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The N-Terminus and the Chain-Length Determination Domain Play a Role in the Length of the Isoprenoid Product of the Bifunctional *Toxoplasma gondii* Farnesyl Diphosphate Synthase

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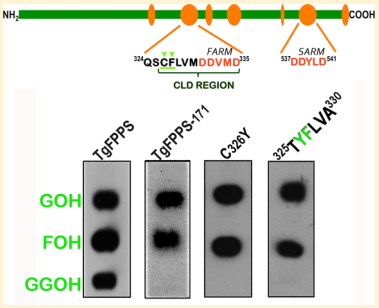
Supporting Information

ABSTRACT: *Toxoplasma gondii* possesses a bifunctional farnesyl diphosphate (FPP)/geranylgeranyl diphosphate (GGPP) synthase (TgFPPS) that synthesizes C_{15} and C_{20} isoprenoid diphosphates from isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). This enzyme has a unique arrangement of the fourth and fifth amino acid upstream from the first aspartic rich motif (FARM) where the fourth amino acid is aromatic and the fifth is a cysteine. We mutated these amino acids, converting the enzyme to an absolute FPPS by changing the cysteine to a tyrosine. The enzyme could be converted to an absolute GGPPS by changing both the fourth and fifth amino acids to alanines. We also constructed four mutated TgFPPSs whose regions around the first aspartate rich motif were replaced with the corresponding regions of FPP synthases from *Arabidopsis thaliana* or *Saccharomyces cerevisiae* or with the corresponding regions of GGPP synthases from *Homo sapiens* or *S. cerevisiae*. We determined that the presence of a cysteine at the fifth position is essential for the TgFPPS bifunctionality. We also found that the length of the N-terminal domain plays a role in determining the specificity and the length of the isoprenoid product. Phylogenetic analysis supports the grouping of this enzyme with other type I FPPSs, but the biochemical data indicate that TgFPPS has unique characteristics that differentiate it from mammalian FPPSs and GGPPSs and is therefore an important drug target.



Isoprenoids make up an extensive group of natural products with diverse structures consisting of various numbers of five-carbon isoprene units. The major building reaction in the pathway is the sequential condensation of isopentenyl diphosphate (IPP) with growing allylic isoprenoid diphosphates. The enzyme farnesyl diphosphate synthase (FPPS) plays a central role in this pathway in eukaryotes by producing farnesyl diphosphate (FPP), an important precursor of sterols, dolichols, ubiquinones, heme *a*, and prenylated proteins. FPPS forms FPP by the sequential condensation of dimethylallyl diphosphate (DMAPP) with two molecules of IPP. The C_{15} isoprenoid units, FPP, could further form GGPP, the C_{20} isoprenoid units, in a reaction catalyzed by the geranylgeranyl diphosphate synthase (GGPPS).

In contrast to mammalian cells, *Toxoplasma gondii*, a major opportunistic pathogen of fetuses from recently infected mothers and of patients with AIDS, possesses a bifunctional FPPS/GGPPS (TgFPPS) that is able to catalyze the formation of both FPP and GGPP.[1] This peculiarity was also recently reported for the *Plasmodium vivax* enzyme.[2] TgFPPS has a long N-terminal extension of ~164 amino acids, 42 of which (26%) are serine, and is localized to the mitochondrion.[1] TgFPPS is also a valid target for drugs because bisphosphonates, diphosphate analogues that are specific FPPS inhibitors, inhibit parasite growth in vitro and in vivo.[3−8] Lipophilic bisphosphonates that block prenyl synthases also have potent activity against *Plasmodium* liver stages.[9]

FPPS sequences contain seven conserved regions, including two aspartate rich domains named FARM, for first aspartic rich motif, and SARM, for second aspartic rich motif, that are critical for the catalytic action and most likely act as the sites for allylic substrates.[10] The fourth and fifth amino acids upstream of the FARM have been shown to have a critical role in the determination of the length of the isoprenoid products in the reactions catalyzed by FPPSs.[11] These two amino acids have aromatic side chains, like Phe and Tyr, that form the floor of the hydrophobic pocket in the interior of the enzyme that binds the growing hydrocarbon chain.[11] Replacement of these aromatic rings with smaller side chains leads to the formation of longer chain products.[12−14] This domain (from the FARM to the fifth amino acid upstream) was named the chain-length determination (CLD) domain (Figure S1A of the Supporting Information). Some reports indicate that amino acids located in regions other than those defined as CLD also play an important role in the product chain-length determination mechanism of prenyl synthases.[15,16] It is also important to note that the conserved aspartate residues of the two aspartate rich motif DDXXD (FARM and SARM) bind three divalent cations (Mg^{2+}) that are in turn coordinated by the phosphate backbone

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of bisphosphonates, which are potent inhibitors of these enzymes.[17]

When comparing the CLD region of TgFPPS with those of other GGPPSs and FPPSs, we found some interesting differences[1] (Figure S1A of the Supporting Information). The most interesting and relevant for this work is the fact that the fourth amino acid upstream of the FARM region is phenylalanine (F), an amino acid with a bulky aromatic side chain, but the fifth amino acid upstream of the FARM region is a cysteine (C). This arrangement is peculiar when compared to other short chain prenyl synthases because, for example, type I FPPSs have two aromatic amino acids at the fourth and fifth positions upstream of the FARM (Figure S1B of the Supporting Information) and type II FPPSs and type I GGPPS an aromatic amino acid in the fifth position upstream of the FARM and a small side chain-containing amino acid in the fourth position (Figure S1B of the Supporting Information). Interestingly, this peculiar amino acid organization is also found in the *P. vivax*[2] and *Plasmodium falciparum* FPPS orthologs: a phenylalanine in the fourth position and an alanine or a serine in the fifth position upstream of the FARM region, respectively. This uncommon combination of fourth and fifth amino acids may provide the enzymes with the unusual characteristic of being bifunctional, producing both FPP and GGPP as products.[1] However, no mutational study that supports this hypothesis has been conducted with any of these enzymes. This feature may be convenient for the parasites because there is no evidence of the presence of genes with homology to GGPPSs of other organisms in the genomes of either *T. gondii* or *Plasmodium* spp. (http://eupathdb.org/eupathdb/).

