Intersubunit Location of the Active Site of Farnesyl Diphosphate Synthase: Reconstruction of Active Enzymes by Hybrid-Type Heteromeric Dimers of Site-Directed Mutants†

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ABSTRACT: Farnesyl diphosphate synthase is a homodimer of subunits having typically two aspartate-rich motifs with two sets of substrate binding sites for an allylic diphosphate and isopentenyl diphosphate per molecule of a homodimeric enzyme. To determine whether each subunit contains an independent active site or whether the active sites are created by intersubunit interaction, we constructed several expression plasmids that overproduce hybrid-type heterodimers of *Bacillus stearothermophilus* FPP synthases constituting different types of mutated monomers, which exhibit little catalytic activity as homodimers, by combining two tandem *fps* genes for the manipulated monomer subunit with a highly efficient promoter *trc* within an overexpression pTrc99A plasmid. A heterodimer of a combination of subunits of the wild type and of R98E, a mutant subunit which exhibits little enzymatic activity as a dimer form $(R98E)_2$, exhibited 78% of the activity of the wild-type homodimer enzyme, $(WT)_2$. Moreover, when a hybrid-type heterodimeric dimer of FPP synthase mutant subunits (R98E/F220A) was prepared, the FPP synthase activity was 18- and 390-fold of that of each of the almost inactive mutants as a dimeric enzymes, $(R98E)_2$ and $(F220A)_2$ [Koyama, T., et al. (1995) *Biochem. Biophys. Res. Commun. 212*, 681−686], respectively. These results suggest that the subunits of the FPP synthase interact with each other to form a shared active site in the homodimer structure rather than an independent active site in each subunit.

In the biosynthesis of isoprenoid compounds, prenyltransferases, i.e., prenyl diphosphate synthases, catalyze prenyl chain elongation by sequential condensation of isopentenyl diphosphate (IPP)[1] with allylic diphosphates to produce prenyl diphosphates (*1*). These enzymes are extremely interesting from a mechanistic point of view in that they catalyze the repetition of stereospecific condensation of IPP with allylic diphosphates to give prenyl diphosphates with chain lengths depending on the specificities of the individual enzymes (*2*).

Farnesyl diphosphate synthase (EC 2.5.1.10) occupies a central branch point of isoprenoid biosynthetic pathways. It catalyzes the condensations of IPP with dimethylallyl diphosphate (DMAPP) and with geranyl diphosphate (GPP) to produce (*E*,*E*)-farnesyl diphosphate (FPP) as the ultimate reaction product (*2*). During the past 12 years, the genes or cDNAs for FPP synthase have been isolated and characterized from various organisms, including rats (*3*), humans (*4*), yeast (*5*), *Escherichia coli* (*6*), *Bacillus stearothermophilus* (*7*), white lupin (*8*), *Arabidopsis thaliana* (*9*), and rubber tree (*10*). All these FPP synthases are homodimeric enzymes of tightly coupled subunits ranging in size from 32 to 44 kDa as well as the other types of prenyl chain-elongating enzymes except those for the enzymes catalyzing medium-chain prenyl diphosphate synthesis, which consist of two dissociable heteromeric subunits (*11*−*13*).

Poulter and Sacchettini and their co-workers succeeded in determining the crystal structure of the avian FPP synthase to 2.6 Å resolution (*14*). They have shown that the subunits of the dimer are related by a perfect 2-fold axis and that in each of the subunits, 10 core helices arrange around a large central cavity, in which most of the seven highly conserved regions (regions I−VII) typical of prenyltransferases (*7*, *15*) are located in the immediate vicinity of the deep cleft (*14*). Via a number of site-directed mutagenesis studies on the conserved residues in FPP synthases (*16*−*23*), regions II and VI, which contain the typical Asp-rich motifs, DDxxxxDxxxx-RRG and FQxxDDxxD, respectively, were found to be crucial for the catalytic function (*16*, *17*, *19*, *22*, *23*). These facts indicate that there are two large cavities within most of the conserved regions in one FPP synthase molecule, implying the existence of two "catalytic sites" in the enzyme.

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[1] Abbreviations: FPP, (*E*,*E*)-farnesyl diphosphate; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; GPP, geranyl diphosphate; HepPP, heptaprenyl diphosphate; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TLC, thin-layer chromatography; Tris, tris(hydroxymethyl)aminomethane.

