Cytidine diphosphate-diacylglycerol synthesis in *Mycobacterium smegmatis*

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Recent studies have demonstrated that, during infection of macrophages by mycobacteria, phospholipids (PLs) are released from the mycobacterial cell wall within infected macrophages and transported out of this compartment into intracellular vesicles. The release of these PLs may have functions that influence the outcome of mycobacterial infections. Despite their important role, little is known about the biosynthesis of PLs in mycobacteria. In all organisms, PL biosynthesis begins with acylation of *sn*-glycerol 3-phosphate to form phosphatidic acid (PA), which is then converted to the central liponucleotide intermediate, cytidine diphosphate-diacylglycerol (CDP-DAG) via the CDP-DAG synthase (CDS). The present work examines CDS activity in *Mycobacterium smegmatis* extracts, with regard to subcellular localization, pH dependence, bivalent and univalent cation requirement, substrate specificity and regulation by nucleotides. We show that CDS activity, which is mainly found within the cytoplasmic membrane, is Mg^{2+}-dependent and activated by K^{+} ions. Among PAs containing saturated fatty acids, dipalmitoyl-PA is the preferred substrate [$K_m = 0.23 \pm 0.03$ mM for Triton X-100 (v/v)/PA in the ratio 5:1]. Moreover, CDS activity is inhibited by the reaction products PP_i ($IC_{50} = 1.5$ mM), CDP-DAG ($IC_{50} = 0.3$ mM) and the nucleotides ATP, UTP and GTP. This study contributes to the delineation of PL biosynthesis in mycobacteria.

Key words: kinetics, phospholipid biosynthesis, regulation, substrate specificity.

INTRODUCTION

Pathogenic mycobacteria are able to survive and multiply within phagosomes of host phagocytes. Survival is believed to be modulated by the mycobacterial cell wall, which contains various complex lipids [1]. Mycobacteria have been reported to contain the phospholipids (PLs), phosphatidyl-*myo*-inositol (PI), PI mannosides (PIMs), cardiolipin (CL) and phosphatidylethanolamine [2]. PI and PIMs are the most prominent and characteristic PLs of the mycobacterial cell wall [1,2].

It has been established that PI, an essential component [3], and PIMs induce granuloma formation and recruitment of natural killer T cells in mice [4]. In addition, they provide the lipid anchor for the immunosuppressive molecule lipoarabinomannan [5], a key virulence factor [6]. PIMs and lipoarabinomannan have been shown to be released from the mycobacterial cell wall within infected macrophages and transported out of this compartment into intracellular vesicles [7,8]. Similarly, it has been shown that during the infection of macrophages by *Mycobacterium bovis* Bacille Calmette–Guérin, CL is exported from phagosomes after the cleavage of mycobacterial CL by a macrophage-derived lysosomal phospholipase A_2 [9]. The release of these PLs may have functions that influence the eventual outcome of mycobacterial infections.

Very little is known of the biosynthesis of PLs in mycobacteria. In all organisms, PL biosynthesis begins with acylation of *sn*-glycerol 3-phosphate to form phosphatidic acid (PA), which is then converted to the central liponucleotide intermediate cytidine diphosphate-diacylglycerol (CDP-DAG) via the CDP-DAG synthase (CTP:phosphatidate cytidylyltransferase, EC 2.7.7.41) (CDS) [10,11]. CDP-DAG constitutes the common precursor for the biosynthesis of PLs [12]. In mycobacteria, biosynthesis of PA has been studied in *M. butyricum* (now termed *M. smegmatis*) [13]. PA appeared to be synthesized mainly through a 2-acylglycerophosphate pathway [13]. The biosynthesis of PI has been studied with cell wall fractions of *M. smegmatis*, where CDP-DAG was found to be the key metabolic precursor [14]. CDP-DAG was also shown to be a substrate for CL biosynthesis in cell-free extracts of *M. smegmatis* [15].

CDP-DAG is situated at a key branch point in lipid biosynthetic pathways, leading to PL synthesis and, thus, the control of its synthesis and utilization is likely to play an important role in the regulation of PL biosynthesis [10,11]. In the present study, we report the characterization of CDS activity in *M. smegmatis*, contributing to the delineation of the biosynthesis of CDP-DAG, a central metabolite in mycobacterial PL biosynthetic pathways.

