Characterization of cytidylyltransferase enzyme activity through high performance liquid chromatography

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ABSTRACT

The cytidylyltransferases are a family of enzymes that utilize cytidine 5'-triphosphate (CTP) to synthesize molecules that are typically precursors to membrane phospholipids. The most extensively studied cytidylyltransferase is CTP:phosphocholine cytidylyltransferase (CCT), which catalyzes conversion of phosphocholine and CTP to cytidine diphosphocholine (CDP-choline), a step critical for synthesis of the membrane phospholipid phosphatidylcholine (PC). The current method used to determine catalytic activity of CCT measures production of radiolabeled CDP-choline from ^{14}C -labeled phosphocholine. The goal of this research was to develop a CCT enzyme assay that employed separation of non-radioactive CDP-choline from CTP. A C18 reverse phase column with a mobile phase of 0.1 M ammonium bicarbonate (98%) and acetonitrile (2%) (pH 7.4) resulted in separation of solutions of the substrate CTP from the product CDP-choline. A previously characterized truncated version of rat CCT α (denoted CCT α 236) was used to test the HPLC enzyme assay by measuring CDP-choline product formation. The V_{max} for CCT α 236 was 3850 nmol/min/mg and $K_{0.5}$ values for CTP and phosphocholine were 4.07 mM and 2.49 mM, respectively. The HPLC method was applied to glycerol 3-phosphate cytidylyltransferase (GCT) and CTP:2-C-methyl-D-erythritol-4-phosphate cytidylyltransferase synthetase (CMS), members of the cytidylyltransferase family that produce CDP-glycerol and CDP-methylerythritol, respectively.

Keywords: Cytidylyltransferase, Phosphatidylcholine, Enzyme, Assay, Chromatography.

1. Introduction

The cytidylyltransferase family is comprised of enzymes that transfer a cytidine 5'-phosphate from cytidine 5'-triphosphate (CTP) to a second acceptor molecule, typically for synthesis of molecules that are precursors to membrane phospholipids. Previously characterized cytidylyltransferases include CTP: phosphoethanolamine cytidylyltransferase (ECT) [1], CTP: glycerol-3-phosphate cytidylyltransferase (GCT) [2], 2-C-methyl-D-erythritol-4-phosphate cytidylyltransferase synthetase (CMS) [3], and CTP: phosphocholine cytidylyltransferase (CCT) [4-6]. CCT is a vital enzyme for phosphatidylcholine (PC) biosynthesis, the major phospholipid in mammalian cell membranes. In mammals, PC can be synthesized via the CDP-choline pathway by the sequential catalytic action of three enzymes: choline kinase utilizes choline and ATP to produce phosphocholine, CCT converts phosphocholine to CDP-choline, and choline phosphotransferase produces PC from CDP-choline and diacylglycerol. In humans there are two genes that encode CCT. *Pcyt1a* encodes the CCT α protein, predominantly localized to the nucleus, and *Pcyt1b* encodes the CCT β protein, which is cytoplasmic [6].

CCT has three domains that make up the overall structure, the central catalytic domain, amino acid residues 1-236 in rat CCT α , the membrane lipid activation domain, residues 238-299, and the C-terminal phosphorylated domain, residues 315-367 [7,8]. In a cell, CCT is activated upon translocation to the membrane and is thought to be activated through association with membrane lipids [9]. Since expression of the catalytic domain, amino acids 1-236, resulted in a form of CCT that is constitutively active it was proposed that a mechanism for activation involves a conformational change in the membrane lipid

activation domain of CCT that exposes the active site [10]. Structural studies and molecular dynamics later illustrated the membrane lipid activation domain acts as an autoinhibitory amphipathic alpha helix that interacts with an alpha helix at the opening to the active site and inhibits catalysis in the absence of lipids [11]. Mutagenesis studies on rat CCT α indicated histidine and arginine residues found in the conserved HxGH motif and RTEGISTT sequence were critical for catalysis [12,13]. In addition, mutation of lysine 122 of rat CCT α resulted in a catalytically compromised enzyme [13]. Determination of the three dimensional structure of CCT α 236 confirmed the presence of these amino acids in the active site and characterized interactions of each residue with CDP-choline. Also, two additional amino acids, His168 and Tyr173, were identified as active site amino acids and shown to be important for catalysis by site-directed mutagenesis [14].