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Reed and Rilling investigated precisely the characteristics of the substrate binding sites of avian FPP synthase (*24*). They demonstrated that the homodimeric enzyme binds two molecules of each of the three allylic diphosphates, DMAPP, GPP, and FPP, whereas four molecules of the homoallylic substrate, IPP, can bind to the enzyme, indicating that each subunit of avian liver FPP synthase has a single allylic binding site accommodating DMAPP, GPP, FPP, and even IPP, and one specific binding site for IPP.

On the other hand, further X-ray structural analysis of avian FPP synthase by soaking crystals in the substrate solutions has demonstrated that the allylic diphosphates were bound through Mg^{2+} ions to the Asp residues of the first DDXXD motif, which is in region II, with the hydrocarbon tails of all the ligands growing down the hydrophobic pocket of the large cavity (*25*).

However, it is still difficult to explain the actual feature of a dynamic phenomenon which is conducted in the catalytic site during the catalysis even with all of the detailed findings on the structure of the prenyltransferase. This paper presents evidence of intersubunit participation in catalysis, which does not appear to be suggested by the structure itself.

EXPERIMENTAL PROCEDURES

Materials. [1-^{14}C]IPP (1.95 GBq/mol) was purchased from Amersham Corp. Nonlabeled IPP, DMAPP, and GPP were the same preparations as used in the previous work (*23*). Alkaline and acid phosphatases were purchased from Boehringer Mannheim. Precoated reversed phase thin-layer chromatography (TLC) plates (LKC-18) were products of Whatman. Restriction enzymes and other DNA-modifying enzymes were purchased from Takara Shuzo Co., Ltd. (Ohtsu, Japan), and Toyobo Co., Ltd. (Osaka, Japan), unless otherwise stated. All other chemicals were analytical grade.

General Procedures. Restriction enzyme digestion, transformation, and other standard molecular biology techniques were carried out as described by Sambrook et al. (*27*). The protein concentration was determined with a protein assay kit (Bio-Rad) using bovine serum albumin as the standard protein.

Site-Directed Mutagenesis. Site-directed mutagenesis was conducted essentially in a manner similar to that described in our previous paper (*20*).

The single-stranded DNA template of pEX47 (*20*) was isolated with a helper phage, M13KO7. The mutagenic oligonucleotide employed for the mutagenesis to produce the R98E mutant subunit was 5′-TTGCGGGAGGGCAAG-3′ (the mismatched base is underlined). Introduction of the mutation was confirmed by sequencing the whole nucleotide sequence with a sequencer (Perkin-Elmer model 373A). The plasmid for production of the F220A mutant, pEXF220A, was the same preparation as described in our previous paper (*22*).

Overproduction and Purification of FPP Synthase Mutants. The procedures employed for the overproduction and purification of several sets of FPP synthase mutants, $(R98E)_2$,[2] WT/R98E, and R98E/F220A, as well as the wild-type enzyme, $(WT)_2$, were essentially similar to that for $(WT)_2$ purification previously reported by us (*7*).

FPP Synthase Assay. The enzymatic activity was measured by determination of the amount of [1-^{14}C]IPP incorporated into butanol-extractable prenyl diphosphates. In a standard assay procedure, the incubation mixture contained, in a final volume of 200 μL, 50 mM Tris-HCl buffer (pH 8.5), 20 mM $MgCl_2$, 50 mM NH_4Cl, 50 mM 2-mercaptoethanol, 25 μM allylic diphosphate (DMAPP or GPP), 0.46 μM [1-^{14}C]-IPP (1.95 GBq/mol), and an appropriate amount of enzyme protein to be examined. The incubation was carried out at 55 °C for an appropriate period of time, and then the reaction mixture was treated with 1-butanol after addition of 200 μL of H_2O saturated with NaCl to extract the product of the prenyltransferase reaction. The radioactivity in the butanol extract was measured with an Aloka LSC-1000 liquid scintillation counter.

For kinetic studies, the concentrations of the enzymes were kept at 50, 50, 1500, and 20 000 ng/200 μL [for $(WT)_2$, WT/R98E, R98E/F220A, and $(R98E)_2$, respectively], and the incubation periods were 5, 10, 50, and 60 min, respectively.

Product Analysis. After the enzymatic reaction at 55 °C for an appropriate period of time, the radioactive prenyl products in the reaction mixture were hydrolyzed to the corresponding alcohols with potato acid phosphatase according to our method (*28*). The alcohols were extracted with pentane and analyzed by TLC on a reversed phase LKC-18 plate in a solvent system of acetone and H_2O (9:1). The positions of authentic standard alcohols were visualized with iodine vapor, and the distribution of radioactivity was determined by autoradiography. The TLC plates were exposed on a Fuji imaging plate at room temperature for 1 day, and analyzed with a Fuji BAS 1000 Mac bioimage analyzer.

