Farnesyl Diphosphate Synthase. Altering the Catalytic Site To Select for Geranyl Diphosphate Activity†

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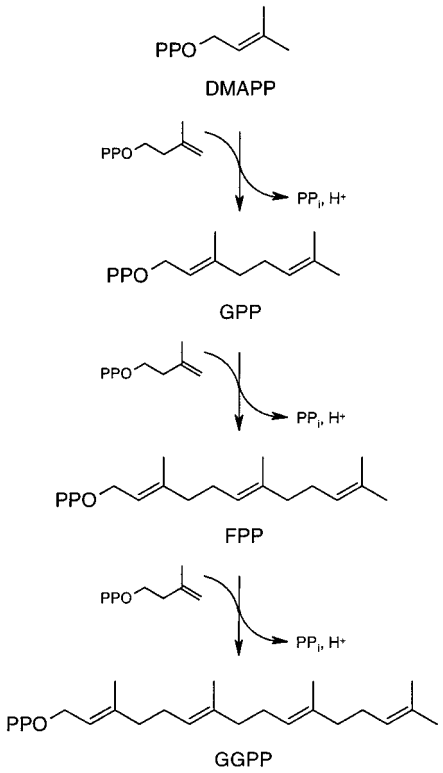
ABSTRACT: Farnesyl diphosphate synthase (FPPase) catalyzes chain elongation of the C_5 substrate dimethylallyl diphosphate (DMAPP) to the C_{15} product farnesyl diphosphate (FPP) by addition of two molecules of isopentenyl diphosphate (IPP). The synthesis of FPP proceeds in two steps, where the C_{10} product of the first addition, geranyl diphosphate (GPP), is the substrate for the second addition. The product selectivity of avian FPPase was altered to favor synthesis of GPP by site-directed mutagenesis of residues that form the binding pocket for the hydrocarbon residue of the allylic substrate. Amino acid substitutions that reduced the size of the binding pocket were identified by molecular modeling. FPPase mutants containing seven promising modifications were constructed. Initial screens using DMAPP and GPP as substrates indicated that two of the substitutions, A116W and N144′W, strongly discriminated against binding of GPP to the allylic site. These observations were confirmed by an analysis of the products from reactions with DMAPP in the presence of excess IPP and by comparing the steady-state kinetic constants for the wild-type enzyme and the A116W and N114W mutants.

The major building steps in the isoprenoid biosynthetic pathway are the sequential chain elongation reactions where the hydrocarbon moieties in allylic diphosphate substrates are added to isopentenyl diphosphate (IPP).[1] A large family of isoprenyl diphosphate synthases, which are selective for the length of the hydrocarbon chains and the stereochemistry of the newly formed double bonds in the products, has evolved to mediate these reactions (*1*). The *E* double bond selective short chain length synthases constitute a subgroup that synthesizes geranyl diphosphate (GPP), *E*,*E*-farnesyl diphosphate (FPP), and *E*,*E*,*E*-geranylgeranyl diphosphate (GGPP) (see Scheme 1). The *E*-selective farnesyl diphosphate (FPPase) and geranylgeranyl diphosphate synthases (GGPPase) are closely related enzymes (*2*). Both are homodimers and have a high level of similarity in their amino acid sequences. The catalytic sites in FPPase and GGPPase contain two aspartate-rich motifs. The first, a DDXXD or DDXXXXD sequence, binds the diphosphate residue in the allylic substrates, while the second, a DDXXD motif, binds IPP.

Scheme 1: Reactions Catalyzed by Short Chain Length Isoprenyl Diphosphate Synthases



Geranyl diphosphate synthase (GPPase) activity has been reported in plant tissues, and the genes for the heterodimeric enzyme from spearmint were recently cloned and characterized (*3*). One of the subunits in GPPase has substantial similarity to plant GGPPases, including the two aspartate-rich motifs typically seen in these enzymes. The other subunit only has a modest degree of similarity with other FPP and GGPP synthases and lacks both of the aspartate-rich motifs.