In this work, mutational studies of TgFPPS were performed to investigate the mechanism responsible for the bifunctionality of TgFPPS. We found that the CLD arrangement of amino acids does have a role in the bifunctionality of the enzyme. Unexpectedly, we found that the length of the N-terminal domain has a role in determining the length of the isoprenoid product of the TgFPPS reaction. In spite of its bifunctionality, our phylogenetic analysis shows that TgFPPS groups with other type I FPPSs and not with GGPPSs.

MATERIALS AND METHODS

Materials. Oligonucleotide primers were obtained from Integrated DNA Technologies (Coralville, IA). TaqDNA polymerase, the site-directed mutagenesis kit, and restriction enzymes were from Invitrogen. The ECL chemiluminescence detection kit and nylon membranes were from Amersham Biosciences. The plasmid miniprep kit, the gel extraction kit, and the plasmid maxiprep kit were from Qiagen Inc. (Chatsworth, CA). IPP, DMAPP, GPP, FPP, and GGPP were from Isoprenoids, Lc. Anti-FLAG M2 affinity resin was from Sigma. [4-^{14}C]Isopentenyl diphosphate triammonium salt (55.0 mCi/mmol) was from PerkinElmer Life Sciences. All other reagents were analytical grade.

Cell Cultures. Tachyzoites of *T. gondii* strain RH were cultivated in human fibroblasts and purified as described previously.[18] Host cells were cultivated in Dulbecco's modified minimal essential medium supplemented with 10% fetal bovine serum. Host cells were infected with tachyzoites at a ratio of 5:1 (parasite:host). Tachyzoites were collected 2−3 days post-infection. Cell cultures were maintained at 37 °C at 5% CO_2.

Construction of *TgFPPS* Mutants. The construction of TgFPPS-171 has been described previously.[1] The *TgFPPS-141* and *TgFPPS-103* expression cassettes were generated by polymerase chain reaction (primers 15 and 16 in Table S1 of the Supporting Information). The truncated *TgFPPSs* were cloned into the NcoI and XhoI sites of pET-28a using routine protocols. Site-directed mutagenesis was performed following the manufacturer's instructions (Invitrogen). pET-28a-*TgFPPS-141* was used as a template, and the oligonucleotides used are included in Table S1 of the Supporting Information (primers 1−14). All the mutants were confirmed by sequencing.

Expression and Purification of Recombinant *TgFPPS* in *Escherichia coli*. The *TgFPPS-103*-, *TgFPPS-141*-, and *TgFPPS-171*-expressing plasmids were introduced into *E. coli* strain BL21(DE3). The expression of recombinant TgFPPSs was induced by addition of 0.4 mM isopropyl β-thiogalactopyranoside at 18 °C overnight. The recombinant proteins were purified using His-Bind 900 cartridges (Novagen) following the manufacturer's instructions. The polyhistidine tag was not removed because this treatment resulted in an almost complete loss of the catalytic activity of FPPSs from other parasites,[19] while the His-tagged enzyme we previously purified from *T. gondii* had excellent activity.[1]

Purification of TgFPPS-FLAG with an Anti-FLAG Resin. Approximately 8×10^9 TgFO cells[1] were purified, washed, and resuspened in lysis buffer [20 mM Hepes, 50 mM KCl, 125 mM sucrose, and 0.5 mM EDTA (pH 7.2)]. A freeze−thaw method was used to break the parasites. The supernatant was collected by centrifugation at 12000*g* for 10 min at 4 °C and then mixed with the anti-FLAG M2 affinity resin for 3 h at 4 °C. After being washed with TBS buffer [50 mM Tris-HCl and 150 mM NaCl (pH 7.4)], the FLAG-tagged protein was eluted with 3× FLAG peptide in TBS buffer at 200 ng/μL.

Sodium Dodecyl Sulfate (SDS) Gel Electrophoresis and Western Blot Analyses. Western blot analyses were performed as described previously.[1] We used an affinity-purified rabbit anti-TgFPPS antibody generated by Cocalico Biologicals (Reamstown, PA) against the recombinant TgFPPS-171 protein.

Enzymatic Activity Assay and Kinetic Studies. The enzymatic assays were performed in a total volume of 100 μL of a mixture containing 10 mM Hepes buffer (pH 7.4), 1 mM $MgCl_2$, 2 mM dithiothreitol, 100 μM [4-^{14}C]IPP (10 μCi/μmol), allylic substrate (100 μM DMAPP, 27 μM GPP, or 13 μM FPP), and 4−40 ng of protein. The reactions were conducted at 37 °C for 30 min and terminated by the addition of 10 μL of 6 M HCl. The mixture was made alkaline by addition of 15 μL of 6 M NaOH, extracted with 1 mL of hexane, washed with water, and then transferred to a scintillation vial for counting.

For kinetic studies, the concentration of DMAPP, GPP, FPP, or IPP was varied, whereas the corresponding countersubstrate was kept at a saturating concentration. A nonlinear regression analysis in Sigma Plot 10.0 was used to estimate the kinetic parameters.

Reverse Phase TLC. The radioactive prenyl products synthesized during the enzymatic reactions were hydrolyzed to the corresponding alcohols by overnight incubation with an alkaline phosphatase (Life sciences, 18011-015) at room temperature. The alcohols were subsequently extracted with hexane and separated by thin layer chromatography using HP-TLC-RP18 plates with reversed phase Unibond octadecyl-modified silica gel (AnalTech) using an acetone/H_2O mixture (6:1, v/v) as the running solvent. The position of the standard prenyl alcohol was visualized using iodine vapor. Radioactivity

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was visualized by autoradiography or using a phosphor-Imaginer.

Phylogenetic Analysis. The prenyl synthase protein sequences were retrieved from the National Center for Biotechnology Information (NCBI) and OrthoMCL (version 1.0) databases. The phylogenetic tree from Wang and Ohnuma[11] was used as a guide to search for known farnesyl diphosphate (FPPS), geranylgeranyl diphosphate (GGPPS), and C_{30}−C_{50} diphosphate synthase protein sequences. The *E. coli* FPPS sequence was used to query the NCBI database using the genomic Basic Local Alignment Search Protein (BLASTP) tool by selecting for organisms ranging from prokaryotes to eukaryotes. The default BLAST parameters (expected threshold of 10, low-complexity filter, BLOSSUM 62 substitution matrix) were used.

