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2C-Methyl-D-erythritol 4-Phosphate Enhances and Sustains Cyclodiphosphate Synthase IspF Activity

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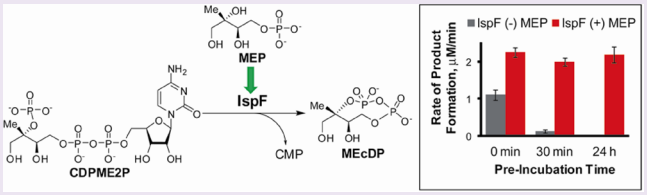
Supporting Information

ABSTRACT: There is significant progress toward understanding catalysis throughout the essential MEP pathway to isoprenoids in human pathogens; however, little is known about pathway regulation. The present study begins by testing the hypothesis that isoprenoid biosynthesis is regulated *via* feedback inhibition of the fifth enzyme cyclodiphosphate synthase IspF by downstream isoprenoid diphosphates. Here, we demonstrate recombinant *E. coli* IspF is not inhibited by downstream metabolites isopentenyl diphosphate (IDP), dimethylallyl diphosphate (DMADP), geranyl diphosphate (GDP), and farnesyl diphosphate (FDP) under standard assay conditions. However, 2C-methyl-D-erythritol 4-phosphate (MEP), the product of reductoisomerase IspC and first committed MEP pathway intermediate, activates and sustains this enhanced IspF activity, and the IspF-MEP complex is inhibited by FDP. We further show that the methylerythritol scaffold itself, which is unique to this pathway, drives the activation and stabilization of active IspF. Our results suggest a novel feed-forward regulatory mechanism for 2C-methyl-D-erythritol 2,4-cyclodiphosphate (MEcDP) production and support an isoprenoid biosynthesis regulatory mechanism *via* feedback inhibition of the IspF-MEP complex by FDP. The results have important implications for development of inhibitors against the IspF-MEP complex, which may be the physiologically relevant form of the enzyme.



Isoprenoids represent a large class of natural products and metabolites that include but are not limited to perfumes, drugs, lipids, and hormones.[1] Despite the impressive structural diversity of this natural product class, isoprenoids are all derived from dimethylallyl diphosphate (DMADP) and isopentenyl diphosphate (IDP), which are products of two independent metabolic pathways, the mevalonate (MVA) pathway and the 2C-methyl-D-erythritol 4-phosphate (MEP) pathway. The MVA pathway, first identified in the late 1950s,[2] was thought to be the sole source of IDP and DMADP until the MEP pathway was discovered in the early 1990s.[3−5] The MEP pathway is utilized by higher plants, algae, bacteria, and many pathogenic organisms including *Mycobacterium tuberculosis* and the apicomplexan parasites *Plasmodium falciparum* and *Taxoplasma gondii*. In contrast, mammals utilize the MVA pathway as the exclusive source of isoprenoids; thus, the MEP pathway enzymes have gained attention as promising targets for development of anti-infective agents and herbicides.[6−8]

The MEP pathway to IDP and DMADP is composed of seven enzymes starting from 1-deoxy-D-xylulose 5-phosphate (DXP) synthase (Figure 1), which catalyzes formation of DXP from pyruvate and D-glyceraldehyde 3-phosphate (GAP). Formation of 2C-methyl-D-erythritol 4-phosphate (MEP) from DXP is catalyzed by reductoisomerase IspC and represents the first committed step in non-mammalian isoprenoid biosynthesis. MEP then undergoes cytidylation (IspD) and phosphorylation (IspE) to form 4-diphosphocytidyl-2C-methyl-D-erythritol 2-phosphate (CDPME2P). Cyclodiphosphate synthase IspF, which is the focus of this study, catalyzes the conversion of CDPME2P to the cyclic diphosphate 2C-methyl-D-erythritol 2,4-cyclodiphosphate (MEcDP) with concomitant release of CMP. MEcDP undergoes reductive ring-opening catalyzed by IspG to form linear diphosphate (*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate (HMBDP), which is finally converted into IDP and DMADP by the action of the reductase IspH.

The fifth enzyme in the MEP pathway, IspF, catalyzes a unique cyclization reaction that has generated interest as a potential point of inhibition of isoprenoid biosynthesis toward the development of new anti-infective agents. Accordingly, this enzyme has been shown to be indispensable in *M. tuberculosis* and *B. subtilis* in studies to validate it as a drug target.[9−11] In this reaction, the 2-phosphate group of CDPME2P displaces CMP to form cyclic diphosphate MEcDP (Figure 2). X-ray crystallography and mechanistic studies have demonstrated that IspF is active in its trimeric form,[12] with three active sites at the monomer interfaces. The enzyme requires Zn^{2+} and Mg^{2+} to position the substrate and stabilize the developing charge in the pentavalent transition state.[11,13−17]

Several structural studies have now confirmed the presence of a hydrophobic cavity along the 3-fold symmetry axis of the IspF homotrimer[14,16−18] that appears to accommodate downstream isoprenoid intermediates (IDP/DMADP, GDP or