Preparation of Expression Plasmids for Heterodimers of FPP Synthase. *WT/R98E*. The *Apa*I−*Hin*dIII fragment of pEX11, which contains the *trc* promoter and the *fps* gene of *B. stearothermophilus* (*7*), was inserted into the *Apa*I−*Hin*dIII site of pBluescriptKS(+). After *Eco*RV digestion of the resulting plasmid, the DNA fragment containing *trc* and *fps* was inserted into the *Eco*RV site residing upstream of the *trc* promoter of pR98E, to produce pwt/R98E as illustrated in Figure 1.

R98E/F220A. In a manner similar to that described above, pR98E/F220A was prepared from pR98E and pEXF220A (*22*).

Separation between the Homodimer and Heterodimer of FPP Synthase Mutants. Overproduction and purification of the mixtures of the homo- and heterodimers of FPP synthase mutants were carried out in a manner similar to that described in the preceding section except that the incubation temperature of the *E. coli* harboring the heterodimer expression plasmid, pwt/R98E or pR98E/F220A, was changed to 37 °C, and IPTG was added to each of the culture media at A_{600} values of 0.4−0.5. FPP synthase fractions after Mono Q chromatography, which were found to be near homogeneous via SDS−PAGE, were applied to a Mono-P HR5/5 column (Pharmacia Biotech) that had been equilibrated with 25 mM *N*-methylpiperazine-HCl buffer (pH 5.72). Elution was carried out with a descending pH gradient from 7 to 4 formed by Polybuffer 74 (Pharmacia Biotech) as an eluent (Figure 2).

[2] To prevent confusion, homodimers are described by a monomer subunit in parentheses with a subscript 2. Thus, $(R98E)_2$ means a homodimer of R98E subunits, and $(WT)_2$ is the wild-type enzyme.

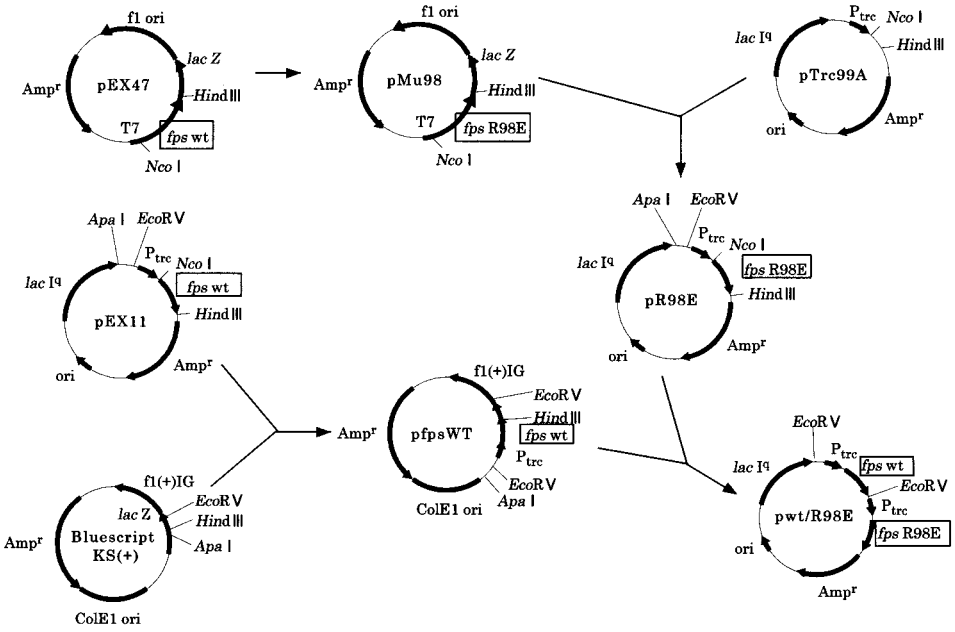

FIGURE 1: Construction of expression plasmids for homodimeric mutant FPP synthase (pR98E) and heterodimeric FPP synthase (pwt/R98E).