Protein sequences were used to create multiple-sequence alignments to construct phylogenetic trees of prenyl synthases. Multiple-sequence alignments were created using the default (slow/accurate) multiple-alignment parameters of Clustal W.[20,21] To optimize the alignments, Multiple EM for Motif Elicitation (MEME)[22] was used to search for protein motifs and allow deletion of nonconserved regions. The default expected motif distribution was applied, which specifies the ZOOPS (zero or one per sequence − zero or one instance of the pattern per sequence). The GeneDoc (version 2.6.002) Multiple Sequence Alignment Editor and Shading Utility[23] was used to visualize the motifs identified by MEME. The phylogenetic analyses were conducted with MEGA5[24] using the Dayhoff matrix-based model for amino acid substitutions.[25] The evolutionary divergence between sequences was estimated to construct 100 phylogenetic trees by the neighbor-joining method.[26]

RESULTS

Heterologous Expression and Activity of Truncated TgFPPS. With the aim of increasing the solubility of the recombinant TgFPPS expressed in bacteria, we constructed expression vectors with truncated versions of the long N-terminal extension (absent in other FPPSs). Using this approach, we expressed TgFPPS proteins lacking 103, 141, or 171 amino acids of the N-terminal extension (Figure S1C of the Supporting Information). This approach improved the solubility of the recombinant proteins. The protein lacking the first 171 amino acids was the most soluble and was expressed at higher levels in *E. coli*. We also overexpressed the FLAG-tagged native enzyme in *T. gondii* and purified it using anti-FLAG affinity resins as described previously.[1] Figure 1 shows the Western blot analyses of the different proteins obtained using affinity-purified polyclonal antibodies against TgFPPS. It is interesting to note that the endogenous protein (Figure 1B) has a molecular mass (~58 kDa) similar to that of the FLAG-tagged native and recombinant truncated versions of the protein (Figure 1A and ref 1), while expression of the full-length recombinant TgFPPS in bacteria has a molecular mass of ~75 kDa (data not shown), which is closer to the calculated mass of 69.6 kDa (646 amino acids). This result agrees with the prediction using Mitoprot (http://ihg.gsf.de/ihg/mitoprot.html) that the mitochondrial targeting signal is processed at approximately 150 amino acids. These results suggest that the endogenous TgFPPS is processed in *T. gondii* losing at least part of its N-terminal extension.

We tested the activity of these proteins (native TgFPPS, TgFPPS-171, TgFPPS-141, and TgFPPS-103) and analyzed the

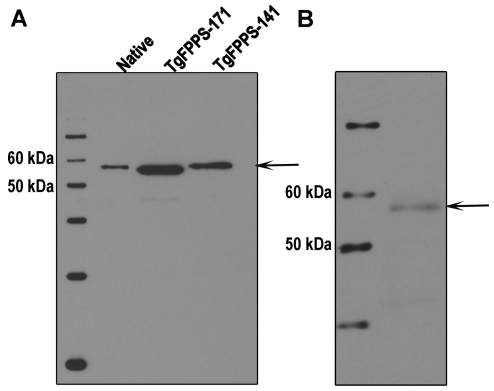

Figure 1. Western blot analysis of affinity-purified FLAG-tagged native, His-tagged truncated recombinant, and endogenous TgFPPS. (A) Purified native FLAG-tagged TgFPPS expressed in *T. gondii* (Native) has a size similar to the sizes of the two N-terminally truncated versions without 171 (TgFPPS-171) or 141 (TgFPPS-141) amino acids. TgFPPS-FLAG was expressed in *T. gondii* and purified by affinity column chromatography. His-tagged TgFPPS-141 and TgFPPS-171 were expressed in *E. coli* and purified using nickel columns. The purified proteins were subjected to sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) on 10% polyacrylamide gels, transferred to nylon membranes, and probed with an affinity-purified polyclonal antibody against TgFPPS (1:2000). (B) Western blot analysis of the endogenous TgFPPS from total lysates of *T. gondii* RH tachyzoites. Membranes were probed with the affinity-purified polyclonal antibody against TgFPPS (1:2000). Arrows show the band corresponding to the endogenous protein (~58 kDa).

products formed using TLC, and the data are shown in panels A−D of Figure 2, respectively. When native TgFPPS (Figure 2A) was used, a mixture of GPP, FPP, and GGPP was produced. However, the small amount of protein obtained precluded further analysis of this enzyme. We investigated the activity of the TgFPPP-103 or TgFPPS-141 recombinant enzyme with DMAPP as the substrate, and the reaction produced a mixture of GPP, FPP, and GGPP (Figure 2B,C). Using GPP as the substrate yielded FPP and GGPP, and FPP yielded only GGPP. These results are in agreement with a stepwise or partially processive mechanism of chain elongation. Unexpectedly, TgFPPS-171 showed mostly FPPS activity and formed GPP and FPP with DMAPP as the substrate or FPP with GPP as the substrate (Figure 2D). This mutant was not efficient at elongating FPP (Figure 2D), suggesting that the extra amino acids that are missing in this sequence have a role in determining the length of the isoprenoid product. Interestingly, the lack of this N-terminal extension did not affect the overall enzyme activity with DMAPP as the substrate while greatly decreased its activity with FPP as the substrate (Figure 2D and Table 1). Because TgFPPS-141 produced the same isoprenoid products as the native enzyme and the yield of the native enzyme (likely processed at 150 amino acids) was too low for further characterization, we used this truncated version for the subsequent studies.