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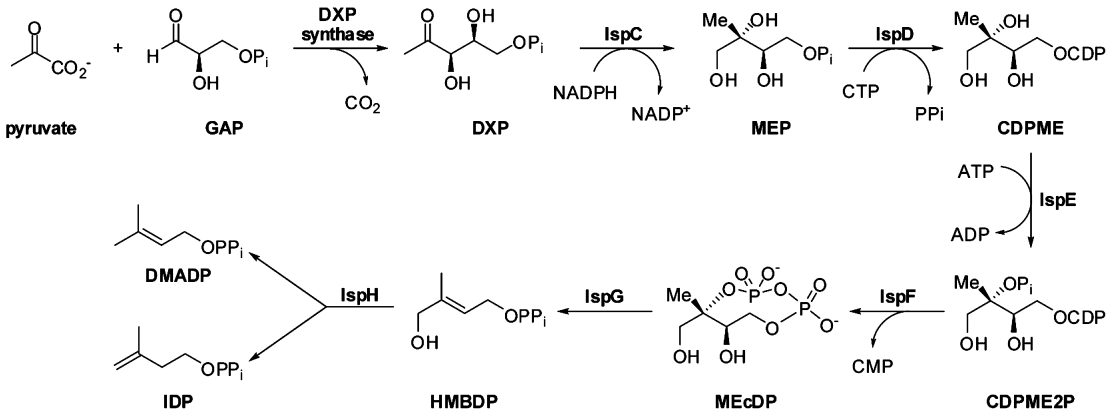

Figure 1. Methylerythritol phosphate (MEP) pathway.

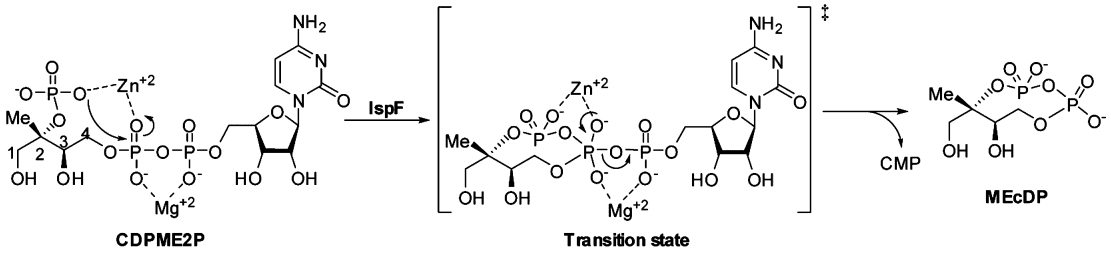

Figure 2. Proposed IspF reaction mechanism depicting substrate positioning and charge stabilization by Zn^{2+} and Mg^{2+}.

FDP), suggesting the possibility of feedback regulation of the MEP pathway *via* inhibition of IspF. However, to our knowledge, the biochemical evaluation of IDP/DMADP, FDP, or GDP as inhibitors of IspF has not been reported. In the present study to understand regulation of isoprenoid biosynthesis and identify new mechanisms of inhibition of this unique enzyme, we show that IspF is not inhibited by IDP, DMADP, GDP, or FDP under standard assay conditions. Contrary to our expectations, we have observed apparent enhancement of enzyme activity and subtle stabilizing effects on IspF activity by several downstream isoprenoid diphosphates. This unexpected outcome led to analysis of all MEP pathway intermediates as possible modulators of IspF activity, revealing the greatest enhancement of IspF activity and most striking activity-sustaining effects by the first committed pathway intermediate, methylerythritol phosphate (MEP). Here, we report the biochemical characterization of MEP-induced activation/stabilization of IspF and show that the 2*C*-methyl-D-erythritol scaffold itself drives this effect. Our studies come full circle to demonstrate biochemical inhibition of IspF by FDP, only in the presence of MEP. This work is of interest because it reveals a possible feed-forward regulatory mechanism for MEcDP production *via* MEP-induced activation/stabilization of IspF and supports an isoprenoid biosynthesis regulatory mechanism *via* feedback inhibition of the IspF-MEP complex by downstream isoprenoids.

RESULTS AND DISCUSSION

Characterization of IspF Activity by HPLC and NMR.

The biochemical evaluation of IspF is challenging because of the difficulties in accessing the substrate, CDPME2P, coupled with the instability of the substrate under a variety of assay conditions. In addition, the IspF product MEcDP (Figure 2) does not bear a chromophore and therefore is not detectable by HPLC-UV analysis. Thus, IspF-catalyzed formation of MEcDP from CDPME2P was confirmed using a two-dimensional H–P–P NMR method developed in our lab[19,20] (Supplementary Figure S2a,b). Formation of CMP in this reaction was confirmed by HPLC comparison to an authentic sample. An HPLC-based assay monitoring enzyme-catalyzed CMP formation from CDPME2P was previously developed for characterization of IspF.[13] Here, we have optimized assay conditions to prevent degradation of the CDPME2P and ensure reproducible HPLC retention times for substrate and product (Supplementary Figure S2c). First, we have determined that use of HEPES, Tris, MOPS, TEA, and Tricine buffers promotes degradation of the substrate CDPME2P to CDP at both 37 and 20 °C. Substrate degradation in these buffers is enhanced with increasing BSA or $MgCl_2$ concentrations. However, 50 mM phosphate buffer causes only minor substrate degradation at 37 °C, and no degradation is observed at 20 °C. Thus, the standard assay conditions reported here include the use of phosphate buffer at 20 °C.