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[1] Abbreviations: BME, β-mercaptoethanol; BSA, bovine serum albumin; DEA, diethanolamine; DMAPP, dimethylallyl diphosphate; EDTA, ethylenediaminetetraacetic acid; FPP, farnesyl diphosphate; FPPase, farnesyl diphosphate synthase; GGPP, geranylgeranyl diphosphate; GGPPase, geranylgeranyl diphosphate synthase; GPP, geranyl diphosphate; GPPase, geranyl diphosphate synthase; HexPPase, hexaprenyl diphosphate synthase; IPP, isopentenyl diphosphate; IPTG, isopropyl β-thiogalactopyranoside; LB, Luria−Bertani; N144′, asparagine at position 144 in the other subunit of the FPPase homodimer; orf, open reading frame; PMSF, phenylmethanesulfonyl fluoride; SolPPase, solanesyl diphosphate synthase; TBME, *tert*-butyl methyl ether; wt, wild type.

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Table 1: FPPase Mutants

mutation	plasmid	sense primer sequence	restriction site for screening	codon(s) changed
F113W	pF113W	5′-G TTC CAG GCC TTC *TGG* CTG GTG GCT GAT GAC ATC ATG GAT C-3′	*Eco*RV site removed	TTC → TGG
A116W	pA116W	5′-GCC TTC TTC CTG GTG *TGG* GAT GAC ATC ATG GAT CAG TCC-3′	*Eco*RV site removed	GCT → TGG
N144′W	pN144′W	5′-GGT TTG GAT GCC ATC *TGG* GAC TCC TTC CTC CTG GAA TCC TCT GTG TAC-3′	*Xho*I site removed	AAC → TGG
T181H	pT181H	5′-CAG ACC GCC TAC CAA *CAC* GAG CTC GGC CAG ATG CTG GAC CTC-3′	*Pfl*MI introduced	ACT → CAC
T181Y	pT181Y	5′-CAG ACC GCC TAC CAG *TAC* GAG CTC GGC CAG ATG CTG GAC CTC-3′	*Pfl*MI introduced	ACT → TAC
F109W/F113W	pF109/113W	5′-GC ATC CAG TTG *TGG* CAG GCC TTC *TGG* CTG GTG GCT GAT GAC ATC GAT C-3′	*Eco*RV site removed	TTC → TGG TTC → TGG
F112W/F113W	pF112/113W	5′-G TTG TTC CAG GCC *TGG TGG* CTG GTG GCT GAT GAC ATC AGT GAT C-3′	*Eco*RV site removed	TTC → TGG TTC → TGG

The biochemical properties of the recombinant enzyme were briefly described.

The active site of avian FPP synthase consists of a large central cavity with the two aspartate-rich regions located on opposite walls near the opening (*4*). The binding pocket for the hydrocarbon chain in the allylic substrate extends from the first aspartate-rich motif into the interior of the protein. The aromatic ring of F113 forms the "floor" of the pocket and is buttressed by the aromatic ring of F112. Replacing the aromatic rings in F112 and F113 with smaller side chains by site-directed mutagenesis produces FPPase variants that selectively synthesize GGPP (F112A), geranylfarnesyl diphosphate (F113S), or longer chain length prenyl diphosphates (F112A/F113S) (*5*). An X-ray structure of the double mutant shows that substitution of the aromatic rings by hydrogen and hydroxyl groups, respectively, removes the floor of the binding pocket and creates a channel that extends from the top of the binding pocket where the DDXXD motif is located, through the center of the protein, into the dimer interface, and out to the solvent. We have extended our mutagenesis studies of avian FPPase by shortening the binding pocket and now report the construction of two variants that show substantial selectivity for synthesis of GPP.

MATERIALS AND METHODS

Materials

[1-^{14}C]IPP was purchased from Amersham. IPP, DMAPP, and GPP were synthesized by the procedure of Davisson et al. (*6*). *Escherichia coli* strains JM109 (Promega), BMH71-18 (Promega), and DH5α (Life Technologies, Inc.) were used for cloning. *E. coli* strain XA90 was used for protein expression. Restriction enzymes were purchased from either New England Biolabs or Life Technologies. DNA sequencing and oligonucleotide primer synthesis were performed at the Huntsman Cancer Institute, Protein/DNA Core Facility. Isopropyl β-thiogalactopyranoside (IPTG) was from Life Technologies. Protease inhibitors, ampicillin (Amp), and β-mercaptoethanol (BME) were from Sigma. DE-52 ion-exchange resin was from Whatman. Hydroxyapatite Bio-Gel HT resin was from Bio-Rad. Centriprep-10 microfiltration units were from Amicon. Merck 20 × 20 cm silica gel 60 thin-layer chromatography (TLC) plates were purchased from Fisher. All solvents for TLC and ligroine for activity assays were from Fisher. CytoScint scintillation cocktail was from ICN. Calf mucosa alkaline phosphatase was from Sigma.