MATERIALS AND METHODS

Strains and growth conditions

M. smegmatis mc²155 was a gift from W. R. Jacobs (Albert Einstein College of Medicine, Bronx, New York, NY, U.S.A.). *M. smegmatis* cells were routinely grown in Luria–Bertani (LB) medium supplemented with 0.05% Tween 20 (Sigma) at 37 °C.

Materials

Fine chemicals were purchased from Sigma unless otherwise stated. [5-^{3}H]CTP (tetrasodium salt) (20 Ci · $mmol^{-1}$) was purchased from Dupont NEN Research products.

Preparation of cells extracts

M. smegmatis was grown as described above, harvested, washed with 50 mM Tris/HCl (pH 7.5) and stored at −20 °C. Bacteria

Abbreviations used: C_{16}, palmitic acid; C_{19}, tuberculostearic acid; CDP-DAG, cytidine diphosphate-diacylglycerol; CDS, CDP-DAG synthase; CL, cardiolipin; LB, Luria–Bertani; PA, phosphatidic acid; PA_{C16}, dipalmitoyl-PA; PI, phosphatidyl-*myo*-inositol; PIM, PI mannoside; PL, phospholipid.

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(10 g wet weight) were resuspended in 50 mM Tris/HCl (pH 7.5), containing 10 mM $MgCl_2$ and 5 mM 2-mercaptoethanol (buffer A) at 4 °C and subjected to probe sonication (Soniprep 150, 1 cm probe; MSE Sanyo Gallenkamp, Crawley, Sussex, U.K.) for a total time of 10 min in 60 s pulses with 90 s cooling intervals between pulses. The sonicated material was centrifuged at 27000 ***g*** for 20 min at 4 °C to obtain the cell wall fraction, which was resuspended in buffer A. The resulting 27000 ***g*** supernatant was further centrifuged at 100000 ***g*** for 2 h at 4 °C to yield membranes (pellet) and cytosolic (supernatant) fractions. Protein concentrations in the various fractions were determined using the bicinchoninic acid Protein Assay kit (Pierce, Rockford, IL, U.S.A.).

CDS enzyme assays

Standard assay

CDS activity was assayed by measuring the conversion of [^{3}H]CTP to [^{3}H]CDP-DAG. Reaction mixtures consisted of 1 mM [5-^{3}H]CTP (3.5×10^3 c.p.m. · nmol^{-1}), 1.5 mM PA, 7.5 mM Triton X-100, 3 mM $MgCl_2$, 50 mM KCl, 1 mg/ml BSA, 0.25 mM dithiothreitol, 20 μg bacterial protein in 50 mM Tris/HCl (pH 8) in a final volume of 100 μl. The mixture was incubated at 37 °C for 30 min and the reaction terminated by the addition of 4 ml of chloroform/methanol/water (10:10:3, by vol.). A phase separation was obtained by adding 1.75 ml of chloroform and 0.75 ml of water. The organic phase was washed twice by adding 2 ml of chloroform/methanol/water in the proportion (3:47:48, by vol.). Since no labelled product other than CDP-DAG was found in the washed lipid extract, quantification of the reaction was performed directly by scintillation counting of the washed lipid extract. CDS activity was determined by subtracting counts present in control assays (either no enzyme or boiled enzyme).

Specific assays

Unless otherwise stated, the experiments were performed using PA from egg phosphatidylcholine (PA_{eggs}).

To determine the K_m value, PA concentration was varied from 0 to 1.7 mM, while the ratio of Triton X-100/PA was held constant at 5:1. For the determination of the time-, enzyme concentration- and the Mg^{2+}-dependent enzyme activity profile, pH optimum, metal-ion dependence, K^+ and Na^+ stimulation, substrate specificity, product inhibition and nucleotide effect the corresponding factors were varied.

CDP-DAG inhibition experiments were performed using a Triton X-100 concentration of 30 mM. The effect of pH on CDS activity was determined using the following buffers: 50 mM sodium acetate (pH 6.0), 50 mM Tris/HCl (pH 7.0–9.0) and 50 mM glycine/NaOH (pH 10.0).