Prokaryotes also express cytidylyltransferases, such as GCT [2], which catalyzes the formation of CDP-glycerol from CTP and glycerol-3-phosphate, a reaction that is part of the metabolic route to poly(glycerol phosphate), the major component of teichoic acids found in gram positive bacterial cell walls [15]. GCT from the organism *Bacillus subtilis* consists of just a catalytic domain that is approximately 30% identical to the catalytic domains of other members of the cytidylyltransferase family. The similar catalytic mechanism and the smaller size have made GCT an attractive target for catalytic, structural, and modeling studies that can be applied to the other members of the family [16]. CMS is a cytidylyltransferase also found in bacteria, as well as protozoans, archaea, and plant cells. The enzyme catalyzes an intermediate step in the mevalonate-independent pathway of isoprenoid biosynthesis known as the 1-deoxy-D-xylulose 5-phosphate / 2-C-

methyl-D-erythritol 4-phosphate (DXP/MEP) pathway [3]. CMS catalyzes production of 4-diphosphocytidyl-2-C-methyl-D-erythritol (CDP-MEP) from the substrates CTP and 2-C-methyl-D-erythritol-4-phosphate (MEP) for the eventual synthesis of isopentenyl diphosphate, a precursor for the synthesis of isoprenoids, molecules essential for cell proliferation and differentiation [17]. Vertebrate species produce isoprenoids using a mevalonate-dependent pathway, suggesting that the DXP/MEP pathway could be a target for antibacterial, herbicidal, and antimalarial molecules [3].

The major goal of this research was to develop a method utilizing high performance liquid chromatography (HPLC) to separate the CCT substrate cytidine 5'-triphosphate (CTP) from the product cytidine diphosphate-choline (CDP-choline). Successful separation would establish a basis for quantifying CCT enzyme activity and provide an alternative to the current CCT enzyme assay that depends on conversion of [^{14}C]phosphocholine to [^{14}C]CDP-choline [18]. In addition, if a separation method could be developed for CCT, it was the hope that other radioisotope-dependent cytidyltransferase assays could be replaced with non-radioactive methods.

2. Materials and methods

2.1 Protein purification

Rat CCT α 236 was expressed and purified through the CM-sepharose step as described previously [10]. *Enterococcus faecalis* GCT was expressed and purified as described previously [19]. Plasmid DNA encoding *Listeria monocytogenes* 6x His-tagged

CMS was a gift from Alisha Morganthaler (Illinois State University, manuscript in preparation). Protein concentration was determined using the method of Bradford [20] with a BSA standard curve and the Bio-Rad reagent following manufacturer's instructions.

2.2 Rat CCT α 236 Enzyme Assay

CCT α 236 assay tubes contained a total volume of 100 μ L, including 10 μ L of 10x assay buffer (500 mM imidazole, 250 mM magnesium acetate, pH 7), 10 μ L of 100 mM cytidine 5'-triphosphate (CTP), and 10 μ L of 40 mM phosphocholine (PoC) chloride calcium salt tetrahydrate. Reagents were purchased from Sigma-Aldrich. The enzymatic reaction was initiated by the addition of CCT α 236 protein, incubated at 37°C for 15 minutes, and terminated by placing the tubes in boiling water for 5 minutes. CTP and phosphocholine concentrations were varied in order to determine $V_{\max}$ and $K_{0.5}$ values for CCT α 236. Kinetic data were plotted as specific activity versus concentration of either CTP or phosphocholine and fit to the Hill equation using Sigma Plot (Systat Software).

2.3 Listeria monocytogenes CMS Enzyme Assay

CMS assay tubes contained a total volume of 20 μ L, including 2 μ L of 10x assay buffer (500 mM imidazole, 250 mM magnesium acetate, pH 7), 2 μ L of 20 mM CTP, and 2 μ L of 20 mM 2-C-methyl-D-erythritol-4-phosphate (MEP). Reagents were purchased from Sigma-Aldrich. The enzymatic reaction was initiated by the addition of CMS, incubated at 37°C for 15 minutes, and terminated by placing the tubes in boiling water for 5 minutes.