Second, quenching of the enzyme reaction with EDTA or organic solvent (MeOH) results in unreliable retention times and peak areas of the product, CMP. This problem is resolved through the use of 0.1% SDS to quench reactions.[21−24] Under these optimized assay conditions, a pH/rate analysis indicates the *E. coli* IspF enzyme exhibits an apparent maximal rate at pH 7.4 in 50 mM phosphate buffer (Supplementary Figure S3).

Detailed kinetic analysis of IspF-catalyzed formation of CMP suggests that IspF follows hyperbolic kinetics, and kinetic parameters ($K_m^{CDPME2P}$ = 339 ± 32 μM and k_{cat} = 61 ± 3 min^{-1}; Table 1 and Supplementary Figure S4) are comparable to those previously reported.[25] Various inhibitors of IspF have been reported[25−27] including CDP.[26] For further validation of this HPLC-based assay, we tested inhibition of IspF by CDP. Under optimized conditions and in the presence of 100 μM CDPME2P, an IC_{50} of 768 μM was determined for CDP (Supplementary Figure S5).

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Table 1. Summary of the Kinetic Parameters of *E. coli* Cyclodiphosphate Synthase IspF at 20°C in the Absence or Presence of 2C-Methyl-D-erythritol 4-Phosphate (MEP) or 2C-Methyl-D-erythritol (ME)

	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (μM^{-1} min^{-1})	AC_{50} (μM)
IspF	339 ± 32	60.6 ± 3	0.18	
IspF + MEP	93.8 ± 11	80.7 ± 5	0.86	133 ± 33
IspF + ME	119 ± 18	87.4 ± 7	0.74	106 ± 14

Evaluation of IDP/DMADP, GDP, and FDP as Inhibitors of IspF. Crystallographic studies of IspF by several groups have reported the presence of electron density in the hydrophobic intersubunit cavity of the enzyme.[14,18] Subsequent mass spectrometry experiments identified the downstream isoprenoid products IDP/DMADP, GDP, and FDP bound to IspF in an approximate ratio of 1:4:2, respectively.[18] These findings suggested the possibility of feedback regulation of the MEP pathway through inhibition of IspF by downstream isoprenoids. However, in the present study, we are unable to demonstrate biochemical inhibition of IspF by IDP, DMADP, GDP, or FDP at concentrations up to 500 μM (Figure 3),

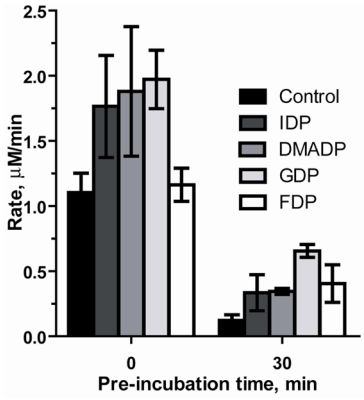

Figure 3. Rate of IspF-catalyzed CMP formation when enzyme reactions are initiated by addition of IspF (0 min) or CDPME2P (30 min) following a 30-min preincubation of IspF with 500 μM IDP, DMADP, GDP, or FDP.

under our standard assay conditions. To examine the possibility of a feedback inhibition mechanism requiring a particular composition of downstream isoprenoids,[18] we tested the inhibition of IspF in the presence of isoprenoid mixtures containing a 1:4:2 ratio of IDP, GDP, and FDP. Again, inhibition of IspF was not observed, even in the presence of 200:800:400 μM IDP/GDP/FDP (Supplementary Table S1). Interestingly, under standard assay conditions an apparent enhancement of IspF reaction rate, albeit subtle, was observed in the presence of IDP (1.6-fold increase in initial rate at 500 μM), DMADP (1.7-fold increase in initial rate at 500 μM), and GDP (1.8-fold increase in initial rate at 500 μM), compared to control (Figure 3). In the absence of any observed inhibitory effects by downstream metabolites, we reasoned that inhibition of IspF by these isoprenoids might instead be time-dependent. Thus, we re-evaluated IDP, DMADP, GDP, and FDP as inhibitors under conditions where these downstream metabolites were preincubated with IspF for 30 min, and initial reaction rates were measured after initiation with the substrate CDPME2P (Figure 3). In the absence of added isoprenoids, only 11% activity is retained following preincubation at 20 °C. In the presence of 500 μM IDP or DMADP, 19% and 18% activity is retained, respectively, following a 30-min preincubation. Interestingly, preincubation of IspF with 500 μM GDP or FDP prevents loss of IspF activity to some degree. In the presence of GDP, ~33% activity is retained following preincubation, representing a 5.4-fold enhancement in the rate of CMP formation, compared to control after the same preincubation. In the presence of FDP, 35% activity is retained, and the rate of CMP formation is 3.3-fold higher than control after the same preincubation. Sustained activity was also evident following a 10-min preincubation, and rate enhancements at this time point fall within expected trends.