RESULTS

Characterization of CDS activity in *M. smegmatis*

M. smegmatis was grown in LB medium and harvested in mid-exponential phase. Different subcellular fractions were prepared and assayed for CDS activity by measuring the conversion of [^{3}H]CTP to [^{3}H]CDP-DAG (Figure 1). Approximately 80 % of the CDS activity was recovered in the membrane fraction, with weaker CDS activity observed in the cell wall and cytosol fractions (Figure 1). CDS enzymes are known to be membrane proteins; the synthesis of CDP-DAG in the cell wall and cytosol fractions was probably due to cross contamination with membranes. The product synthesized in the membrane fraction was identified as CDP-DAG, based on co-migration with a commercial standard in two TLC solvent systems. As shown in Figure 2(A), only one labelled product was found in the lipid extract. Consequently, quantification of the reaction products

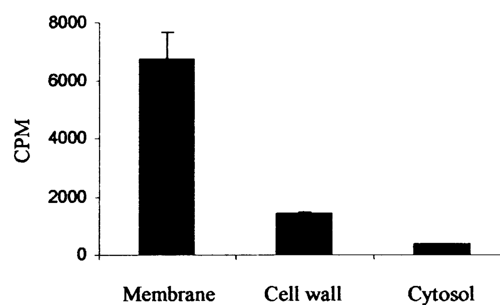

Figure 1 Synthesis of CDP-DAG by different subcellular fractions of *M. smegmatis*

M. smegmatis cells grown in LB medium were harvested and the protein fractions prepared as described in the Material and methods section. CDS activity was assayed by measuring the conversion of [^{3}H]CTP to [^{3}H]CDP-DAG. Reaction mixtures contained, in a total volume of 100 μl, 1 mM [5-^{3}H]CTP (3.5×10^3 c.p.m. · nmol^{-1}), 1.5 mM PA, 7.5 mM Triton X-100, 0.25 mM dithiothreitol, 3 mM $MgCl_2$, 50 mM KCl, 1 mg · ml^{-1} BSA and 20 μg of bacterial protein in 50 mM Tris/HCl (pH 8). The reactions were incubated at 37 °C for 30 min.

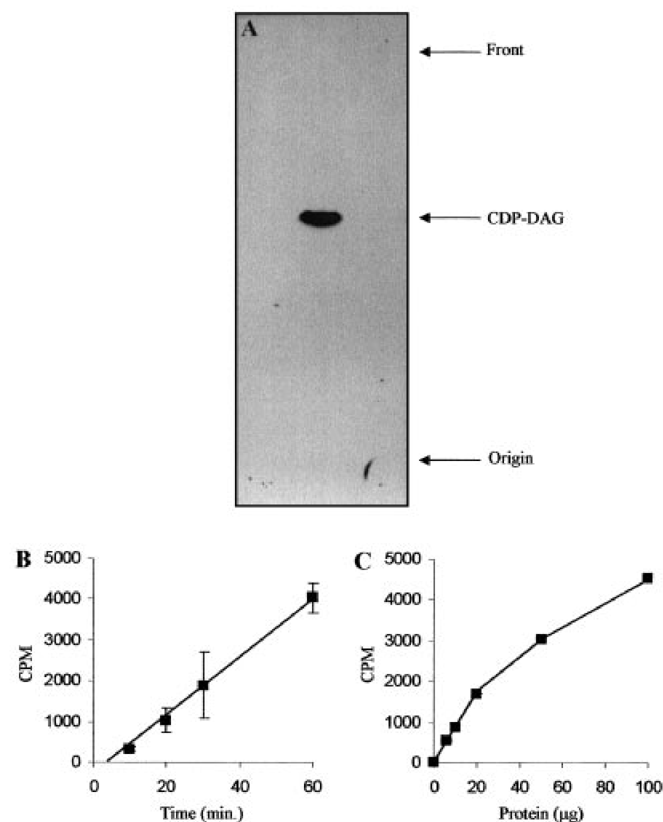

Figure 2 Synthesis of CDP-DAG in the membrane fraction

(**A**) TLC analysis of the reaction product. Lipids (approx. 3×10^5 c.p.m.) were extracted, analysed by TLC using chloroform/methanol/acetic acid/water (50:28:4:8, by vol.) [16], and TLC plates were exposed to Kodak X-omat AR film. (**B**) Time dependence of CDP-DAG synthesis. (**C**) Enzyme concentration dependence of CDP-DAG synthesis.