2.4 Enterococcus faecalis GCT Enzyme Assay

GCT assay tubes contained a total volume of 100 μL , including 10 μL of 10x assay buffer (500 mM imidazole, 250 mM magnesium acetate, pH 7), 10 μL of 25 mM CTP, and 10 μL of 40 mM glycerol phosphate. Reagents were purchased from Sigma-Aldrich. The enzymatic reaction was initiated by the addition of GCT protein, incubated at 37°C for 15 minutes, and terminated by placing the tubes in boiling water for 5 minutes.

2.5 HPLC and Sample Preparation

The HPLC used for this research was a 3 piece system by Finnigan Surveyor, obtained from Thermo Fisher Scientific, that consisted of a LC Pump Plus, an Autosampler Plus, a PDA Plus Detector and a ZORBAX Rx-C18 4.6 mm x 15 cm column. Elution of CTP and CDP-choline from the column was monitored by UV absorbance of the cytosine ring at 254 nm. Output from the instrument was in milli absorbance units (mAU). The experimental data were analyzed using the software program ChromQuestTM. The autosampler on the HPLC needs a minimum of 300 μL in the sample vial to withdraw 10 μL of the sample and make an effective injection onto the column. After termination of the enzyme-catalyzed reaction, samples were centrifuged at 16,000 x g for 15 minutes to pellet any insoluble material. Each microcentrifuge tube from the CCT α 236 and GCT enzyme assays contained 100 μL of sample, which were diluted 10-fold by placing 50 μL of the assay sample into 450 μL of deionized water in a 12 x 32 mm glass vial. CMS had a smaller reaction volume of 20 μL , so 4 μL was

removed and placed in 396 μL of deionized water. In each case, a 10 μL injection was made onto the column.

2.6 Preparation of a CDP-choline Standard Curve

Twenty 12 x 32 mm glass vials were prepared, ranging in concentration from 0.001 mM to 0.1 mM CDP-choline. Each vial was placed into the autosampler of the HPLC, along with a control vial of 0.1 mM CTP. The column was equilibrated with the mobile phase (0.1 M ammonium bicarbonate (98%) and acetonitrile (2%) pH 7.4) for 15 minutes. The mobile phase had an isocratic flow rate of 0.5 mL/minute for 5 minutes per trial. The area under the peak was plotted versus nanomoles of CDP-choline injected on the column.

3. Results and Discussion

3.1 Separation of CTP from CDP-choline

In order to develop an HPLC method for a CCT enzyme assay, the substrate CTP needed to be separated from the product CDP-choline, and each molecule detected through absorbance at 254 nm. The method that resulted in successful separation of CTP and CDP-choline was similar to that used by Ip et al [21], who separated various nucleotides and nucleosides using C18 Reverse Phase HPLC and a mobile phase of 0.1 M triethylammonium bicarbonate (98%) and acetonitrile (2%) at pH 7.4. The only modification made to the method of Ip et al [21] was to use ammonium bicarbonate in place of triethylammonium bicarbonate. Initially, solutions containing either CTP or

CDP-choline were passed through the HPLC column and differences in retention time observed. Next, stock solutions containing both CTP and CDP-choline (0.001 mM, 0.01 mM, or 0.1 mM CTP and CDP-choline) were passed through the reverse phase C18 column with the mobile phase of 0.1 M ammonium bicarbonate (98%) and acetonitrile (2%) at a flow rate of 0.5 ml/min at pH 7.4. A 10 μ l injection of each stock solution containing equal concentrations of both CTP and CDP-choline resulted in separation (Fig. 1). The detection was fairly sensitive, with injection of 10 μ l of the solution containing 0.001 mM CTP and CDP-choline (10 picomoles of each compound) producing a peak that was able to be analyzed for peak area. The separation may be attributed to the difference in hydrophobicity of the target compounds; CTP has a greater negative charge and hydrophilic character in comparison to CDP-choline, possibly causing CTP to elute before CDP-choline.

3.2 Correlation of Peak Area to nanomoles of CDP-choline

In order to quantitate the nanomoles of CDP-choline produced in an enzyme-catalyzed reaction, a correlation between peak area and nmoles of compound needed to be established. Therefore, a standard curve was created by analyzing solutions with a known amount of CDP-choline, the product of the CCT-catalyzed reaction. Figure 2A illustrates the direct relationship between peak area and nmoles of CDP-choline, the basis for quantification of the product CDP-choline. Figure 2B shows the increase in the peak height and area as the concentration of CDP-choline is increased. The linear relationship between peak area and nanomoles of CDP-choline provides a means for determination of nanomoles of CDP-choline produced in a cytidylyltransferase-catalyzed reaction.