Evaluation of Activity-Enhancing and Activity-Sustaining Effects of DXP, MEP, CDPME, and HMBDP. The moderate enhancement of IspF activity by downstream isoprenoids and the apparent stabilization effects of GDP and FDP on enzyme activity through a 30-min preincubation prompted the analysis of the activity-enhancing and/or activity-sustaining effects of other MEP pathway intermediates. Upstream MEP pathway intermediates DXP and CDPME, products of DXP synthase and IspD, respectively, neither increase initial rate nor prevent the loss of IspF activity (Figure 4a). The product of IspG, HMBDP, exhibits subtle enhancing effects (1.4-fold rate increase at 500 μM HMBDP) and sustains IspF activity comparably to IDP and DMADP with 18% residual activity of IspF after a 30-min preincubation (Figure 4a). However, MEP, the product of reductoisomerase IspC and first dedicated intermediate in non-mevalonate isoprenoid biosynthesis, demonstrates the most striking effects of any MEP pathway intermediate tested in this study. MEP enhances the IspF-catalyzed rate of CMP formation by 2-fold (at 500 μM MEP), and notably, this activity is sustained for >24 h. Interestingly, analysis by size exclusion chromatography indicates the quaternary structure of IspF appears unchanged over time (data not shown), in the presence or absence of MEP, and inclusion of the reducing agent TCEP in the enzyme assay buffer has no effect on the time-dependent deactivation of IspF (data not shown). Thus, changes in quaternary structure or oxidation state of IspF neither explain the observed time-dependent instability in the absence of MEP nor account for the stabilization effects of MEP. In addition, the inactivated form of IspF cannot be reactivated by MEP (following a 30 min preincubation at 20 °C), suggesting MEP induces rate enhancement and prevents loss of activity of a more physiologically relevant form of IspF.

Methylerythritol Scaffold Is Essential To Enhance and Sustain IspF Activity. The absence of IspF enhancing effects of the monophosphate-containing bioprecursor DXP suggests the methylerythritol scaffold itself contributes to the particular effects exhibited by MEP. We prepared 2C-methyl-D-erythritol (ME) and D-erythritol 4-phosphate (EP) (Figure 4b)[28] to assess the contribution of either the phosphoryl or 2C-methyl group, respectively, in the activity-enhancing and stabilizing properties of MEP. Under the same reaction conditions, ME exhibits an activating effect comparable to that of MEP (2.0-fold at 500 μM, Figure 4b), and IspF activity in the presence of ME, like MEP, is retained for >24 h. Interestingly, the 2C-desmethyl analogue EP only subtly activates IspF (1.3-fold at 500 μM, Figure 4b) and does not sustain IspF activity despite sharing a structure similar to that of MEP. These results suggest these effects are specific and driven by the 2C-methylerythritol scaffold that is unique to this pathway. The lack of activating or

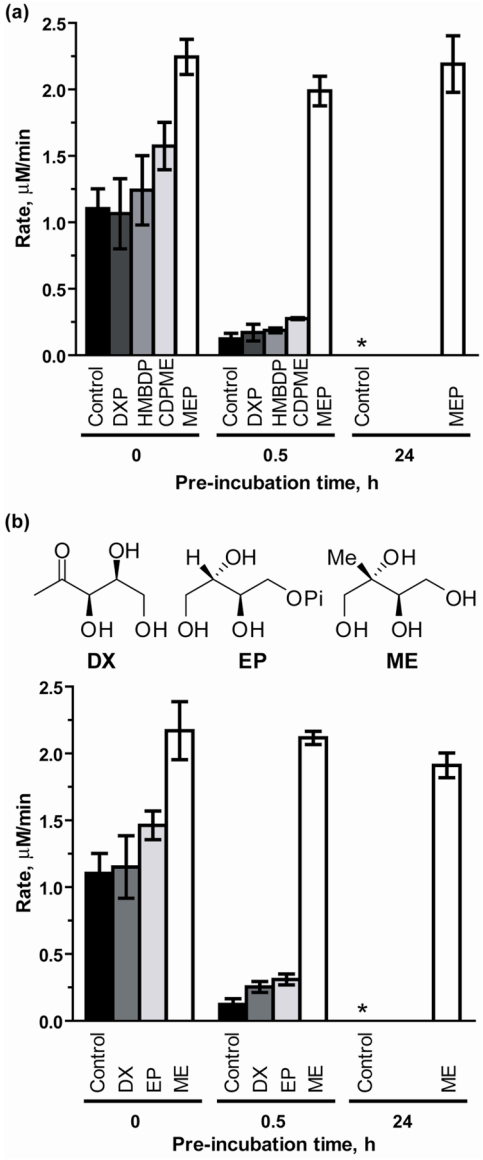

Figure 4. (a) Rate of CMP formation in the presence of 500 μM DXP, CDPME, HMBDP, DX, EP, MEP, and ME. (b) Structures of D-erythritol 4-phosphate (EP), 2*C*-methyl-D-erythritol (ME), and deoxyxylulose. *No appreciable rate after 24 h.

stabilizing properties of other polyhydroxylated compounds, deoxyxylulose (DX) and the widely used enzyme stabilizers glycerol or DDM (Supplementary Figure S6), further supports this specific effect of MEP.