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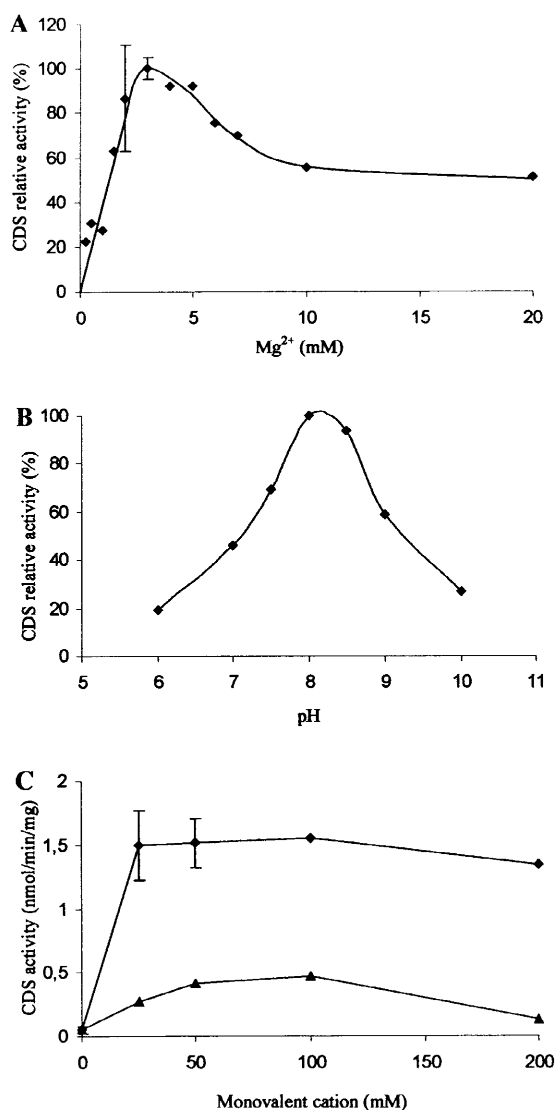

Figure 3 Concentration and pH dependence of mycobacterial CDS activity

(**A**) The effect of Mg^{2+} concentration (**A**), pH (**B**) and K^+ (◆) and Na^+ (▲) concentrations (**C**) were determined (at 37 °C, for 1.5 mM PA, 1 mM CTP in 50 mM Tris/HCl). Activities were normalized to the value for the assay with 3 mM Mg^{2+}. (**B**) Activities were normalized to the value for the assay at pH 8. (**C**) CDS activity was determined at pH 8, using 3 mM Mg^{2+}.

was performed directly by scintillation counting of the lipid extract [16]. CDS activity was linear for at least 60 min (Figure 2B) and up to 20 μg of bacterial protein (Figure 2C). To determine the CDS activity parameters, a time span of 30 min and 20 μg of protein were used.

Kinetics of CDS activity

Mg^{2+} is essential for CDS activity [17–19]. The optimal Mg^{2+} concentration was approx. 3 mM (Figure 3A) and at concentrations higher than 3 mM, Mg^{2+} became inhibitory. This value is slightly lower than values determined for rat liver (20 mM) [17], *Saccharomyces cerevisiae* (20 mM) [18] and *Escherichia coli* (approx. 10 mM) [19] CDS enzymes. The pH for optimal activity was 8 (Figure 3B). *E. coli* CDS has been reported to be activated by K^+ ions with an optimal activity at 200 mM KCl [19]. Similarly, *M. smegmatis* CDS was strongly stimulated by K^+ ions with approx. 30-fold increase in activity at concentrations between 25 and 100 mM KCl (Figure 3C). NaCl at the same concentrations was also stimulatory but to a lesser extent than that observed with KCl (Figure 3C). Activation of CDS activity by the univalent cations K^+ and Na^+ may be due in part to an increase in the ionic strength but the difference in magnitude of stimulation observed between the two cations suggests that K^+ ions may exert a stimulatory effect on the enzyme itself.