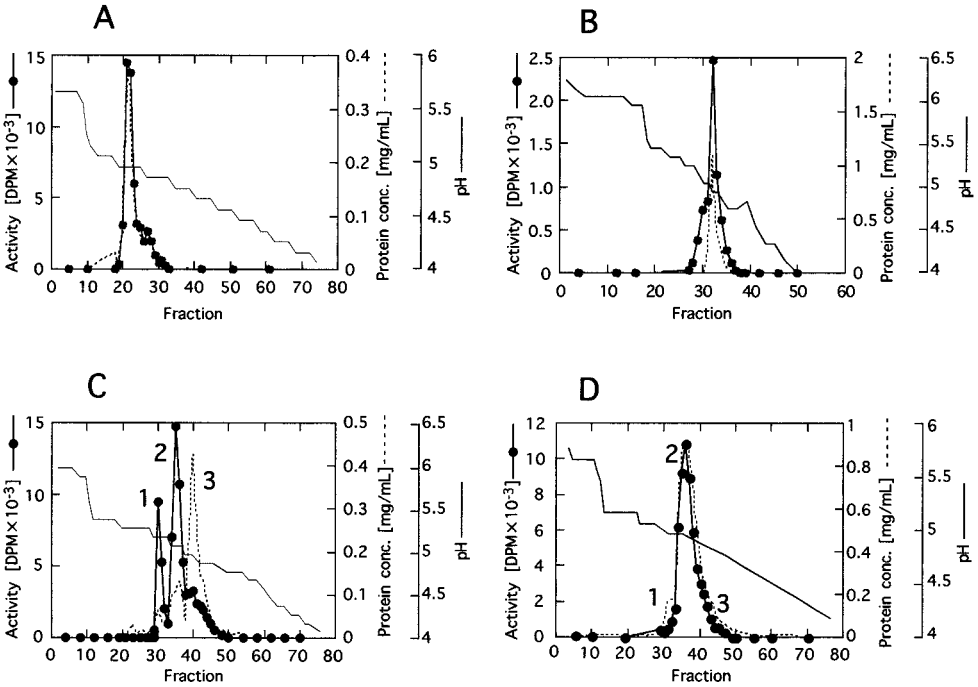

FIGURE 2: Mono-P chromatograms of homo- and heterodimeric FPP synthases. FPP synthase fractions obtained from each of the Mono-Q chromatographic runs were subjected to Mono-P chromatography as described in Experimental Procedures: (A) wild-type enzyme, $(WT)_2$, derived from *E. coli* JM 109/pEX11, (B) $(R98E)_2$ derived from *E. coli* JM 109/pR98E, (C) FPP synthase mixtures derived from *E. coli* JM 109/pwt/R98E, and (D) mutated FPP synthase mixtures derived from *E. coli* JM 109/pR98E/F220A.

Native PAGE Analysis. The enzyme samples were electrophoresed on a 14% polyacrylamide gel prepared essentially according to the standard methods for SDS−PAGE (*29*) except that SDS and 2-mercaptoethanol were omitted in all buffers, and the samples were not boiled. Native gels were pre-electrophoresed for 20 min; the enzyme fractions after Mono P chromatography were loaded and electrophoresed for 6−9 h at a constant current of 20 mA. After the electrophoreses, gels were stained either with Coomassie Brilliant Blue or with a silver staining kit (Daiichi Pure Chemicals, Tokyo, Japan).

RESULTS

Preparation of $(R98E)_2$. To prepare a mutated FPP synthase that has little catalytic activity and, in addition, has a p*I* value different from that of the wild type, we replaced the Arg at position 98 of *B. stearothermophilus* FPP synthase (*7*) with Glu. Arg-98 of the bacterial enzyme is the equivalent to Arg-126 of the avian FPP synthase (*15*), whose sequence is 25% identical to that of the bacterial enzyme, and resides in the conserved basic motif RRG located downstream from the DDXXD motif in region II. This motif is found in the outside loop between central helices D and E according to

Table 1: Kinetic Parameters of Wild-Type and Mutant Homo- and Heterodimeric FPP Synthases[a]

FPP synthase	K_m(IPP)[b] (μM)	K_m(GPP) (μM)	K_m(DMAPP) (μM)	k_{cat}[c] $\times 10^4$ (s^{-1})	k_{rel}[d]
wild-type, $(WT)_2$	9.10 ± 1.23	5.54 ± 1.19	11.1 ± 1.52	90700 ± 3800	1
$(R98E)_2$	20.1 ± 2.93	12.4 ± 0.64	27.6 ± 0.20	26.3 ± 0.8	2.90×10^{-4}
$(F220A)_2$[e]	24.9 ± 3.10	3.5 ± 0.70	22.5 ± 8.6	1.18 ± 0.11	1.30×10^{-5}
WT/R98E	11.6 ± 3.21	13.5 ± 0.02	19.1 ± 0.75	71100 ± 4000	0.784
R98E/F220A	28.5 ± 5.37	9.84 ± 0.57	17.7 ± 2.40	466 ± 6.12	5.14×10^{-3}

[a] Each value represents the mean $\pm$ the standard deviation of two determinations. [b] For the reaction with GPP. [c] k_{cat} was calculated from the V_{max} value for the reaction with IPP and GPP. [d] k_{cat} value relative to that of the wild type. [e] Data from ref *22*.