3.3 HPLC Method Application to Rat CCT α 236

At this point a successful method for separation of standard solutions of the CCT236 substrate (CTP) and product (CDP-choline) had been established. This method was applied to reaction tubes from a CCT α 236 enzyme assay that, presumably, contained both CTP and CDP-choline. Initial assays varied the concentration of purified CCT α 236, which provided evidence that the HPLC method developed using standard solutions of CTP and CDP-choline solutions was applicable to an enzyme assay. It also established that increasing the amount of CCT α 236 enzyme results in an increase in product formation. Varying the amount of enzyme added also gave an indication of the optimal amount of enzyme needed to conduct enzyme kinetics experiments. The amount of CCT α 236 added to each assay tube is indicated in Figure 3, which illustrates the increase in CDP-choline formation when increasing the amount of enzyme. The activity of recombinant, purified CCT α 236 was easily measured since it is a constitutively active truncation of CCT. The full length CCT requires activation via association with lipids and has been reported to have activity 50-fold lower in the absence of lipids when compared to the activity of CCT α 236 [10]. In theory, the HPLC assay should be able to be utilized to assay full length CCT in the absence of lipids if the assay samples were not diluted 10-fold before injection on the HPLC and the amount of enzyme was increased as necessary. Cytidylyltransferase activity found in a tissue extract, such as a liver homogenate, could also, in theory, be measured using the HPLC assay since as little as 10 picomoles of CDP-choline was detectable and able to be used to determine enzyme activity. It is possible that accurate determination of tissue cytidylyltransferase activity would require

an increase in sample volume or incubation time to produce sufficient CDP-choline, greater than 10 picomoles.

3.4 Determination of the Kinetic Properties of Rat CCT α 236

The activity of CCT α 236 is dependent on the concentration of its substrates phosphocholine and CTP. Increasing either substrate concentration will result in an increase in specific activity, defined as the nanomoles of product (CDP-choline) produced per minute per mg of enzyme. The concentration of the substrates CTP and phosphocholine were varied in individual assays while keeping a constant amount of enzyme (1.16 μ g) in each reaction tube (Fig. 4). The data were fit to the Hill equation using nonlinear regression analysis to obtain the $K_{0.5}$ and $V_{\max}$ values as well as the Hill coefficient, which provides information about cooperativity with respect to each substrate. CCT α 236 has been postulated to be a dimer in solution [10]. In an enzyme with multiple subunits, cooperativity is the effect that binding one molecule of substrate in the active site has on the binding affinity for additional substrate molecules by the other subunits. A Hill coefficient that has a value less than one indicates negative cooperativity, while a coefficient greater than one indicates positive cooperativity. Varying CTP concentration resulted in a $K_{0.5}$ value of 4.07 ± 0.67 mM and a $V_{\max}$ of 4348 ± 380 nmol/min/mg. The Hill coefficient was 1.59, indicating positive cooperativity, suggesting when one molecule of CTP binds to one monomer of CCT α 236 the affinity of the second monomer for CTP will increase. CCT has been previously reported to exhibit cooperativity towards CTP, with a reported value for the Hill coefficient of 1.4 [13]. Varying phosphocholine concentration resulted in a $K_{0.5}$ value of 2.49 ± 0.33 mM and a

$V_{\max}$ of 3341 ± 158 nmol/min/mg. The Hill coefficient was 0.992, indicating little or no cooperativity with respect to phosphocholine. The average $V_{\max}$ from the kinetic analysis using the HPLC assay method was approximately 3850 nmol/min/mg, reasonably close to the reported specific activity of 6420 nmol/min/mg for CCT α 236 purified through the CM-sepharose chromatography step and assayed using the radioisotope method [10]. The K_m for CTP using the radioisotope assay was reported to be 3.15 mM, similar to the $K_{0.5}$ of 4.07 mM reported here for CTP, and the K_m for phosphocholine was reported to be 0.90 mM, lower than the $K_{0.5}$ of 2.49 mM in this study for phosphocholine [10].