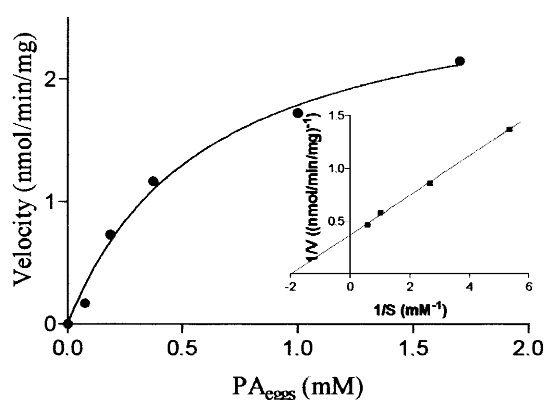

Figure 4 Kinetic analysis of PA_{eggs} towards CDS activity

The initial velocities of CDP-DAG production were measured by varying the concentration of PA_{eggs} from 0 to 1.7 mM. The linear fit was used to calculate the K_m value of PA_{eggs}, which was determined to be 0.61 ± 0.12 mM.

CDS enzymes are membrane-bound proteins, and non-ionic detergents are necessary for their solubilization [10]. As a result, kinetic parameters of CDS enzymes are influenced by the surface concentration of the substrate PA in the detergent micelle [10]. To obtain a reasonable linear double-reciprocal plot for the determination of the K_m value, PA concentration was varied in the presence of a fixed PA-to-detergent ratio. Consequently, kinetics experiments were conducted using a uniform mixed micelle substrate of Triton X-100/PA in the ratio 5:1. An K_m (app) of 0.61 ± 0.12 mM was found for PA_{eggs} (Figure 4). This value is similar to that found for yeast (0.5 mM) [18], *E. coli* (0.28 mM) [19] and *Plasmodium falciparum* (0.9 mM) [20] CDS, but lower than that determined for guinea pig liver (2.5 mM) [11] and rat liver (7.4 mM) [17] CDS activities.

Ability of other metal ions to activate mycobacterial CDS activity

We examined whether other bivalent metal ions were able to replace Mg^{2+}. Ca^{2+} at a concentration of 3 mM gave a partial activity of approx. 55 % compared with that obtained with 3 mM Mg^{2+} (Figure 5). Fe^{2+} and Mn^{2+} also led to partial activities of almost 30 % of that obtained with Mg^{2+}, whereas all the other metal ions examined gave activities less than 6 %.

Substrate specificity of CDS activity

Results presented in this paper were obtained using PA_{eggs}, which is known to have good solubility in detergent solutions because of the presence of unsaturated fatty acids, in contrast with PA, which contains only saturated fatty acids [19]. Nevertheless, mycobacterial PLs mainly contain saturated fatty acids, palmitic acid (C_{16}) and tuberculostearic acid (C_{19}) [2]. Consequently, various PAs containing saturated fatty acids, dilauryl-PA ($C_{12:0}$), dimyristoyl-PA ($C_{14:0}$), dipalmitoyl-PA ($C_{16:0}$; PA_{C16}) and distearoyl-PA ($C_{18:0}$) were examined as substrates at 1.5 mM. Each substrate

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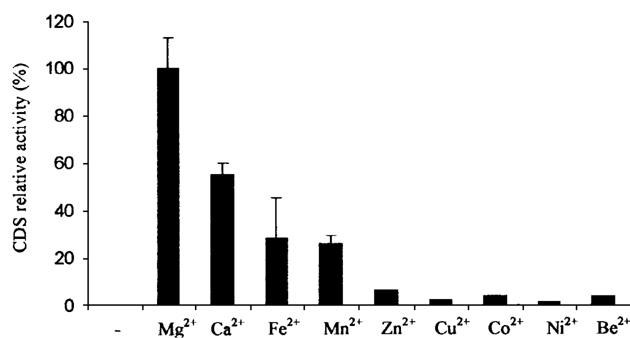

Figure 5 Metal-ion dependence of mycobacterial CDS activity

The activities were determined using 3 mM of each cation and were normalized to the value obtained with 3 mM $MgCl_2$; -, no metal ion.