Enzymatic Activity of Mutated TgFPPSs. FPPSs have been divided into two types [type I (eukaryotic) and type II (eubacterial)], while GGPPSs have been divided into three types (type I GGPPS, which includes archaeal GGPPS, type II GGPPS, from eubacteria and plants, and type III GGPPS, which includes yeast and mammalian GPPS) (Figure S1B of the Supporting Information). The CLD region, especially the amino acids occupying the fourth and fifth positions upstream

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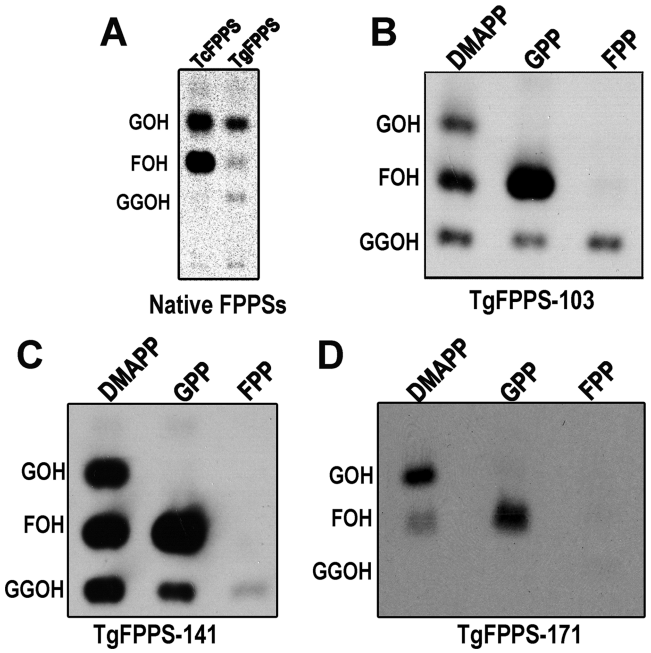

Figure 2. Reverse phase thin layer chromatography of the alcohols obtained by enzymatic hydrolysis of the products formed by the mutated TgFPPSs. Truncated versions of His-tagged TgFPPSs were expressed in *E. coli*, purified using nickel affinity chromatography, and incubated (40 ng of each enzyme purified from *E. coli* or 4 ng of FLAG-TgFPPS purified from *T. gondii* lystates) with [1-^{14}C]IPP and the different allylic substrates (DMAPP, GFP, and FPP) at 37 °C for 1 h. Other conditions are as described in Materials and Methods. Abbreviations: GOH, geraniol; FOH, farnesol; GGOH, geranylgeraniol. (A) Comparison of the products formed by the native TgFPPS purified from a *T. gondii* lysate as described in Materials and Methods. *Trypanosoma cruzi* FPPS (TcFPPS)[19] was used as a control. Note that there was only 4 ng of FLAG-TgFPPS used in this study while there was 40 ng of TcFPPS. (B) Products formed by the recombinant enzyme lacking 103 amino acids, expressed and purified from *E. coli*. IPP was used at saturating concentrations for all the assays, and the second substrate is indicated at the top. (C) Products formed by the enzyme TgFPPS-141 in the presence of saturating concentrations of IPP and one of the three substrates indicated at the top. (D) Products formed by the enzyme TgFPPS-171 in the presence of saturating concentrations of IPP and one of the three substrates indicated at the top.

of the FARM as well as two amino acid insertions within the FARM region (Figure S1B of the Supporting Information), has been reported to determine the final products of the reaction it catalyzes.[11] TgFPPS and *P. vivax* FPPS[2] have just one aromatic amino acid (phenylalanine) at the fourth position and a cysteine and alanine (or serine in the case of *P. falciparum*), respectively, at the fifth position upstream of the FARM (Figure S1B of the Supporting Information).[1] To investigate the role of the region around the FARM in the product specificity, we replaced the six-residue sequence between positions 325 and 330 of TgFPPS-141 (^{325}SCFLVM^{330}) with the corresponding sequences of two eukaryotic FFPSs, those from *Arabidopsis thaliana* (^{325}TYFLVL^{330}) or *Saccharomyces cerevisiae* (^{325}TYFLVA^{330}).[27] The mutant enzymes were overexpressed in *E. coli*, purified, and assayed for enzyme activity. Their K_m for DMAPP was not affected as compared to that of TgFPPS-141, while only the enzyme with the *S. cerevisiae* sequence had a lower V_{max} (Table 1). Both mutants behave as FPPSs generating GPP and FPP when DMAPP is used as the cosubstrate (Figure 3) but were unable to elongate FPP to GGPP (Figure 4). This mutagenesis analysis indicates that the bifunctional role of TgFPPS is also determined by this CLD.

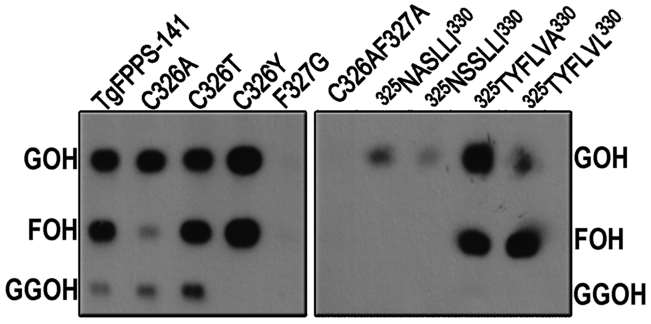

Figure 3. Conversion of TgFPPS to a FPPS by site-directed mutagenesis. Reverse phase thin layer chromatography of the alcohols obtained by enzymatic hydrolysis of the products formed by mutated TgFPPS-141. The His-tagged TgFPPS mutants were constructed as described in Materials and Methods. Each of the mutants was expressed in *E. coli* and purified using nickel affinity columns. The enzyme reactions were performed by incubation of 40 ng of each protein with DMAPP and IPP as substrates at 37 °C for 1 h. Other conditions are as described in Materials and Methods.

To determine whether the fourth or fifth amino acid upstream of the FARM played a significant role in the activity or in the determination of the chain length of the products of TgFPPS, the normal (TgFPPS-141) and mutant enzymes, in which an alanine, threonine, or tyrosine replaced the cysteine at the fifth position, or a glycine replaced the phenylalanine at the

Table 1. Enzymatic Properties of TgFPPS

	DMAPP		FPP	
mutation[a]	K_m (μM)	V_{max} [μmol min^{-1} (mg of protein)$^{-1}$]	K_m (μM)	V_{max} [μmol min^{-1} (mg of protein)$^{-1}$]
WT (-141)	18.7 ± 7.0	31.8 ± 5.5	2.3 ± 1.1	4.8 ± 0.4
WT (-171)	7.6 ± 2.0	19.6 ± 1.6	NA[b]	NA[b]
^{325}SCFLVM^{330}-^{325}TYFLVA^{330}	18.2 ± 3.1	14.4 ± 0.5	NA[b]	NA[b]
^{325}SCFLVM^{330}-^{325}TYFLVL^{330}	19.0 ± 3.8	37.1 ± 0.3	NA[b]	NA[b]
C326A	16.2 ± 4.7	22.1 ± 3.4	ND[c]	2.7 ± 1.2
C326Y	9.3 ± 0.3	20.4 ± 0.3	NA[b]	NA[b]
^{325}SCFLVM^{330}-^{325}NASLLI^{330}	5.6 ± 0.3	0.3 ± 0.2	4.7 ± 0.6	10.7 ± 0.2
^{325}SCFLVM^{330}-^{325}NSSLLI^{330}	44.9 ± 36.9	1.5 ± 0.8	7.2 ± 0.6	6.1 ± 0.5

[a]The recombinant proteins containing these mutated amino acids were expressed and purified and their activities measured as described in Materials and Methods. [b]No activity. [c]Not determined.