Methods

General Procedures. Molecular modeling was performed using Insight II (Biosym/MSI, San Diego). Plasmid DNA for restriction analysis was obtained by the boiling method as described by Sambrook et al. (*7*). Plasmid DNA for transformations was obtained using the Wizard Prep Plus MiniPreps DNA purification system (Promega). Large-scale plasmid preparations utilized the Midi Preparation DNA purification kit from Qiagen. DNA concentrations were determined by measuring absorbance at 260 nm on a Cary 4 UV/visible spectrophotometer. Procedures for the preparation of $CaCl_2$-competent *E. coli* cells, *E. coli* transformations, and restriction digests were conducted as described by Sambrook et al. The Gene Editor in vitro site-directed mutagenesis kit from Promega was used to construct site-directed mutants. Unless otherwise stated, transformed *E. coli* strains were grown at 37 °C in Luria−Bertani (LB) media containing 125 μg/mL ampicillin. FPP synthase activity was assayed by the acid-lability procedure (*8*). Radioactivity was measured in CytoScint scintillation cocktail using a Tri-carb 2300TR liquid scintillation analyzer (Packard). Proteins were purified at 4 °C using a Pharmacia FPLC system. SDS−polyacrylamide gel electrophoresis (SDS−PAGE) was performed using the discontinuous buffer system of Laemmli (*9*). Gels were stained with Coomassie Brillant Blue R (Sigma). Protein concentrations were determined by the method of Bradford (*10*) using bovine serum albumin as a standard. Storage phosphor autoradiography was performed on a Molecular Dynamics Storm 840 phosphorimager.

Site-Directed Mutagenesis. Plasmid pMJY189, a pTTQ18N-based expression vector (Amersham) with a tac promoter and the open reading frame for avian FPP synthase, was used as the template for mutagenesis reactions according to protocols similar to those described earlier (*5*). The sequences of mutagenic primers are listed in Table 1. Silent mutations in the mutagenic primers were used to introduce or remove diagnostic restriction sites to facilitate screening, and colonies containing the desired mutations were identified by restriction analysis. All mutations were confirmed by DNA sequencing.

Protein Synthesis and Purification. *E. coli* XA90 was transformed with wt or mutant plasmid and plated on LB/AMP (125 μg/mL). Overnight cultures were grown from

single colonies and used to inoculate 500 mL of media. Cultures were grown at 37 °C until the OD_{600} reached 0.5, at which time they were induced with IPTG (1 mM). After 4−5 h, the cells were harvested by centrifugation at 7000*g* for 15 min and stored at −80 °C until needed.

Approximately 3 g of frozen cell paste was thawed on ice and suspended in 25 mL of ice-cold sonication buffer (5 mM potassium phosphate buffer, pH 7.0, 10 mM BME, 1 mM PMSF, 0.5 mM EDTA, 10 mg/mL leupeptin, and 1 mg/mL pepstatin A). The suspension was disrupted by sonication on ice, and cellular debris was cleared by centrifugation at 30000*g* for 20 min. The cleared cell lysate was loaded at a rate of 2 mL/min onto a 2.5 × 30 cm DE-52 cellulose column previously equilibrated with 5 mM potassium phosphate, pH 7.0, and 10 mM BME (buffer A). The column was washed with buffer A at a rate of 4 mL/min until the absorbance at 280 nm returned to baseline and eluted with a 360 mL linear gradient from buffer A to buffer B (55 mM potassium phosphate, pH 7.0, and 10 mM BME) at a flow rate of 4 mL/min. FPPase eluted in the last third of the gradient, at approximately 40 mM potassium phosphate. Fractions containing FPPase activity were combined, filtered, and loaded at 0.5 mL/min onto a 1.5 × 15 cm hydroxyapatite column that had been preequilibrated with 40 mM potassium phosphate, pH 7.0, and 10 mM BME (buffer C). The column was washed with 3 column volumes of buffer C and eluted with a linear gradient over 3 column volumes to 500 mM potassium phosphate pH 7.0, and 10 mM BME (buffer D) at flow rate of 1 mL/min. A sharp peak in the middle of the gradient contained FPPase activity. Fractions containing FPPase were combined and dialyzed against 1 L of protein storage buffer [20 mM BHDA-KOH, pH 7.0, 10 mM BME, and 30% glycerol (v/v)] for 16 h. The protein was concentrated by ultrafiltration and stored at −20 °C. Protein purified and stored in this manner retained greater than 90% of its activity after 3 months.