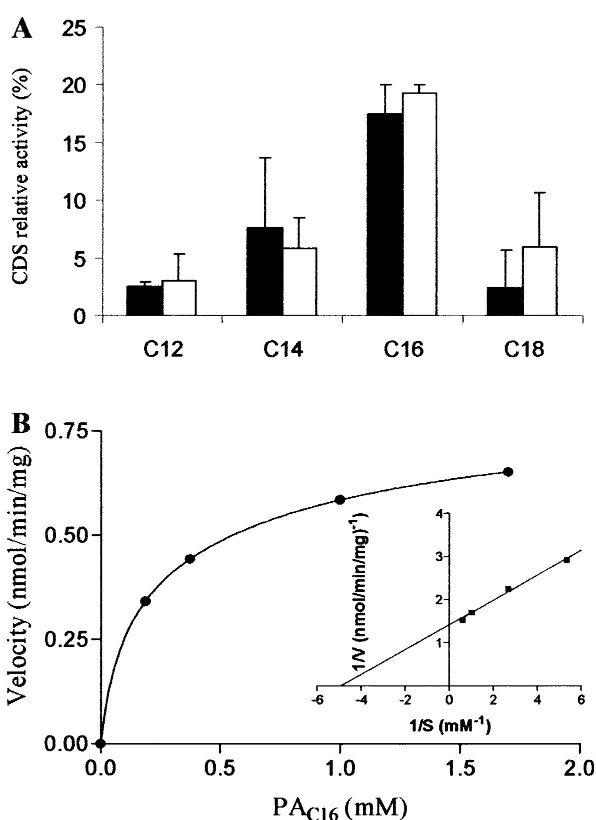

Figure 6 Substrate specificity of mycobacterial CDS activity

(**A**) CDS activity was assayed using dilauryl-PA (C12), dimyristoyl-PA (C14), dipalmitoyl-PA (C16) or distearoyl-PA (C18) in the presence of 7.5 mM (black bars) or 30 mM (white bars) Triton X-100. Activities were normalized to the value for the assay with PA_{eggs} as substrate. (**B**) The initial velocities of CDP-DAG production were measured by varying the concentration of PA_{C16} from 0 to 1.7 mM. The linear fit was used to calculate the K_m value of PA_{C16}, which was determined to be 0.23 ± 0.03 mM.

was tested using 7.5 or 30 mM Triton X-100 to take into account differences in solubility, which may arise from differences in the fatty acyl chain length. PA_{C16} was the best substrate giving rise to an activity of 19 % of that obtained with PA_{eggs}, whereas the other PAs gave activities less than half of those obtained with PA_{C16} (Figure 6A). The results are in agreement with the fact that

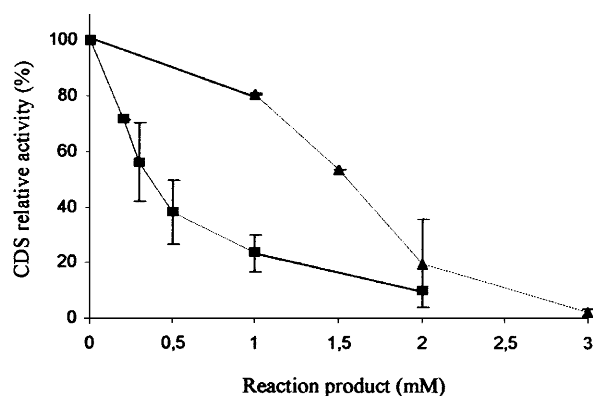

Figure 7 Inhibition of mycobacterial CDS activity

The reaction products, namely PP_i (▲) and CDP-DAG (■) were the inhibitors of the activity [at 37 °C, for 1.5 mM PA, 1 mM CTP, 3 mM $MgCl_2$, 50 mM KCl in 50 mM Tris/HCl (pH 8)]. Activities were normalized to the value for the assay without any product added. IC_{50} (PP_i) = 1.5 mM; IC_{50} (CDP-DAG) = 0.3 mM.

mycobacterial PLs mainly contain C_{16} and C_{19} fatty acid substituents. Using Triton X-100/PA_{C16} in the ratio 5:1, a K_m (app) of 0.23 ± 0.03 mM was found for PA_{C16} (Figure 6B). This value is slightly lower than that found for PA_{eggs} indicating that CDS enzyme may have a stronger affinity for PA_{C16} than PA_{eggs}. The difference in velocities may arise from a difference of solubility between the two PA types in Triton X-100, as described previously [19].