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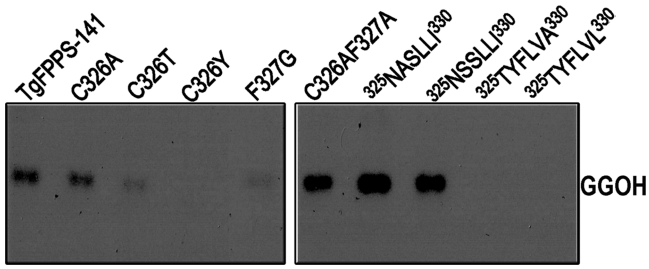

Figure 4. Conversion of TgFPPS to a GGPPS by site-directed mutagenesis. Reverse phase thin layer chromatography of the alcohols obtained by enzymatic hydrolysis of the products formed by mutated TgFPPS-141 in the presence of FPP and IPP. The TgFPPS mutants were those described in the legend of Figure 3. The enzyme reactions were performed by incubation of 40 ng of proteins with FPP and IPP as substrates at 37 °C for 1 h. Other conditions are as described in Materials and Methods.

fourth position, were also overexpressed in *E. coli*, purified, and assayed for activity. While substitution of the cysteine (^{326}Cys) with either alanine or threonine did not prevent the formation of GGPP using either DMAPP (Figure 3) or FPP (Figure 4) as the substrate, substitution with the bulky amino acid tyrosine prevented the generation of GGPP in agreement with the postulated role of the amino acid in the fifth position upstream of the FARM region in limiting the product length of FPPSs.[13] In either case, the V_{max} was decreased by ~30% while only the K_m for DMAPP was significantly decreased when tyrosine replaced ^{326}Cys, in agreement with its conversion into an exclusive FPPS. Conversion to an exclusive FPPS in the case of TgFPPS-171 also resulted in a similar reduction in the K_m for DMAPP (Table 1). Substitution of the bulky phenylalanine at the fourth position with a glycine greatly decreased the activity with either DMAPP (Figure 3) or FPP (Figure 4) as the cosubstrate. Presumably, this mutation causes additional structural changes that affect the affinity of the enzyme for the cosubstrate.

Conversion of TgFPPS into a Synthase Capable of Producing GGPP. To further confirm the role of the region around the FARM in product specificity, we replaced the sequence between positions 325 and 330 of TgFPPS-141 (^{325}SCFLVM^{330}) with the corresponding sequences of two eukaryotic GGPPSs, those from *Homo sapiens* (^{325}NASLLI^{330}) and *S. cerevisiae* (^{325}NSSLLI^{330}) (Figure S1B of the Supporting Information). The mutant enzymes were overexpressed in *E. coli*, purified, and assayed for enzyme activity. The mutants were able to produce GPP only when DMAPP was used as the cosubstrate (Figure 3) with very low activity (Table 1), while they have good activity with FPP as the cosubstrate (Table 1) and were able to generate GGPP (Figure 4). A double mutation of the fourth and fifth amino acids upstream of the FARM (^{326}CF^{327} to ^{326}AA^{327}) also converted TgFPPS into a straight GGPPS (Figures 3 and 4). The analysis of these mutants further supports the idea that the unique combination of the fourth and fifth amino acids upstream of the FARM is responsible for the bifunctionality of TgFPPS.

Phylogenetic Analysis of TgFPPS. Taking into account the bifunctionality of the TgFPPS, we were interested in analyzing its phylogenetic profile to see if the sequence grouped with other FPPSs or with GGPPSs. We used the *E. coli* FPPS protein sequence to search the NCBI database for the (*E*)-isoprenyl diphosphate synthases, FPPS, GGPPS, and C_{30}−C_{50} PP synthases (HEPPS, OPPS, and DPPS) (Table S2 of the Supporting Information). We also searched for TgFPPS orthologs using the OrthoMCL database. We recovered orthologs from *P. falciparum*, *Cryptosporidium hominis*, and *Cryptosporidium parvum*. We selected a set of sequences that included archaebacteria, eubacteria, and eukaryotic species.

MEME analysis of the selected sequences identified the seven conserved domains of FPPS and GGPPS and also the FARM and SARM. Some peculiarities are also highlighted after analysis of the alignments (Figure S2 of the Supporting Information). Amino acid residues of domains IV and V are mostly conserved in type I FPPSs. Type I FPPSs and most type II GGPPSs share domain VII, but different amino acids are found in the sequences of most type II FPPSs, type I GGPPSs, type III GGPPSs, C_{30}−C_{50} PP synthases, and bifunctional enzymes.

The phylogeny of the prenyl synthase was constructed using MEGA5, and it is shown in Figure 5. We built 100 phylogenetic trees using 47 sequences, which included FPPS, GGPPSs, and C_{30}−C_{50} PP synthases. The final tree displays bootstrap values of ≥50%. Most evolutionary relationships are supported by significant bootstrap values (>75%). Lower bootstrap values can be attributed to an incomplete phylogenetic inventory (missing species) and sequence divergence among species. Our phylogenetic tree shows that the Apicomplexan putative FPPSs cluster with type I FPPS enzymes. Other putative isoprenoid biosynthetic enzymes found in Apicomplexa cluster with long chain prenyl synthases.