FPPase Assays. FPPase was assayed by the acid-lability procedure in 200 μL (total volume) of 20 mM BHDA buffer, pH 7.0, 1 mM $MgCl_2$, 10 mM BME, and 0.1% (v/v) BSA. For routine assays, 200 μM allylic substrate (DMAPP or GPP) and 20 μM [^{14}C]IPP (10 μCi/μmol) were used. Reactions were initiated by addition of 20 μL of enzyme and incubated at 37 °C for 10 min before 0.2 mL of HCl−methanol (1:4 v/v) was added. After an additional 10 min at 37 °C, the acid-labile material was extracted by vortexing vigorously with 1.0 mL of ligroine (bp 90−120 °C) for 15 s. A 0.5 mL portion of the organic phase was removed, added to 10 mL of CytoScint scintillation cocktail, and analyzed by liquid scintillation spectrometry. Steady-state kinetic constants were determined from initial velocities.

Product Analysis. Samples for analysis by TLC contained 200 μM DMAPP or GPP, 18 μM [1-^{14}C]IPP (50 μCi/μmol), and clarified crude *E. coli* extracts (∼40 μg of total protein) containing overexpressed wt, A116W, or N144′W FPPase in a total volume of 100 μL. The products from a control reaction containing clarified cell lysate from *E. coli* XA-90/pTTQ18N were also analyzed. All samples were run in duplicate. The mixtures were incubated for 20 min at 37 °C before two 5 μL portions were spotted with authentic samples of cold GPP and FPP on a silica gel 60 thin-layer plate. The plates were developed in $CHCl_3$/methanol/water/acetic acid (25:15:4:2 v/v/v/v) and visualized by phosphor autoradiography.

Samples for analysis by GC−MS contained a total of 2.0 μmol of IPP, 10 μmol of allylic substrate, and 100−150 μg of the appropriate purified enzyme in a total volume of 500 μL of 20 mM BHDA buffer, pH 7.0, 0.05% BSA, 2.5 mM BME, and 10 mM $MgCl_2$. A mixture of IPP and the allylic substrates was added in four equal portions at 15 min intervals, and the reactions were incubated at 37 °C for a total of 4 h. The precipitant that formed during the incubations was separated from the supernatant by centrifugation at 12000*g* in a microcentrifuge for 10 min. The pellets were each resuspended in 500 μL of 25 mM NH_4HCO_3 and pressed through a 0.5 × 6 cm column of Chelex (NH_4^+ form) with 5 mL of acetonitrile:NH_4HCO_3 (20:80 v/v). Solvent was removed by lyophilization, and the residue was dissolved in 500 μL of 25 mM NH_4HCO_3.

The pH of the supernatants and resuspended pellets was adjusted to 11 by the addition of a diethanolamine (DEA)/HCl buffer (2:1 v/v). Calf mucosa alkaline phosphatase (100 units) and $ZnCl_2$ were added to a final concentration of 1.0 mM. Samples were incubated at 37 °C for 16 h and then extracted with 200 μL of *tert*-butyl methyl ether (TBME). The emulsion that formed was clarified by centrifugation, and the organic layer was removed. The extraction procedure was repeated three times.