Characterization of MEP and ME Effects on IspF Turnover Efficiency. The activity-enhancing properties of MEP and ME were characterized by measuring kinetic parameters of IspF in the presence of MEP or ME and by determining the AC_{50} value for each, the concentration of the additive that elicits 50% of IspF maximal activity.[29,30]

In the presence of 100 μM CDPME2P, an AC_{50}^{MEP} of 133 ± 33 μM was determined (Table 1, Supplementary Figure S7), and the maximal initial rate under these conditions was found to be ~2-fold the initial rate measured in the absence of MEP. It was reasoned that MEP may enhance turnover efficiency as a consequence of changes in $K_m^{CDPME2P}$, k_{cat}, or both. To gain further insight into MEP-mediated IspF activation, we determined the kinetic parameters of IspF in the presence of 500 μM MEP, conditions producing a near-maximal rate enhancement. In the presence of MEP, $K_m^{CDPME2P}$ = 93.8 ± 11 μM (Table 1, Supplementary Figure S4), representing a 3.6-fold increase in affinity of the substrate ($K_m^{CDPME2P}$ = 339 ± 32 μM in the absence of MEP). A comparable k_{cat} (1.3-fold) was also observed in the presence of MEP (k_{cat} = 80.7 ± 5 min^{-1}). The addition of MEP therefore results in a 4.8-fold increase in the efficiency of turnover (k_{cat}/K_m), consistent with the idea that MEP induces a more active form of IspF (Table 1).

Similarly, an AC_{50}^{ME} of 106 ± 14 μM was measured (Table 1, Supplementary Figure S7). Further, ME appears to induce an IspF conformation displaying a 2.9-fold increase in affinity for the substrate ($K_m^{CDPME2P}$ = 119 ± 18 μM in the presence of 500 μM ME, Table 1, Supplementary Figure S4), comparable to the effect observed in the presence of MEP. The k_{cat} in the presence of ME (80.7 ± 5 min^{-1}, 1.4-fold) is also comparable to that of MEP reaction. Thus, the efficiency of turnover (k_{cat}/K_m) increases 4.1-fold in the presence of ME, an increase comparable to that observed in the presence of MEP (Table 1, Supplementary Figure S4).

Inhibition of the IspF-MEP Complex. The idea that MEP enhances activity of IspF and prevents enzyme deactivation raises questions about the influence of IspF inhibitors on the IspF-MEP complex and, in particular, the relevance of the IspF-MEP complex in a feedback inhibition mechanism to regulate isoprenoid biosynthesis. To ascertain the effects of MEP on inhibition of IspF, we re-evaluated the known inhibitor CDP as well as downstream metabolites that could act as feedback inhibitors (HMBDP, IDP, DMADP, GDP, and FDP). In the absence of MEP, CDP inhibits IspF with an IC_{50} of 768 μM (Supplementary Figure S5). However, in the presence of 500 μM MEP, comparable initial rates of CMP formation were observed in the presence or absence of 700 μM CDP (Figure 5), suggesting the putative IspF–MEP complex is less sensitive to the effects of CDP.

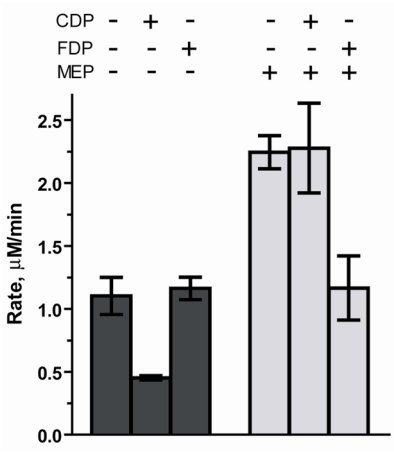

Figure 5. Evaluation of inhibitory effects of CDP (700 μM) and FDP (500 μM) in the presence or absence of MEP (500 μM).

In the absence of MEP, downstream metabolites HMBDP, IDP, DMADP, and GDP are not inhibitors of IspF (Figure 3). Likewise, these downstream metabolites do not inhibit the IspF-MEP complex (Supplementary Table S2). However, while FDP shows no IspF inhibitory activity in the absence of MEP (Figure 3), inhibition of the IspF–MEP complex (43%) by FDP is observed (Figure 5, Supplementary Table S2),

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suggesting a MEP-stabilized conformation may be more sensitive to the effects of FDP.

Discussion. The MEP pathway has drawn tremendous interest as a target for drug discovery since it is absent in humans and is the sole source of essential isoprenoid building blocks in many pathogenic organisms.[3−8] In addition, the pathway has the potential to be manipulated through bioengineering methods to enable increased and sustained production of isoprenoids with medicinal value.[31−34] Understanding enzyme function and regulation in the MEP pathway is critical in the endeavor to target these enzymes in the development of new anti-infective agents or optimize isoprenoid production in bioengineering efforts. However, relatively little is known about regulation of the MEP pathway in pathogenic organisms.[35,36] Studies carried out by Brown *et al.* demonstrated that overexpression of the *dxs* gene (encoding DXP synthase) in *M. tuberculosis* leads to accumulation of downstream MEP pathway product HMBDP, *via* upregulation of IspC (Dxr) and IspG (GcpE).[35] Their findings suggest that DXP synthase (Dxs) is involved in transcriptional control and therefore controls flux at the starting point of the pathway. In addition, the authors proposed IspG (GcpE) as a rate-limiting step of the pathway. Other studies have focused on the interesting IspDF bifunctional enzyme[36−39] as a potential regulatory mechanism in isoprenoid biosynthesis. IspDF is known to catalyze the first and third steps in the conversion of MEP to MEcDP in several bacterial species, and early reports proposed this organization of enzymes could enhance flux through the MEP pathway by substrate channeling through a IspDF/IspE complex;[38] however, studies by Lherbert *et al.*[40] could not provide evidence of enhanced metabolic flux through this complex, suggesting the IspDF bifunctional enzyme may serve some other regulatory role.