■ DISCUSSION

In most organisms, the short chain prenyl GPP, FPP, and GGPP synthases elongate DMAPP to products of C_{10}, C_{15}, and C_{20}, respectively. FPPS usually synthesizes FPP (the C_{15} product) from DMAPP (C_5 product) or GPP (C_{10} product), and GGPPS uses FPP as the cosubstrate to synthesize GGPP (C_{20} product). It has been demonstrated that the chain length of the final product does not exceed the limit determined by the specificity of the enzyme.[11] Previous studies by Ohnuma et al.,[13] using random chemical mutagenesis aimed at altering the chain-length selectivity of prenyl synthases, have shown that *Bacillus stearothermophilus* FPP synthase could be converted into a GGPPS by replacing only one amino acid, tyrosine 81 of the FPP synthase, situated at the fifth position upstream of the FARM. Similar results were reported by Tarshis et al.,[28] who mutated phenylalanine 112 of avian FPP synthase located at the fifth position upstream of the FARM to alanine and detected GGPP formation. Ohnuma et al.[13] showed that the region around the FARM determines the product specificity of FPP and GGPP synthases. Specifically, when both the fourth and fifth positions upstream of the FARM have large bulky aromatic residues like phenylalanine and tyrosine, the final product that FPPSs produce is FPP. This is apparently because these bulky amino acids would block further condensation of compounds with more than 15 carbons. It was proposed that the region between the fourth and fifth amino acids upstream of the FARM all the way to the end of the FARM region determines the final products of all prenyl diphosphate synthases, and it was named the chain-length determination (CLD) region.[11] Interestingly, *T. gondii* FPPS,[1] as the *P. vivax* FPPS (*Pv*GGPPS),[2] is able to synthesize both FPP and GGPP. *T. gondii* apparently relies on TgFPPS for making both products because no other gene encoding an apparent GGPPS has been detected in the genome of this parasite. Upon comparison of the TgFPPS amino acid sequence around the FARM with those

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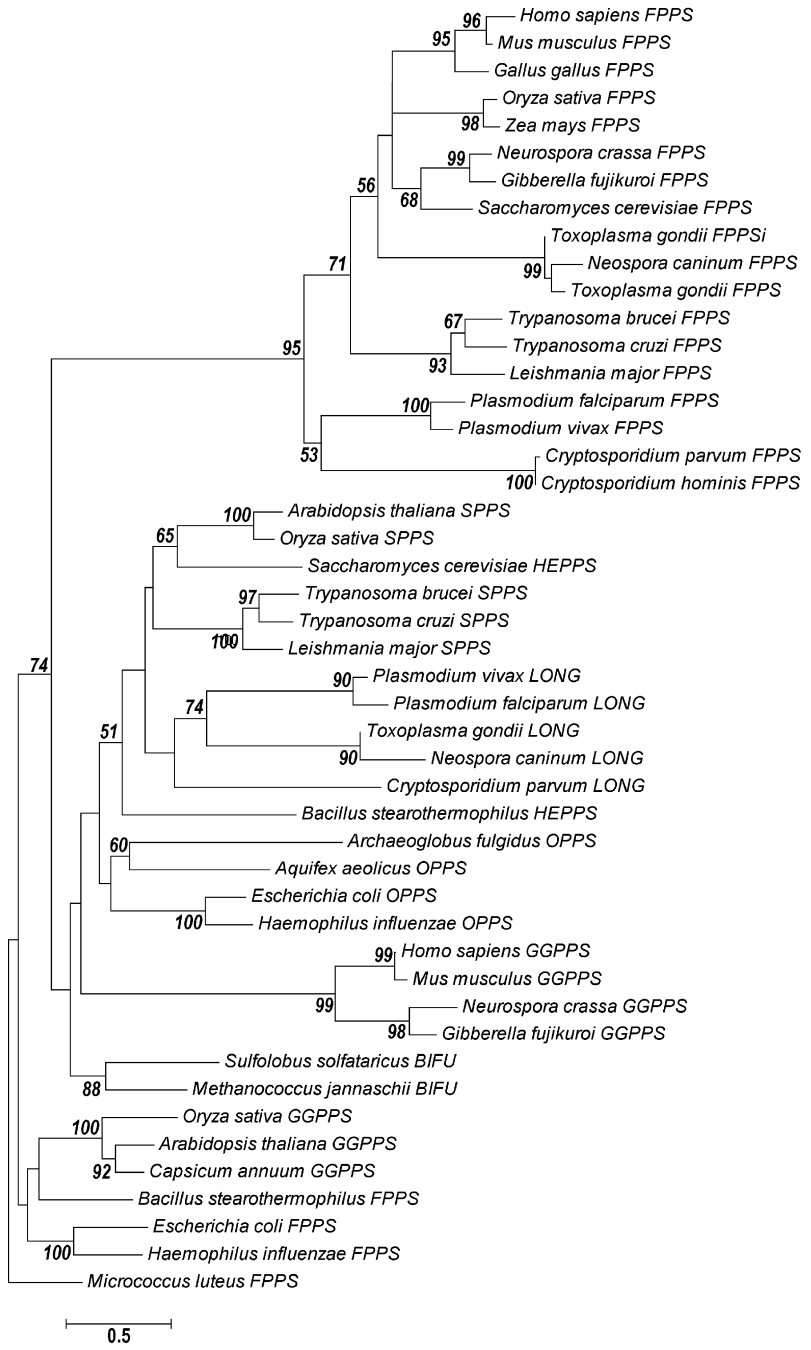

Figure 5. Phylogenetic tree of prenyl synthases. Apicomplexan prenyl synthases cluster in two main groups. FPPSs belong to the type I FPPS group, whereas other Apicomplexan synthases cluster with long chain prenyl synthases. Forty-seven amino acid sequences were analyzed. The alignments are presented in Figure S2 of the Supporting Information, and the sequence identification numbers are listed in Table S2 of the Supporting Information. Bootstrap values of >50% are shown in the tree. Bars indicate 0.5 change per amino acid position. TgFPPSi is the inactive form of the enzyme.[1]

of other FPPSs and GGPPSs, several interesting features were evident. The first was that the fourth amino acid upstream of the FARM is phenylalanine, and the fifth is cysteine.[1] This is different from any of the patterns previously described: (1) both are aromatic amino acids (type I FPPSs); (2) neither of them is an aromatic amino acid (type II and type III GGPPSs); and (3) only the fifth amino acid is aromatic (type II FPPSs and type I GGPPs). In contrast, *Tg*FPPS (and also *Pv*GPPS) has just one aromatic amino acid at the fourth position upstream of the FARM instead of at the fifth position. This organization is unusual although similar to the organization of other Apicomplexan enzymes (Figure S1B of the Supporting Information). The *P. vivax* enzyme has alanine at the fifth position and a phenylanine at the fourth position upstream to the FARM region[2] (Figure S1B of the Supporting Information). This different combination of fourth and fifth amino acids may provide this enzyme its bifunctionality.