The samples were analyzed on a Finnigan MAT 95 high-resolution gas chromatograph/mass spectrometer II equipped with a DB-5 capillary column (J&W Scientific; 30 m, 0.25 mm i.d.). The temperature program used was isothermal 70 °C for 2 min, followed by a linear gradient (10 °C/min) to 250 °C. Both chemical ionization (CH_4) and electron ionization (80 eV) modes were used. Retention times and fragmentation patterns were compared with those of authentic samples of dimethylallyl alcohol, geraniol, nerol, and farnesol.

RESULTS

Design of FPPase Mutants Selective for Synthesis of GPP. Examination of the allylic diphosphate binding pocket in avian FPPase provided important insights about how the protein could be altered to select for synthesis of longer chain length products (*5*). We used a similar approach to identify amino acid substitutions that would lead to an enzyme with selective GPPase activity. The X-ray structure of the cocrystal of avian FPPase containing GPP in the active site (*5*) was analyzed with the aid of Insight II modeling software. Specifically, the side chains of "small" amino acids along the walls of the binding pocket within 6 Å of GPP were replaced by larger side chains, typically Trp, Tyr, or His. The energy of the system was minimized for a subset of residues within 4 Å of the substitution. The substitutions that did not introduce serious steric violations with neighboring residues, but which appeared to block the lower regions of the allylic binding pocket, were selected for mutagenesis. The amino acids thus identified were F109, F112, F113, A116, N144′, and T181. Four of these residues, F109, F112, F113, and A116, are located roughly along one face of the α-helix that terminates in the first aspartate-rich motif (D117−D121). Amino acids of this helix are important in determining the chain length of products with three or more

Table 2: Relative Activities of Avian FPPase and Site-Directed Mutants[a]

enzyme	allylic substrate		selectivity
	DMAPP	GPP	
wt FPPase	1.00	1.00	1.0
F113W	0.50	0.12	4.2
A116W	0.35	0.01	35
N144′W	0.37	0.002	185
T181H	0.016[b]	0.001[b]	16
T181Y	0.020[b]	0.001[b]	20
F109W/F113W	0.26	0.10	2.6
F112W/F113W	0.47	0.06	7.8

[a] Incubations contained 20 μM [^{14}C]IPP and either 200 μM DMAPP or 200 μM GPP. [b] Indistinguishable from *E. coli* background.

isoprenoid residues (*12*−*15*). T181 is across the pocket from F109. N144′ is a residue from the other polypeptide chain in the homodimer and is located near the ω-isoprene unit in GPP and just above T181. Because of the symmetry of the homodimeric structure for FPPase, the two active sites contain residues from the two polypeptide chains. On the basis of the modeling results, we constructed seven FPPase mutants—F113W, A116W, N144′W, T181H, T181Y, F109W/F113W, and F112W/F113W.

Construction, Initial Characterization, and Purification of FPPase Mutants. Avian FPPase mutants were constructed from pMJY189, a pTTQ18N derivative with the orf for the enzyme under control of a *tac* promotor. The mutants, along with the codons that were changed, are listed in Table 1. In addition, the mutants were tagged with additional silent mutations that removed or introduced restriction sites within the orf. The constructs were cloned into *E. coli* XA90, and the resulting strains gave levels of FPPase proteins similar to those obtained for the wt enzyme.

The mutants were initially screened for selectivity toward synthesis of GPP relative to wt FPPase by comparing the activities of cell-free extracts when DMAPP (synthesis of GPP) or GPP (synthesis of FPP) was the allylic substrate for condensation with IPP. The results are listed in Table 2. The F113W, A116W, and N144′W single mutants and the F109W/F113W and F112W/F113W double mutants all showed substantial activity when incubated with IPP and DMAPP and were more selective toward synthesis of GPP than wt avian FPPase. Of this group, the A116W and N144′W enzymes were the most selective. Both mutants were approximately 35% as active as wt in the DMAPP assay. The A116W mutant had only 1% of wt activity in the GPP assay, while the GPP activity for the N144′W enzyme was at background levels. The T181H and T181Y mutants were only marginally active catalysts in both assays.