The present study was motivated by the hypothesis that isoprenoid biosynthesis is regulated through feedback inhibition of IspF.[14,17,18] Our initial results suggested that IspF is not inhibited by downstream isoprenoids IDP, DMADP, GDP, or FDP under standard assay conditions, in the absence of any other additives. Contrary to our initial expectations, the rate of formation of CMP is slightly enhanced in the presence of downstream isoprenoids; further, whereas IspF activity is dramatically decreased over time in the absence of additives, addition of downstream isoprenoid metabolites to the preincubation mixture appears to prevent the loss of IspF activity by varying degrees. It is unknown whether this subtle effect occurs through binding of isoprenoid diphosphates in the hydrophobic cavity of the IspF homotrimer.

Subsequent investigation of all MEP pathway intermediates as potential modulators of IspF activity has revealed the most striking effects by the first committed intermediate in non-mammalian isoprenoid biosynthesis, MEP. In the presence of MEP, the rate of CMP formation is increased to ∼2-fold, and this activity is sustained for >24 h. Upstream metabolites DXP and CDPME neither enhance nor prevent the loss of IspF activity, and HMBDP displays subtle effects similar to those of IDP and DMADP, suggesting that the activating/stabilizing effects are specific to MEP within the context of this pathway. Additional structure−activity relationship studies demonstrate the methylerythritol scaffold itself drives the effects of MEP on IspF, and highlights the particular requirement for the 2*C*-methyl substituent that is unique to the MEP pathway.

An analysis of initial rates of CMP formation at varying MEP concentrations indicates an AC_{50} of 133 ± 33. Zhang *et al.* recently reported MEP levels in *E. coli* to be 0.055 fg/cell (corresponding to ∼394 μM, assuming a cell volume of 0.65 μm^3),[41,42] suggesting the concentration of MEP required to activate IspF is physiologically relevant. Further, detailed kinetic analysis of IspF in the presence of MEP reveals a 4.8-fold increase in k_{cat}/K_m, which is primarily a consequence of increased affinity of IspF for its substrate CDPME2P and is comparable to the effects of other known small molecule activators.[29] Interestingly, ME induces a similar increase in turnover efficiency. While the results of these biochemical analyses suggest MEP- and/or ME-induced activation of IspF is possible *in vivo*, further studies are required to evaluate the effects of MEP depletion on cellular IspF activity and determine the role, if any, of ME *in vivo*.

The finding that MEP increases turnover efficiency and prevents the loss of IspF activity suggests a feed-forward mechanism to maximize MEcDP production and raises interesting questions about the regulatory role of MEP in the cellular requirement for MEcDP. MEcDP is known to accumulate to high levels in many organisms in response to oxidative stress,[43−49] suggesting MEcDP may play a role as antistressor through regulation of metal-dependent enzymes by cation chelation or by regulation of antioxidants. More recently, Grieshaber *et al.*[50] have demonstrated that MEcDP facilitates release of DNA from the histone-like protein Hc1 and thus from chlamydial chromatin during differentiation of elementary bodies (infectious extracellular form) to reticulate bodies (intracellular replicate form). Similarly, Goncharenko *et al.* have also shown that addition of MEcDP to resting ("nonculturable") forms of *M. smegmantis* reactivates growth, suggesting MEcDP may play a role in mycobacterial transition to and from latency[51] through a mechanism involving regulation of chromatin condensation−decondensation. Thus, MEP-induced stabilization of IspF activity would ensure sustained levels of MEcDP for purposes apart from isoprenoid biosynthesis; MEP may act as regulator of MEcDP levels in pathogenesis.

The observation that the IspF−MEP complex is inhibited by the downstream isoprenoid metabolite, FDP, suggests MEP induces a conformational change in IspF and the IspF−MEP complex is more sensitive to inhibition by FDP. Inhibition of the more active IspF−MEP complex by FDP supports the notion that feedback inhibition is a potential regulatory mechanism for isoprenoid biosynthesis under conditions that favor high levels of MEcDP.

The biochemical mechanisms for IspF activation by MEP and inhibition of the IspF-MEP complex by FDP are not well-understood. On the basis that MEP induces enhancement in IspF turnover efficiency by decreasing $K_m^{CDPME2P}$ and shares structural components with the natural substrate, it is tempting to speculate that MEP may act as a feed-forward activator by binding to one of the three active sites. Given that CDPME2P itself exhibits hyperbolic kinetics in the absence of MEP, it is possible that MEP could adopt a binding mode distinct from substrate in at least one of the active sites to increase affinity of CDPME2P and induce a more active form of the enzyme that is also more stable. On the basis of previous reports[14,17,18] and the hydrophobic environment at the trimer interface, it is also reasonable to speculate that FDP may inhibit the IspF−MEP complex through binding in the hydrophobic cavity. However, further biochemical and structural studies are required to shed light on these potential mechanisms of regulation.

The possibility that MEP plays a role in regulating MEcDP levels has important implications for anti-infective drug development. First, if activation and stabilization of IspF is indeed specific to MEP, it follows that inhibition of upstream enzymes to deplete cellular levels of MEP may also expedite loss of IspF activity. It is possible that fosmidomycin, a potent inhibitor of IspC[52] and weak inhibitor of IspD,[41] may also attenuate IspF activity through depletion of enzyme-stabilizing MEP. Thus, inhibitor combinations targeting IspC and IspF may result in potent inhibition of isoprenoid biosynthesis and attenuation of pathogenesis in bacterial pathogens.

