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# A spectrophotometric assay for the determination of 4-diphosphocytidyl-2-*C*-methyl-D-erythritol kinase activity

Cristobal Bernal [a], Eva Mendez [b], José Terencio [b], Albert Boronat [a], Santiago Imperial [a,*]

[a] *Departament de Bioquimica i Biologia Molecular, Universitat de Barcelona, Avda Diagonal 645, 08028 Barcelona, Spain*
[b] *Biochemistry and Molecular Biology Department, Pharmaceutical Research and Development Center, Ferrer International S.A., Juan de Sada 32, 08028-Barcelona, Spain*

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**Abstract**

We report an assay for the determination of the activity of 4-diphosphocytidyl-2-*C*-methyl-D-erythritol kinase, the enzyme which catalyzes the fourth reaction step of the 2-*C*-methyl-D-erythritol 4-phosphate pathway for the synthesis of isoprenoids, which is based on the spectrophotometrical determination of adenosine 5′-diphosphate using pyruvate kinase and L-lactate dehydrogenase as auxiliary enzymes. This method can be adapted to microtiter plates, can be automated, and because of its simplicity and speed can be useful for the functional characterization of the enzyme and for the screening of inhibitors with potential antibiotic or antimalarial action.
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Isopentenyl pyrophosphate (IPP)[1] and its isomer dimethylallyl pyrophosphate [1,2] are the universal five-carbon precursors of one of the largest families of natural products, isoprenoids. Isoprenoids include hopane triterpenes, ubiquinones, and menaquinones in bacteria, carotenoids, plastoquinones, mono-, sesqui-, di-, and triterpenes, and the prenyl side chains of chlorophylls in plants, and quinones, dolichols, steroids, and retinoids in mammals [3]. They account for more than 30,000 naturally occurring molecules of both primary and secondary metabolism.

After the discovery of the mevalonic acid (MVA) pathway in yeast and animals, it was assumed that IPP was synthesized from acetyl-CoA via MVA and then isomerized to DMAPP in all organisms [2–5]. However, an alternative MVA-independent pathway for the biosynthesis of IPP was recently identified by labeling experiments in bacteria [6,7] and plants [8]. This pathway is named after what is currently considered its first committed precursor, 2-*C*-methylerythritol 4-phosphate (MEP) (for recent reviews see [9,10]).

Experimental evidence accumulated since the discovery of the MEP pathway has shown that most organisms use only one of the two pathways for the biosynthesis of their precursors. Thus, the MEP pathway is the only one

* Corresponding author. Fax: +34 93 4021559.
*E-mail address:* imperial@bq.ub.es (S. Imperial).

[1] *Abbreviations used:* ADP, adenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate; CDP-ME, 4-diphosphocytidyl-2-*C*-methyl-D-erythritol; CDP-MEP, 4-diphosphocytidyl-2-*C*-methyl-D-erythritol 2-phosphate; DMAPP, dimethylallyl diphosphate; DTT, dithiothreitol; IPP, isopentenyl pyrophosphate; IPTG, isopropyl β-D-thiogalactoside; LDH, lactate dehydrogenase; MEP, 2-*C*-methyl-D-erythritol 4-phosphate; MVA, mevalonic acid; PEP, phosphoenol pyruvate; PK, pyruvate kinase; SD, standard deviation; SDS, sodium dodecyl sulfate; TLC,thin-layer chromatography.

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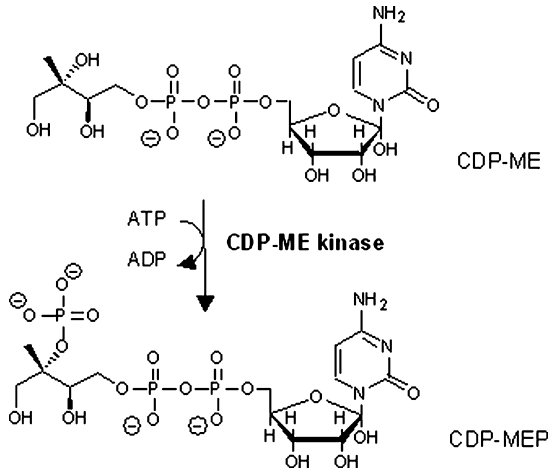


Fig. 1. Reaction catalyzed by CDP-ME kinase.

present in most eubacteria including the causal agents for diverse and serious human diseases such as leprosy, bacterial meningitis, various gastrointestinal and sexually transmitted infections, tuberculosis, certain types of pneumonia, and the malaria parasite *Plasmodium falciparum*, but it is absent from fungi and animals, which synthesize their isoprenoids exclusively through the operation of the MVA pathway. In contrast, plants use both the MEP pathway and the MVA pathway for isoprenoid biosynthesis, although they are localized in different compartments [9–16].