Regulation of CDS activity

Given the position of CDS as a major branch point in the PL biosynthetic pathway, its activity might be expected to be closely regulated [10,11]. However, very little information is available in the literature in this regard [10,11]. The reaction products, PP_i and CDP-DAG, were both inhibitors of CDS activity. CDP-DAG possessed an IC_{50} value of 0.3 mM, whereas PP_i possessed an IC_{50} value of 1.5 mM (Figure 7). Rat liver CDS activity was also reported to be inhibited by CDP-DAG in a similar manner, with an IC_{50} value of 0.5 mM [17].

It was previously reported that GTP, at a concentration of 1–2 mM, activated the rat liver microsomal CDS [21,22], whereas ATP, at a concentration of 5 mM, completely inhibited this activity [21]. In contrast, the mono-, di-, and tri-phosphorylated derivatives of adenosine, cytidine, guanosine and uridine, at concentrations of 1 mM, had no effect on *S. cerevisiae* CDS activity [18]. In the present study, we examined the effect of ATP, GTP and UTP, at concentrations of 2 mM and 4 mM, on *M. smegmatis* CDS activity. The three nucleotides were strongly inhibitory with more than 80 % inhibition at 2 mM (Figure 8). Higher concentrations of ATP and GTP (4 mM) completely abolished CDS activity, whereas a residual activity of 17 % remained using 4 mM UTP (Figure 8). GTP activator effect on rat liver microsomal CDS activity has been tentatively explained by a mechanism involving a covalent modification of the enzyme itself or a protein intimately associated with the CDS enzyme [23,24]. In contrast, the nucleotides may have a strong inhibitory effect on mycobacterial CDS activity, probably by competing with the CTP binding of the CDS enzyme.

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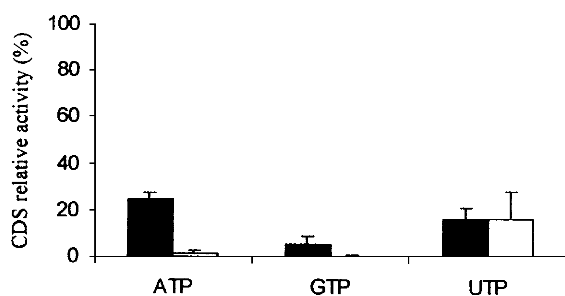

Figure 8 Effect of various nucleotides on mycobacterial CDS

Concentrations of 2 mM (black bars) and 4 mM (white bars) were used on CDS [at 37 °C, for 1 mM CTP, 1.5 mM PA in 50 mM Tris/HCl, 3 mM $MgCl_2$ (pH 8)]. Activities were normalized to the value obtained with the substrate CTP.

DISCUSSION

We have characterized CDS activity in mycobacteria, with regard to subcellular localization, pH dependence, bivalent and univalent cation requirement, substrate specificity, regulation by reaction products and nucleotides. The results presented are an important contribution to the delineation of PL biosynthesis in mycobacteria.

The localization of mycobacterial CDS activity in membranes was not surprising, since these enzymes are known to be membrane-bound and usually require non-ionic detergents for their solubilization and purification. So far, the partial purification of CDS enzymes has only been achieved to near homogeneity for *E. coli* [19] and *S. cerevisiae* [18]. The deduction of the *M. tuberculosis* genome [25] revealed an open reading frame (Rv2881c, *cdsA*), which is homologous with *E. coli* CDS. The predicted gene product is expected to have a molecular mass of 32 kDa and to contain eight putative transmembrane domains [25]. This is in agreement with the localization of CDS activity to membranes. As reported for enzymes from other species, mycobacterial CDS requires Mg^{2+} for activity and is stimulated by K^{+} ions [10,11]. Using a uniform mixed micelle of Triton X-100 and PA, mycobacterial CDS activity exhibited normal saturation kinetics and K_m values for PA were determined.