Lastly, in terms of developing inhibitors of IspF, our results suggest that careful consideration should be given to the conditions under which potential IspF inhibitors are evaluated. In addition to recharacterizing the inhibitory effects of FDP in the presence of MEP, we have evaluated the previously characterized IspF inhibitor CDP in the presence or absence of MEP. Whereas CDP exhibits weak inhibitory activity in the absence of MEP, this effect is diminished in the presence of MEP, providing further evidence that MEP induces a conformational change in IspF that alters its susceptibility to inhibitors. To date, several studies toward development of IspF inhibitors have been reported.[26] It will be of interest to see how inhibitory activities are altered in the presence of MEP and whether compounds that reverse the activating/stabilizing effects of MEP can be identified as possible inhibitors of isoprenoid biosynthesis.

METHODS

Reagents. HMBDP, IDP, DMADP, GDP, and FDP were obtained from commercial sources. DXP and MEP were prepared according the procedures of Taylor *et al.*[53] and Urbansky *et al.*[28] respectively. CDPME was prepared enzymatically from MEP following the procedures of Illarionova *et al.*[54] and Narayanasamy *et al.*[55]

Overexpression and Purification of *E. coli* Cyclodiphosphate Synthase IspF. *E. coli*. BL21 (DE3) competent cells harboring *ispF*-pET24b were grown to $OD_{600} \sim 1.2$ and induced with isopropyl β-D-thiogalactoside (IPTG, 100 μM) at 37 °C, and shaking was continued for an additional 5.5 h. The cells were harvested by centrifugation (2000 × g, 20 min) and stored at −20 °C. The cell pellet was thawed on ice and resuspended in protein purification buffer (2 mL of buffer per gram of cell pellet) containing 125 mM Tris pH 8.0, 157 mM NaCl and 10% v/v glycerol. Cells were lysed by French Press and centrifuged at 4 °C (30 min, 15, 000 rpm) to pellet cell debris and insoluble protein. The supernatant was incubated with Ni^{2+} resin supplemented with 2 mM imidazole for 4.5 h at 4 °C. C-His_6 IspF was eluted from the resin in 5 mL fractions over a stepwise gradient of 5−500 mM imidazole. The fractions were analyzed by 15% SDS-PAGE (stained with Coomassie Brilliant Blue G.), and those containing pure protein were pooled. IspF was dialyzed overnight against a 2 L buffer containing 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA and 10% v/v glycerol. A second dialysis was carried out against a 2 L buffer containing 50 mM Tris-HCl pH 8.0, 100 mM NaCl, 1 mM TCEP and 10% v/v glycerol. Enzyme concentration was determined by Bradford Protein Assay with bovine serum albumin (BSA) as a standard. The C-His_6 IspF stock solution was flash frozen in liquid nitrogen and stored at −80 °C. Yield: 63.4 mg IspF/L of culture (Supplementary Figure S1).

Preparation of 4-Diphosphocytidyl-2*C*-methyl-D-erythritol 2-Phosphate (CDPME2P). CDPME2P was prepared following a modified procedure of Illarionova *et al.*[54] A mixture of MEP (5 mM), CTP (5 mM), ATP (500 μM), phosphoenol pyruvate (7 mM), $MgCl_2$ (5 mM), *E. coli* IspD (2 μM), inorganic pyrophosphatase (7 units), pyruvate kinase (3.5 units), BSA (1 mg mL^{-1}), and *E. coli* IspE (2 μM) in 100 mM Tris (pH 8.0) was incubated at 37 °C for 1.75 h in a total volume of 8 mL. The reaction was quenched with 8 mL of cold acetonitrile, and the mixture was vortexed for 30 s to precipitate proteins. The cloudy mixture was centrifuged at 4000 rpm for 20 min at 4 °C and filtered, and the supernatant containing product was concentrated under reduced pressure at room temperature to remove volatile organics and then subjected to purification by reversed-phase ion-pair HPLC using a Varian Dynamax C18 250 mm × 21.4 mm prep column. The column was developed with a linear gradient of 0 to 30% B at a flow rate of 10 mL/min (where A = 100 mM ammonium acetate buffer, 5 mM tetrabutyl ammonium bisulfate, pH 6.0, and B = acetonitrile containing 5 mM tetrabutyl ammonium bisulfate). Fractions containing the desired compound (λ_{max} = 272 nm, from 37.20 to 44.21 min) were combined and concentrated under reduced pressure to remove organic solvent. The resultant mixture was diluted with cold (4 °C) ddH_2O and lyophilized. Dilution with ddH_2O and lyophilization was repeated twice to ensure complete removal of ammonium acetate buffer. The resulting white powder was dissolved in ddH_2O and subjected to ion exchange chromatography to convert the product to the ammonium form, using 8 g of NH_4^+-form DOWEX WX8-200 resin. It should be noted that CDPME2P degradation following purification can be prevented by keeping any/all stock solutions on ice. The resultant compound, 12.2 mg (91%), was identical to previously reported CDPME2P.[19]