Given the essential nature of the MEP pathway and its absence in mammals, the enzymes comprising the MEP pathway represent potential targets for the generation of selective antibacterial, antimalarial, and herbicidal molecules [10,15–18].

Strategies for the systematic screening of inhibitors of the MEP pathway should rely on the availability of simple, sensitive, and nonhazardous assays well suited to automation.

The enzyme 4-(cytidine 5-diphospho)-2-*C*-methylerythritol (CDP-ME) kinase catalyzes the fourth reaction step of the MEP pathway (see Fig. 1). CDP-ME kinase activity was first determined radiometrically using [2-$^{14}$C]CDP-ME as substrate [19]. This method was seen to be sensitive and specific and allowed identification of the reaction product CDP-MEP. However, like many radiochemical assays it has several disadvantages which would not make it the method of choice for the analysis of large number of samples and automation: it demands additional manipulations to separate the labeled product from the remaining labeled substrate at the end of the incubation (e.g., by thin-layer chromatography (TLC) [19,20]).

CDP-ME kinase activity has also been determined by reverse-phase HPLC using an Asahipak GS-320 HQ [21] or a Multospher 120 RP 18-AQ-5 [22] column and UV detection. This method is not easy to use also as it requires HPLC equipment and expertise. Here we report an assay for the determination of CDP-ME kinase activity based on the spectrophotometrical determination of adenosine 5′-diphosphate using pyruvate kinase and L-lactate dehydrogenase as auxiliary enzymes. The method is easy to perform and sensitive and can be used for the functional characterization of the enzyme and for the automated high-throughput screening analysis of chemical compound collections for the search of inhibitors with antibiotic or antimalarial actions.

## Materials and methods

### *Materials*

CDP-ME was enzymatically synthesized from MEP as follows: reaction mixtures contained 38 mM MEP, 38 mM CTP, 10 mM $MgCl_2$, 2.5 U bakers' yeast inorganic pyrophosphatase (Sigma), and 60 μg of *Escherichia coli* CDP-ME synthase in Tris–HCl buffer, pH 7.5, in a final volume of 500 μl. Reaction mixtures were incubated at 37 °C for 4 h. Reactions were stopped by heating at 80 °C for 5 min, centrifuged, and filtered through Biomax-5K NMWL ultrafree filters (Millipore). CDP-ME preparations were purified by HPLC: 100-μl batches were consecutively applied to a Synergi $C_{18}$ (4.6 × 150 mm-4μ Hydro) column and separated as described below for the HPLC assay. Fractions containing CDP-ME were pooled, lyophylized, redissolved in distilled water, and stored at −20 °C until use. The CDP-ME preparations obtained had a final concentration of 30–35 mM. CDP-ME used as standard was purchased from Echelon Biosciences.

MEP and recombinant *E. coli* CDP-ME synthase were obtained as previously described [23]. Other enzymes, substrates, and cofactors were purchased from Sigma. The premixed solutions of pyruvate kinase (PK) and L-lactate dehydrogenase (LDH) from rabbit skeletal muscle which were used as auxiliary enzymes was also a Sigma product (reference P0294). Proteinase inhibitors (Complete mini EDTA-free tablets) were obtained from Roche.

### *Overexpression and purification of recombinant E. coli CDP-ME kinase*

A DNA fragment containing the coding region of the *E. coli ychB* gene was PCR-amplified and subcloned into the expression vector pQE30 (Qiagen). The resulting plasmid was designated pQE30-*ychB*. M15[pREP4] *E. coli* cells transformed with pQE30-*ychB* were grown at 37 °C in 2× TY medium supplemented with ampicillin (100 μg/ml) and kanamycin (50 μg/ml) until 0.3–0.4 $OD_{600}$ was reached. Induction was performed with 0.4 mM IPTG for 3 h at 30 °C. Cells were harvested by centrifugation (3000*g*, 10 min) and resuspended in 40 mM Tris–HCl buffer, pH 8.5, containing 5 mM 2-

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