In this work, we describe that the replacement of the six-residue sequence upstream of the FARM region of TgFPPS with the amino acid sequences present in *A. thaliana* or *S.*

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cerevisiae FPPSs or the replacement of the fifth amino acid (cysteine) upstream of the FARM region with a tyrosine caused a change in the enzyme activity so that it is converted into an exclusive FPP synthase. On the other hand, the conversion of *T. gondii* FPPS into a GGPPS was possible when the six amino acid residues upstream of the FARM region were replaced with the residues found in the same position of *H. sapiens* or *S. cerevisiae* GGPPSs. The replacement of amino acids at the fourth and fifth positions upstream of the FARM region with alanine also determined the conversion of TgFPPS into a GGPPS. Unexpectedly, TgFPPS produced more FPP and very little GGPP when the 171 N-terminal amino acids were deleted.

Taken together, our results indicate that the composition of the sequence upstream of the FARM region of TgFPPS and the N-terminal region of the enzyme determine the length of the products of this enzymatic reaction. Although previous work with the *S. cerevisiae* type III GGPPS has shown that the N-terminus of this GGPP protrudes from the helix core into the other subunit and contributes to tight dimer formation, and deletion of some of these amino acids caused the dissociation of the dimer into monomers and abolished the activity of the enzyme,[29] to the best of our knowledge, this is the first report to describe that the N-terminal region of the enzyme has a role in isoprenoid elongation in prenyl synthases. It is interesting to note that the predicted mitochondrial targeting sequence suggests that the targeting signal is processed at approximately 150 amino acids. However, we found the most important differences when we used a truncated version lacking 171 amino acids, thus suggesting that the changes observed are probably due to this extra region of 20 amino acids that is maintained after processing.

We report for the first time the phylogenetic analysis of (*E*)-isoprenyl diphosphate synthases by a multigenome comparison with Apicomplexa. Our biochemical evidence indicates that the TgFPPS is bifunctional, catalyzing the synthesis of both FPP and GGPP.[1] However, the phylogenetic analysis of the TgFPPS classifies it as a type I FPPS. Moreover, the *Cryptosporidium* spp. and *Plasmodium* spp. FPPSs also cluster with type I FPPS. In addition, other putative Apicomplexan prenyl synthases, including one from *T. gondii*, cluster with long chain prenyl synthases, which suggests that these enzymes are involved in the synthesis of C_{30}−C_{50} isoprenoid unit compounds. This is important to mention because the bifunctionality of the *P. vivax* FPPS has been demonstrated,[2] and we provide further evidence that TgFPPS synthesizes both FPP and GGPP, implying that other Apicomplexan FPPSs could also be specific in the synthesis of short chain isoprenoid units.

■ ASSOCIATED CONTENT

Supporting Information

Figures S1 and S2 and Tables S1 and S2. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS

FPPS, farnesyl diphosphate synthase; GGPPS, geranylgeranyl diphosphate synthase; DMAPP, dimethylallyl diphosphate; IPP, isopentenyl diphosphate; GPP, geranyl diphosphate; FPP, farnesyl diphosphate; FOH, farnesol; GGPP, geranylgeranyl diphosphate; GGOH, geranylgeraniol; FARM, first aspartic rich motif; SARM, second aspartic rich motif; CLD, chain-length determination; TLC, thin layer chromatography.

■ REFERENCES

(1) Ling, Y., Li, Z. H., Miranda, K., Oldfield, E., and Moreno, S. N. (2007) The farnesyl-diphosphate/geranylgeranyl-diphosphate synthase of *Toxoplasma gondii* is a bifunctional enzyme and a molecular target of bisphosphonates. *J. Biol. Chem. 282*, 30804−30816.

(2) Artz, J. D., Wernimont, A. K., Dunford, J. E., Schapira, M., Dong, A., Zhao, Y., Lew, J., Russell, R. G., Ebetino, F. H., Oppermann, U., and Hui, R. (2011) Molecular characterization of a novel geranylgeranyl pyrophosphate synthase from *Plasmodium* parasites. *J. Biol. Chem. 286*, 3315−3322.

(3) Martin, M. B., Grimley, J. S., Lewis, J. C., Heath, H. T., III, Bailey, B. N., Kendrick, H., Yardley, V., Caldera, A., Lira, R., Urbina, J. A., Moreno, S. N., Docampo, R., Croft, S. L., and Oldfield, E. (2001) Bisphosphonates inhibit the growth of *Trypanosoma brucei*, *Trypanosoma cruzi*, *Leishmania donovani*, *Toxoplasma gondii*, and *Plasmodium falciparum*: A potential route to chemotherapy. *J. Med. Chem. 44*, 909−916.

(4) Yardley, V., Khan, A. A., Martin, M. B., Slifer, T. R., Araujo, F. G., Moreno, S. N., Docampo, R., Croft, S. L., and Oldfield, E. (2002) In vivo activities of farnesyl pyrophosphate synthase inhibitors against *Leishmania donovani* and *Toxoplasma gondii*. *Antimicrob. Agents Chemother. 46*, 929−931.

(5) Ling, Y., Sahota, G., Odeh, S., Chan, J. M., Araujo, F. G., Moreno, S. N., and Oldfield, E. (2005) Bisphosphonate inhibitors of *Toxoplasma gondi* growth: In vitro, QSAR, and in vivo investigations. *J. Med. Chem. 48*, 3130−3140.

(6) Rosso, V. S., Szajnman, S. H., Malayil, L., Galizzi, M., Moreno, S. N., Docampo, R., and Rodriguez, J. B. (2011) Synthesis and biological evaluation of new 2-alkylaminoethyl-1,1-bisphosphonic acids against *Trypanosoma cruzi* and *Toxoplasma gondii* targeting farnesyl diphosphate synthase. *Bioorg. Med. Chem. 19*, 2211−2217.