HPLC-Based IspF Assay. IspF reaction mixtures contained 50 mM phosphate buffer, pH 7.4, 5 mM $MgCl_2$, CDPME2P, 50 nM IspF and 50 μg/mL BSA in a total volume of 160 μL. The reactions at were initiated by addition of IspF to the mixture at 20 °C. *Sample preparation and analysis:* To terminate the IspF reaction, 40 μL of reaction mixture was added to 80 μL of cold 0.1% SDS at 2, 4, and 6 min. Quenched mixtures were briefly vortexed and incubated on ice for 15 min. To remove proteins prior to HPLC analysis, the quenched reaction mixture was passed through 3K MWCO (molecular weight cut-off) Nanosep centrifugal devices from Pall Corporation. Samples (90 μL) were injected onto a Beckman HPLC equipped with low-retention PEEK tubing to reduce sample-to-metal interaction[56−58] and analyzed by reversed-phase ion-pair HPLC using an Altima C18 3 μ, 53 mm × 7 mm rocket column. The column was developed with a linear gradient of 0 to 100% B at a flow rate of 3 mL/min (where A = 100 mM phosphate buffer, 5 mM tetrabutyl ammonium bisulfate, pH 6.0 and B = 100 mM phosphate buffer, 5 mM tetrabutyl ammonium bisulfate in 30% acetonitrile (retention times: CMP = 1.10 min, CDPME2P = 3.53 min, Supplementary Figure S2c). The CMP and CDPME2P peak areas were measured, and the concentration of CMP was calculated as a fraction of the total peak area. The data were analyzed using GraFit version 7 software.

Determination of IspF Kinetic Parameters. IspF reactions contained 50 mM phosphate buffer, pH 7.4, 5 mM $MgCl_2$, 4-diphosphocytidyl-2*C*-methyl-D-erythritol 2-phosphate (25−1000 μM in the absence of MEP or 25−700 μM in the presence of 500 μM MEP or 500 μM ME), 50 nM IspF, and 50 μg/mL BSA in a total volume of 160 μL. Samples were then analyzed as described above (Supplementary Figure S4).

IspF Inhibition Assay. IspF reactions contained 50 mM phosphate buffer, pH 7.4, 5 mM $MgCl_2$, 100 μM CDPME2P, varying concentrations of inhibitor, 50 nM IspF, and 50 μg/mL BSA in a total volume of 160 μL. For evaluation of CDP as an inhibitor (Supplementary Table S2, Figure S5), a concentration range of 0.1−1000 μM CDP was used (Supplementary Figure S4). For the initial evaluation of downstream isoprenoids as feedback inhibitors, each compound was tested at a final concentration of 500 μM (Supplementary Table S1). For reactions involving isoprenoid mixtures, IDP, GDP, and FDP were added to the reaction to a final ratio of 1:4:2, respectively (Supplementary Table S1). Reactions were initiated by addition of IspF to the mixture at 20 °C. Initial rates were measured using the HPLC-based assay described above. For determination of IC_{50}^{CDP} (Supplementary Figure S5), initial rates were plotted as a function of CDP concentration, and the IC_{50} was determined using GraFit version 7 software.

Assays To Evaluate Time-Dependent Effects. IspF was preincubated with the compound under evaluation for 30 min or 24 h at 20 °C, and the reaction was initiated with 4-diphosphocytidyl-2*C*-methyl-D-erythritol 2-phosphate (100 μM). For evaluation of down-

stream isoprenoids as time-dependent inhibitors, DMADP, IDP, GDP, and FDP were preincubated with IspF at a final concentration of 500 μM. For evaluation of other MEP pathway intermediates, DXP, CDPME, MEP, and HMBDP were preincubated with IspF at a final concentration of 500 μM. Initial rates were then measured using the HPLC-based assay described above (Supplementary Table S1).

MEP and ME AC_{50} Determination. IspF reactions contained 50 mM phosphate buffer, pH 7.4, 5 mM $MgCl_2$, 100 μM CDPME2P, 50 μg/mL BSA, and varying concentrations of MEP (1−1500 μM) or ME (1−2000 μM) in a total volume of 160 μL. Initial rates were measured using the HPLC-based assay described above, and plotted as a function of MEP concentration to determine the AC_{50}^{MEP} or AC_{50}^{ME} (Supplementary Figure S7). The data was analyzed by Kaleidagraph software version 4.03, using eq 1:[59]

$$y = \frac{A_f - A_0}{1 + \left(\frac{AC_{50}}{x}\right)} \quad (1)$$

A_f is fractional activity at test concentration x, A_0 is relative basal activity, AC_{50} is the concentration of activator (MEP) that gives 50% of maximal activity, and s is the slope factor.

Inhibition of MEP-Stabilized IspF. IspF reaction mixtures were prepared as described above for IspF inhibition assays. In addition, MEP (500 μM) was added to each mixture to promote formation of the MEP-stabilized form of IspF. CDP (tested at a concentration of 700 μM, Figure 5, Supplementary Table S2), HMBDP, IDP, DMADP, GDP (Supplementary Table S2), and FDP (tested at a final concentration of 500 μM, Figure 5, Supplementary Table S2) were then evaluated as inhibitors of IspF using the protocol described above. Initial rates of CMP formation were measured using HPLC.

■ ASSOCIATED CONTENT

Supporting Information

E. coli C-His_6-IspF Ni^{2+} affinity purification, IspF reaction product characterization by 2-D NMR and HPLC, pH/rate profile, IspF inhibition by CDP, tabulated rates and assessment of IspF stabilization by glycerol and DDM. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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