Given the position of CDS activity as a key branch point in the PL biosynthetic pathway, its activity might be expected to be closely regulated [10,11]. However, very little information is available to support this notion further [10,11]. One possible mechanism of regulation has been evoked by the finding that GTP, but not ATP or UTP, at a concentration of 2 mM activates CDS activity in rat liver microsomal fractions [24]. In contrast, we observed that mycobacterial CDS activity was dramatically inhibited by ATP, GTP and UTP at 2 mM. This is probably explained by the competition for the CTP binding site of the CDS enzyme and may represent a regulatory mechanism for mycobacterial CDS activity. Feedback inhibition by the reaction products can also be expected, since we have found that both PP_i and CDP-DAG are effective inhibitors of CDS activity. Another proposed regulatory mechanism would be selectivity governed by the fatty acid composition of the substrate PA [11]. Indeed, the composition of mammalian CDP-DAG closely resembles that of PI, in contrast with that of PA [26], suggesting that CDS might use a particular PA molecular species for CDP-DAG biosynthesis, which is further transformed into PI. However, examination of the effect of fatty acid composition of PA on mammalian CDS activity has revealed little or no selectivity [27]. In contrast, the fatty acid composition of PA, CDP-DAG and PI in potato tuber, pea leaf and soya-bean microsomes revealed that PA and CDP-DAG contain the same molecular species in similar amounts, whereas PI contains only two well-defined molecular species [28]. These results indicate that, in this case, the CDS enzyme does not possess any selectivity, whereas the regulatory step is at the PI synthase level [28]. The situation in mycobacteria is unclear, since there is no data concerning the structure of endogenous PA or CDP-DAG. PI, PIM and phosphatidylethanolamine are predominantly acylated by the saturated fatty acids C_{19} at *sn*-1 and C_{16} at *sn*-2 positions of the glycerol unit [2,4,29,30]. In contrast, CL is predominantly acylated with octadecenoic acid ($C_{18:1}$), C_{19} being present only in a smaller proportion, at *sn*-1 and with C_{16}, in the case of *M. tuberculosis* and *M. bovis* Bacille Calmette–Guérin, but with hexadecenoic acid ($C_{16:1}$) in the case of *M. smegmatis*, at *sn*-2 positions [2,29,30]. We have observed that among PA-containing saturated fatty acids, PA_{C16} was the best substrate with a K_m value of 0.23 ± 0.03 mM. This is in agreement with the fatty acid composition found in mycobacterial PLs with regard to acyl chain length, although we did not possess 1-tuberculostearoyl-2-palmitoyl-PA for a direct comparison. In addition, a direct comparison between PA bearing unsaturated and saturated fatty acids was precluded because of the relative insolubility of the latter in detergent solution [19]. This may explain the difference observed in velocities of PA_{eggs} and PA_{C16} (5-fold weaker for the latter), although the affinity of the mycobacterial CDS enzyme appeared stronger for PA_{C16} than for PA_{eggs} (K_m of 0.23 ± 0.03 mM compared with 0.61 ± 0.12 mM). Nevertheless, a major regulatory event determined by the fatty acid composition of mycobacterial PLs seems to be situated at the PA biosynthetic step. Indeed, it was shown that in *M. butyricum* (*M. smegmatis*), in the presence of palmitoyl-CoA and oleoy-CoA, 1-oleoyl-2-palmitoyl-glycerophosphate was predominately synthesized [13], which is in agreement with the positional distribution of these two fatty acids found in the CL of this mycobacterial strain [29]. A regulatory mechanism seems to be present at the PI synthase level, since the *M. smegmatis* PI synthase was shown to have a strict substrate specificity towards endogenous substrates [14]. The specificity could be abrogated by adding CHAPS to the PI synthase assay, a phenomenon already reported for PI synthase from maize coleoptiles [31].

In conclusion, this study provides an important contribution to the delineation of the biosynthesis of mycobacterial CDP-DAG, a central metabolite involved in PL biosynthesis.

This work was supported by the Medical Research Council (G9901077 and G9901078), the Wellcome Trust (058972/Z/99/Z), the National Institutes of Health (NIH) grant AI-38087 from the National Cooperative Drug Discovery Groups for the Treatment of Opportunistic Infections (NCDDG-OI), National Institutes of Allergy and Infectious Diseases (NIAID), NIH. G.S.B. is currently a Lister Institute Jenner Research Fellow. We gratefully acknowledge Dr L. Gannoun and Dr H. Vial (Université Montpellier II) for advice concerning the CDS assay.

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Received 5 March 2002/28 June 2002; accepted 10 July 2002
Published as BJ Immediate Publication 10 July 2002, DOI 10.1042/BJ20020370

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