(7) Szajnman, S. H., Garcia Linares, G. E., Li, Z. H., Jiang, C., Galizzi, M., Bontempi, E. J., Ferella, M., Moreno, S. N., Docampo, R., and Rodriguez, J. B. (2008) Synthesis and biological evaluation of 2-alkylaminoethyl-1,1-bisphosphonic acids against *Trypanosoma cruzi* and *Toxoplasma gondii* targeting farnesyl diphosphate synthase. *Bioorg. Med. Chem. 16*, 3283−3290.

(8) Szajnman, S. H., Rosso, V. S., Malayil, L., Smith, A., Moreno, S. N., Docampo, R., and Rodriguez, J. B. (2012) 1-(Fluoroalkylidene)-1,1-bisphosphonic acids are potent and selective inhibitors of the enzymatic activity of *Toxoplasma gondii* farnesyl pyrophosphate synthase. *Org. Biomol. Chem. 10*, 1424−1433.

(9) Singh, A. P., Zhang, Y., No, J. H., Docampo, R., Nussenzweig, V., and Oldfield, E. (2010) Lipophilic bisphosphonates are potent inhibitors of *Plasmodium* liver-stage growth. *Antimicrob. Agents Chemother. 54*, 2987−2993.

(10) Gabelli, S. B., McLellan, J. S., Montalvetti, A., Oldfield, E., Docampo, R., and Amzel, L. M. (2006) Structure and mechanism of the farnesyl diphosphate synthase from *Trypanosoma cruzi*: Implications for drug design. *Proteins 62*, 80−88.

(11) Wang, K., and Ohnuma, S. (1999) Chain-length determination mechanism of isoprenyl diphosphate synthases and implications for molecular evolution. *Trends Biochem. Sci. 24*, 445−451.
(12) Ohnuma, S., Nakazawa, T., Hemmi, H., Hallberg, A. M., Koyama, T., Ogura, K., and Nishino, T. (1996) Conversion from farnesyl diphosphate synthase to geranylgeranyl diphosphate synthase by random chemical mutagenesis. *J. Biol. Chem. 271*, 10087−10095.
(13) Ohnuma, S., Narita, K., Nakazawa, T., Ishida, C., Takeuchi, Y., Ohto, C., and Nishino, T. (1996) A role of the amino acid residue located on the fifth position before the first aspartate-rich motif of farnesyl diphosphate synthase on determination of the final product. *J. Biol. Chem. 271*, 30748−30754.
(14) Ohnuma, S., Hirooka, K., Hemmi, H., Ishida, C., Ohto, C., and Nishino, T. (1996) Conversion of product specificity of archaebacterial geranylgeranyl-diphosphate synthase. Identification of essential amino acid residues for chain length determination of prenyltransferase reaction. *J. Biol. Chem. 271*, 18831−18837.
(15) Hemmi, H., Noike, M., Nakayama, T., and Nishino, T. (2003) An alternative mechanism of product chain-length determination in type III geranylgeranyl diphosphate synthase. *Eur. J. Biochem. 270*, 2186−2194.
(16) Noike, M., Katagiri, T., Nakayama, T., Koyama, T., Nishino, T., and Hemmi, H. (2008) The product chain length determination mechanism of type II geranylgeranyl diphosphate synthase requires subunit interaction. *FEBS J. 275*, 3921−3933.
(17) Aripirala, S., Szajnman, S. H., Jakoncic, J., Rodriguez, J. B., Docampo, R., Gabelli, S. B., and Amzel, L. M. (2012) Design, Synthesis, Calorimetry and Crystallographic analysis of 2-Alkylaminoethyl-1,1-Bisphosphonates as inhibitors of *Trypanosoma cruzi* Farnesyl Diphosphate Synthase. *J. Med. Chem. 55*, 6445−6454.
(18) Miranda, K., Pace, D. A., Cintron, R., Rodrigues, J. C., Fang, J., Smith, A., Rohloff, P., Coelho, E., de Haas, F., de Souza, W., Coppens, I., Sibley, L. D., and Moreno, S. N. (2010) Characterization of a novel organelle in *Toxoplasma gondii* with similar composition and function to the plant vacuole. *Mol. Microbiol. 76*, 1358−1375.
(19) Montalvetti, A., Bailey, B. N., Martin, M. B., Severin, G. W., Oldfield, E., and Docampo, R. (2001) Bisphosphonates are potent inhibitors of *Trypanosoma cruzi* farnesyl pyrophosphate synthase. *J. Biol. Chem. 276*, 33930−33937.
(20) Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994) CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res. 22*, 4673−4680.
(21) Thompson, J. D., Plewniak, F., and Poch, O. (1999) BAliBASE: A benchmark alignment database for the evaluation of multiple alignment programs. *Bioinformatics 15*, 87−88.
(22) Bailey, T. L., and Elkan, C. (1994) Fitting a mixture model by expectation maximization to discover motifs in biopolymers. *Proc. Int. Conf. Intell. Syst. Mol. Biol. 2*, 28−36.
(23) Nicholas, K. B., Nicholas, H. B., and Deerfield, D. W. (1997) GeneDoc: Analysis and Visualization of genetic variation. In EMBNEW.NEWS 4:14.
(24) Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. (2011) MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol. 28*, 2731−2739.
(25) Eck, R. V., and Dayhoff, M. O. (1966) Evolution of the Structure of Ferredoxin Based on Living Relics of Primitive Amino Acid Sequences. *Science 152*, 363−366.
(26) Saitou, N., and Nei, M. (1987) The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol. Biol. Evol. 4*, 406−425.
(27) Ohnuma, S., Hirooka, K., Ohto, C., and Nishino, T. (1997) Conversion from archaeal geranylgeranyl diphosphate synthase to farnesyl diphosphate synthase. Two amino acids before the first aspartate-rich motif solely determine eukaryotic farnesyl diphosphate synthase activity. *J. Biol. Chem. 272*, 5192−5198.
(28) Tarshis, L. C., Proteau, P. J., Kellogg, B. A., Sacchettini, J. C., and Poulter, C. D. (1996) Regulation of product chain length by isoprenyl diphosphate synthases. *Proc. Natl. Acad. Sci. U.S.A. 93*, 15018−15023.
(29) Chang, T. H., Guo, R. T., Ko, T. P., Wang, A. H., and Liang, P. H. (2006) Crystal structure of type-III geranylgeranyl pyrophosphate synthase from *Saccharomyces cerevisiae* and the mechanism of product chain length determination. *J. Biol. Chem. 281*, 14991−15000.