3.5 Application of HPLC Enzyme Assay to CMS and GCT

In general, cytidylyltransferase family members use CTP as a substrate and produce a cytidine diphosphate derivative as a product. In theory, the HPLC assay method developed using CCT α 236 should be able to be applied to other members of the family. To test this hypothesis we conducted enzyme assays using the HPLC method with CMS from *Listeria monocytogenes* and GCT from *Enterococcus faecalis* [19]. CMS utilizes the substrates CTP and 2-C-methyl-D-erythritol-4-phosphate (MEP) to produce 4-diphosphocytidyl-2-C-methyl-D-erythritol (CDP-ME). The target molecules for HPLC separation, therefore, are CTP and CDP-ME. GCT catalyzes conversion of CTP and glycerol-3-phosphate to CDP-glycerol. The target molecules for HPLC separation for GCT, therefore, are CTP and CDP-glycerol. In each case, these compounds were separated in a manner similar to CTP and CDP-choline (Fig. 5).

3.6 Comparison to Other Cytidylyltransferase Assays

The principle reason for development of an HPLC-based cytidylyltransferase assay was to replace the traditional radioisotope assay, one of the principle methods used to characterize CCT [18]. The HPLC and radioisotope method, in terms of product formation, are similar with the only difference being a percentage of the phosphocholine substrate is ^{14}C labeled instead of non-radioactive phosphocholine. The difference between the methods is in the quantification of the product formed during catalysis. In the radioisotope assay the reaction mixture, containing CTP, unreacted [^{14}C]phosphocholine, and [^{14}C]CDP-choline, is incubated with 500 μL of a 10 mg/mL suspension of activated charcoal for 30 minutes. The charcoal does not bind phosphocholine as well as CDP-choline, providing the basis for separation of the substrate phosphocholine from the product CDP-choline. The charcoal is pelleted by centrifugation and rinsed twice with 500 μL of water to remove the majority of the [^{14}C]phosphocholine from the charcoal. The charcoal pellet is resuspended in 500 μL of 10% (v/v) acetic acid, releasing the CDP-choline from the charcoal. The reaction tube is centrifuged and the supernatant liquid, containing the ^{14}C -labeled product CDP-choline is placed in a scintillation counter to determine the amount of radioactivity in the sample. The scintillation counter quantifies the ^{14}C -labeled CDP-choline in counts per minute (cpm). In order to convert cpm to nanomoles of CDP-choline formed, several controls and corrections must be taken into consideration. First, a background cpm is determined from a tube containing no enzyme and is subtracted from each of the experimental cpm values. A background cpm less than 5% of the experimental value is desirable, however, in cases where there is low enzyme activity this is not always the case, presumably due to some [^{14}C]phosphocholine remaining bound to the charcoal. With the HPLC assay a

control tube is also run with no enzyme, but the peak area for CDP-choline is close to zero and insignificant, eliminating the need to subtract a baseline from the experimental samples. In the radioisotope assay the difference between the experimental cpm and the background cpm is divided by the CDP-choline recovery, assay time (minutes), specific activity of [^{14}C]phosphocholine (cpm/nmol), and amount of enzyme (mg). Before this calculation can take place, however, the CDP-choline recovery is calculated from a control tube that does not contain enzyme but does have a known amount of [^{14}C]CDP-choline. The CDP-choline recovery tube is processed in the same manner as the experimental tubes and the cpm remaining is divided by the initial cpm of [^{14}C]CDP-choline added to the tube, yielding the [^{14}C]CDP-choline recovery.

An advantage of the HPLC assay method is that there is only one step between product formation and product quantification, a simple dilution to increase the volume so that the autosampler can make an injection onto the HPLC. The charcoal binding and multiple washes and centrifugations have been eliminated, making sample preparation simpler. Alternatively, radiolabeled CTP and CDP-choline can be separated using thin layer chromatography (TLC) following the radioisotope assay, eliminating the charcoal binding step, a method that has been employed for several decades to characterize rat CCT [22,23] and was utilized recently to assay *Plasmodium falciparum* CCT [24]. TLC separation allows for a CDP-choline recovery near 100% since the entire band is transferred to a scintillation vial for counting in a scintillation counter. The direct quantification of product in the HPLC method is a distinct advantage over the radioisotope method, but perhaps the major advantage is the cost savings on the purchase of radioactive versus non-radioactive phosphocholine. To run a K_m determination for

CTP using the radioisotope assay, measuring activity in triplicate at ten CTP concentrations, the cost for [^{14}C]phosphocholine would be approximately \$100. However, using non-radioactive phosphocholine and the HPLC assay, the same K_m determination for CTP would have a total phosphocholine cost of 3 cents per triplicate assay.