On the basis of the results of the assays with DMAPP and GPP, the A116W and N144′W proteins were purified in two steps by chromatography on DE-52 and hydroxyapatite. The elution profiles for the mutant proteins were similar to that of the wt enzyme on both columns. After the hydroxyapatite step, the proteins were ~95% pure as judged by SDS−PAGE (data not shown), and 2−3 mg of purified enzyme was typically obtained from a 500 mL culture.

Product Analysis. Wt avian FPPase and the A116W and N144′W mutants were incubated with [1-^{14}C]IPP and DMAPP or GPP, and the products were analyzed by TLC. All three enzymes converted GPP to FPP. The incubations with DMAPP as the allylic substrate gave mixtures of GPP and FPP. However, the amounts of FPP were substantially lower for the two mutants than for wt FPPase.

In a second set of experiments, cold IPP and each allylic substrate were incubated with the wt, A116W, or N144′W enzymes. During the course of the incubation, a white precipitate formed in all of the reactions where GPP was the allylic substrate and in the reaction containing DMAPP and wt FPPase. This precipitate was primarily the Mg^{2+} salt of FPP and is typically encountered in larger scale incubations with FPPase, although GPP will also precipitate at higher concentrations. The precipitate was separated from the supernatant, suspended in NH_4HCO_3 buffer, and passed through a Chelex column (NH_4^+ form) to resolubilize the diphosphates. The supernatants and resolubilized precipitates were treated with alkaline phosphatase, and the hexane-soluble products were analyzed by gas chromatography−mass spectrometry using authentic samples of isopentenol, dimethylallyl alcohol, geraniol, nerol, and farnesol as standards. The results were similar for both fractions. When DMAPP was the starting allylic substrate, geraniol and farnesol were identified as new products. When GPP was the starting allylic substrate, farnesol was detected.

Steady-State Kinetic Constants. The steady-state kinetic constants for wt FPPase and the A116W and N144′W mutants used in the product studies are given in Table 3. Increasing the size of the side chains of A116 and N144′ in the tryptophan mutants had a relatively modest effect on k_{cat} when either DMAPP or GPP was the allylic substrate. Likewise, K_M^{IPP} for wt FPPase only increased 7- and 9-fold for the A116W and N144′W enzymes, respectively. Somewhat larger changes were found for K_M^{DMAPP}, where the constants increased 40-fold for A116W and 91-fold for N144′W. However, the largest changes were seen for K_M^{GPP}. K_M^{GPP} for the A116W and N144′W mutants were 1190−700-fold higher, respectively. These results suggest that the primary effect of the two mutations was to destabilize binding of the allylic substrates and that the loss of binding affinity was greater for GPP than for DMAPP.

DISCUSSION

A family of isoprenyl diphosphate synthases has evolved to synthesize isoprenoid chains of up to approximately 10 isoprene units with *E* double bonds. These enzymes have been classified as short chain length (up to 4 isoprene units) or medium chain length (5 to ~11 isoprene units) *E*-synthases, depending on the length of the final product. Biochemical studies suggest that enzymes fall into four different classes according to their quaternary structures (*16*). The GPPase found in spearmint is a heterodimer (*3*). Two other short-chain enzymes, FPPase and GGPPase, are homodimers (*4*, *5*). Two different quaternary structures have been reported for medium-chain synthases. The hexaprenyl diphosphate synthase (HexPPase) from *Micrococcus luteus* is a heterodimer of two easily dissociable subunits that separate during chromatography (*17*). The solanesyl diphosphate synthase (SolPPase) from *M. luteus* (9 isoprene units) also consists of two components, a stable homodimer and readily dissociable third subunit (*18*).

Genes encoding the short-chain and medium-chain isoprenyl diphosphate synthases have been isolated from a wide

Table 3: Steady-State Kinetic Constants for wt FPPase and the A116W and N144′W Mutants

