations.

FIG. 9. Competitive product inhibition by PPI with respect to CTP (A) and glycerol 3-phosphate (B) as the variable substrates. The PPI concentrations were: 0 mM (■), 1.25 mM (◇), 2.5 mM (○), 5 mM (▲), 7.5 mM (◆), and 10 mM (△).

dylyltransferase will facilitate not only enzymological analysis of the enzyme, but should allow for detailed structural studies by NMR or x-ray crystallography.

REFERENCES
Purification of Glycerol-3-phosphate Cytidylyltransferase