It is important to note that other non-radioactive methods have been employed to assay cytidylyltransferases. These methods employ coupled assays that rely on the enzymatic conversion of pyrophosphate, a product of the cytidylyltransferase reaction, to phosphate by pyrophosphatase. One variation of the coupled assay uses reaction with the dye malachite green to detect phosphate spectrophotometrically at 660 nm [25,26]. Alternatively, after phosphate has been produced from the pyrophosphatase reaction, the enzyme purine-nucleoside phosphorylase catalyzes phosphorolysis of 2-amino-6-mercapto-7-methylpurine ribonucleoside and increases absorbance at 360 nm [27-29]. The pyrophosphatase-coupled assays, just like the HPLC assay, have the advantage of eliminating radioisotopes and spectrophotometric detection is a simple and efficient means employed for many enzyme assays. The disadvantage of the pyrophosphatase-coupled assays is that they measure formation of phosphate from pyrophosphate by pyrophosphatase and do not directly measure the rate of formation of CDP-choline. The HPLC assay, however, has a distinct advantage when compared to pyrophosphatase-coupled assays in that it directly monitors the disappearance of substrate and appearance of product and can likely be used to monitor cytidylyltransferase activity in crude cellular extracts. The pyrophosphatase-coupled assay quantifies free phosphate as the final step,

and in a cell extract there are likely to be multiple sources of phosphate as well as other pyrophosphatases.

3.7 Conclusions

The goal of this research was to develop a cytidylyltransferase enzyme assay that utilized HPLC to separate CDP-choline, a product of the reaction, from CTP, a substrate. Previous methods used to assay cytidylyltransferases used either radioisotopes or relied on the activity of other enzyme-catalyzed reactions in a coupled assay. Exploiting the absorbance of CTP and CDP-choline at 254 nm as a method of detection eliminates the need for detection of radioisotope-labeled product, avoiding the expense associated with the purchase and disposal of radioisotopes. In addition, the HPLC assay method directly measures a product of the cytidylyltransferase reaction, CDP-choline, rather than measuring phosphate produced by pyrophosphatase in a coupled reaction. For these reasons, the HPLC cytidylyltransferase assay is a potentially advantageous alternative to present assay methods.

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Figure Legends

Fig. 1. Separation of CTP and CDP-choline. Stock solutions containing both CTP and CDP-choline were injected onto the HPLC column. (A) 0.01 nanomoles of CTP and CDP-choline were injected onto the column (B) 0.1 nanomoles of CTP and CDP-choline were injected onto the column (C) 1 nanomole of CTP and CDP-choline were injected onto the column.

Fig. 2. Preparation of a CDP-choline standard curve. Increasing amounts of CDP-choline, in nanomoles, was injected onto the HPLC column and the peak area plotted versus the nanomoles of CDP-choline.

Fig. 3. Production of CDP-choline by CCT α 236 using the HPLC enzyme assay. The following amounts of CCT α 236 were added: 0 μ g, 0.15 μ g, 0.31 μ g, 0.62 μ g, 1.08 μ g, 1.39 μ g. In each case, the peak area for CDP-choline increased as the amount of enzyme in the assay was increased. Inset: the linear relationship between the CDP-choline peak area and micrograms of enzyme is illustrated.

Fig. 4. Kinetic analysis of CCT α 236 using the HPLC assay. Top: Specific activity of CCT α 236 as a function of CTP concentration. Bottom: Specific activity of CCT α 236 as a function of phosphocholine concentration. Data are the average of three determinations with standard error indicated by the error bars.

Fig. 5. Separation of the substrate CTP from the reaction product for CCT, GCT, and CMS. Separation of CTP and CDP-choline from the CCT assay is plotted as a dashed line. Separation of CTP and CDP-glycerol from the GCT assay is plotted as a dotted line. Separation of CTP and CDP-methylerythritol from the CMS assay is plotted as a solid black line. A control with no added enzyme is shown as a solid grey line.

Figure

Figure 2

Figure 3

Figure 4

Figure 5