FPPase	k_{cat}^{DMAPP} (s^{-1})	K_M^{IPP} (μM)	K_M^{DMAPP} (μM)	$k_{cat}^{DMAPP}/K_M^{DMAPP}$	k_{cat}^{GPP} (s^{-1})	K_M^{GPP} (μM)	k_{cat}^{GPP}/K_M^{GPP}
wt	0.57 ± 0.01	0.87 ± 0.17	1.10 ± 0.1	0.51	0.89 ± 0.01	0.37 ± 0.02	2.4
A116W	0.46 ± 0.02	8.4 ± 0.5	44 ± 5	1×10^{-2}	0.33 ± 0.01	440 ± 40	7.5×10^{-4}
N144′W	0.41 ± 0.02	6 ± 1	100 ± 20	4×10^{-3}	0.20 ± 0.01	260 ± 30	7.6×10^{-4}

variety of organisms. One of the subunits in the heterodimers and the subunits of the homodimers have several highly conserved regions in their amino acid sequences. Thus, the enzymes are thought to have evolved from a common ancestor (*2*, *19*). In particular, two aspartate-rich regions located in the active site are the putative binding motifs for the diphosphate residues of the two substrates. The depth of the pocket that binds the hydrocarbon chain of the allylic substrate determines the number of isoprene residues that are added to the growing chain. An analysis of the X-ray structure of avian FPPase indicates that the depth of the pocket for FPPase, GGPPase, and the medium chain length synthases is primarily determined by the size of the side chains for amino acids found approximately on one face of the α-helix containing the first aspartate-rich motif (*5*). This hypothesis was confirmed by site-directed mutagenesis to create variants of FPPase that synthesize C_{20}, C_{25}, and higher chain length products (*5*, *12*, *19*). By analogy, it should be possible to create FPPase mutants with a smaller hydrocarbon binding pocket that are selective for synthesis of GPP.

We decided to use the X-ray structure of avian FPPase to guide the construction of GPPase-selective mutants. A group of seven mutants was identified in modeling studies where the size of the binding pocket for the hydrocarbon residue of the allylic substrate was reduced without introducing severe steric interactions into the enzyme−DMAPP complex. Six of the mutations were in the same polypeptide chain that formed most of the walls of the catalytic site. The mutation most selective for GPP synthesis was A116W, which is located immediately adjacent to D117, the first aspartate residue in the allylic diphosphate binding motif. A similar level of selectivity was found for the N144′W mutant. N144′ is across the binding pocket from A116 but, more interestingly, is located on the other polypeptide chain in the homodimer.

A comparison of steady-state kinetic constants for the wt enzyme with those for the A116W and N144′W mutants shows that the substitutions did not substantially alter k_{cat} for the DMAPP → GPP and GPP → FPP steps. K_M^{DMAPP} increased 40- and 90-fold, respectively, in the A116W and N144′W mutants. However, K_M^{GPP} increased 1200- and 700-fold, respectively. Although the alterations in the catalytic site appear to decrease the affinity of FPPase for both of its allylic substrates, the perturbations are much more pronounced for GPP. The only effect on the products of the reaction is the altered chain length selectivity of the enzyme. Addition of IPP to DMAPP or GPP proceeds with high selectivity to give the *E* double bond isomers. If one compares the catalytic efficiencies (k_{cat}/K_M) for condensation of IPP with DMAPP (synthesis of GPP) with those for condensation with GPP (synthesis of FPP), the A116W mutation increases the efficiency of GPP synthesis by a factor of 63 relative to FPP and the N144′W mutation by a factor of 28. For incubations where IPP and DMAPP are the initial substrates and IPP is in excess, the selectivity for GPP synthesis should be even higher than suggested by the k_{cat}/K_M comparisons.

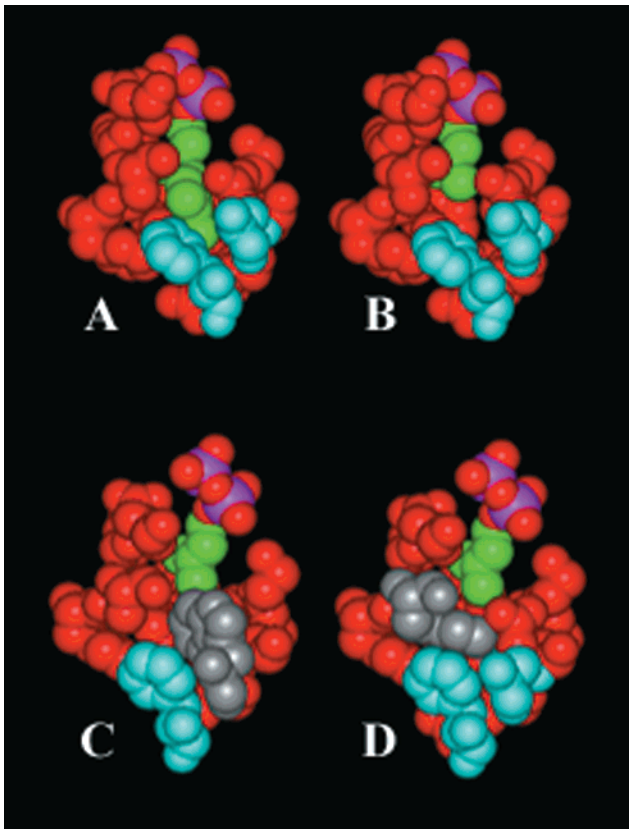

FIGURE 1: Allylic region of the FPPase active site within ca. 5 Å of the bound substrate. The amino acids in the two subunits are colored red and blue, respectively. The atoms of the allylic substrates are colored green (carbon), purple (phosphorus), and red (oxygen). The wt structures with bound allylic substrates are from ref *5*. The structures of the N144′W and A116W enzymes are low-energy conformers of the proteins determined as described in the Results section. DMAPP was docked into the active site with its hydrocarbon residue in the partially filled binding pocket. Structures: (A) wt FPPase with GPP; (B) wt FPPase with DMAPP; (C) N144′W FPPase with the mutant amino acid in gray; (D) A116W FPPase with the mutant amino acid in gray.

Figure 1 shows structures of wt FPPase with GPP (A) and DMAPP (B) bound in the active site, along with structures of the N144′W (C) and A116W (D) mutants with a molecule of DMAPP bound. Both mutations fill in the bottom of the hydrocarbon binding pocket and slightly "lift" DMAPP toward the opening of the active site. Modeling suggests that the hydrocarbon regions of the binding pockets of the two mutants are too restricted to accommodate the C_{10} geranyl moiety in GPP. However, our product analysis clearly shows that both enzymes are capable of binding and elongating GPP to FPP. Thus, their active sites must be sufficiently plastic to accommodate the longer isoprenoid chain, albeit with reduced binding affinity. While the k_{cat}'s for DMAPP → GPP for wt FPPase and the mutant enzymes are similar, substantial reductions are seen in GPP → FPP for the two mutants. This

trend most likely reflects distortions away from the optimized conformation for catalysis to accommodate the additional isoprenoid unit of the allylic substrate in the enzyme−IPP−GPP complex.

While our work was underway, Narita et al. described mutants of the FPPase from *Bacillus stearothermophilus* that were selective for GPP synthesis, giving FPP/GPP product ratios as high as 1:27 (*20*). In this case chain length selectivity was altered by substitutions of amino acids at positions equivalent to F113, L114, and Q115 in avian FPPase along the same α-helix that produced the longer chain length variants. The highest selectivity seen for the *B. stearothermophilus* mutants was when the serine normally found at the position corresponding to 113 in the avian enzyme was replaced by phenylalanine. The corresponding tyrosine mutant was less selective, and the tryptophan mutant was inactive. Interestingly, wt avian FPPase has a phenylalanine at position 113. More recently, Burke et al. reported the isolation, characterization, and functional expression of the genes encoding a heterodimeric GPPase from spearmint (*3*). One of the subunits contains the signature prenyltransferase aspartate-rich motifs and bears a strong resemblance to plant GGPPases. The other subunit does not correlate strongly with other known proteins. Although the steady-state kinetic constants for the enzyme were not well characterized, its specific activity appears to be within an order of magnitude of that reported for avian FPPase.

In summary, wt FPPase catalyzes the stepwise synthesis of GPP and FPP by consecutive additions of IPP. We used the X-ray structure of avian FPPase to design mutants that are highly selective for synthesis of GPP, the normal intermediate in the chain elongation process, by decreasing the size of the binding pocket for the allylic substrate. The most effective mutations, A116W and N144′W, act selectively to reduce the binding of GPP and k_{cat} during the second addition of IPP. It will be interesting to directly compare the chain length selectivity of these FPPase mutants with the GPPase from spearmint (*3*).

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