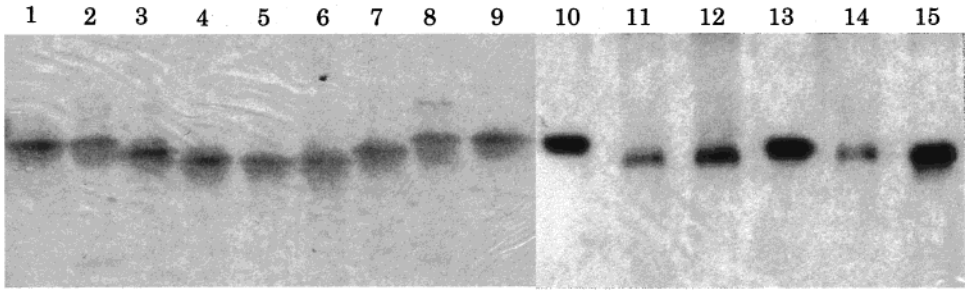

FIGURE 3: Native PAGE of purified homo- and heterodimeric FPP synthases: lanes 1 and 9, $(WT)_2$ (fraction 21 of Figure 2A); lanes 2 and 8, $(WT)_2$ (peak 1, fractions 30 and 31 of Figure 2C, respectively); lanes 3, 7, and 13, WT/R98E (peak 2, fractions 35, 36, and 35 of Figure 2C, respectively); lanes 4 and 6, $(R98E)_2$ (peak 3, fractions 40 and 41 of Figure 2C, respectively); lanes 5 and 15, $(R98E)_2$ (fraction 32 of Figure 2B); lane 10, $(F220A)_2$ (fraction 30 of Figure 2D); and lanes 11, 12, and 14, R98E/F220A (fractions 34−36 of Figure 2D, respectively). Lanes 1−9 and 10−15 were electrophoresed separately. %T = 14 (silver-stained). Because of a delay of the timing on silver staining, some diffusion of the protein bands resulted in digitizations.

the X-ray crystal structure of avian liver FPP synthase (*14*). Overproduction and subsequent purification of the mutant enzyme $(R98E)_2$ were carried out according to procedures similar to those for the wild-type enzyme reported previously (*7*).

During the purification of $(R98E)_2$, the enzyme protein was resolved into two fractions on Mono Q chromatography (data not shown), which was similar to the phenomenon attributed to the oxidoreduction stage of this enzyme observed in the case of the wild-type enzyme and Cys-replaced mutants (*21*). As the latter eluting fraction proved to be homogeneously purified, we employed this fraction for further characterization of $(R98E)_2$. As most of the chromatographic features of the mutated enzyme were quite similar to those of the wild type as well as the mutated enzymes that we have prepared (*7*, *20*−*23*), it is reasonable to consider that the mutations did not grossly alter the structure of the proteins.

As shown in Table 1, the mutant enzyme $(R98E)_2$ exhibited a k_{cat} value approximately 30000-fold smaller than that of the wild type. This result reconfirms that the first Arg residue in the RRG motif in region II is essential for the catalytic function as reported previously for the rat liver enzyme (*17*) as well as for the yeast enzyme (*19*).

Construction of Expression Plasmids for Heterodimers of FPP Synthase. To prepare hybrid-type heterodimers of FPP synthase, WT/R98E, which consist of R98E and wild-type subunits, we designed an expression plasmid pwt/R98E to produce the mRNAs of the mutant FPP synthase and of the wild type at the same time in *E. coli* cells. By combining two tandem overexpression plasmids with a *trc* promotor immediately upstream of each of the *fps* genes, we could obtain a new type of expression plasmid that may yield a mixture of a hybrid type of FPP synthase, WT/R98E, which is composed of a hybrid of R98E and wild-type subunits as well as the homodimer of the mutant enzyme, $(R98E)_2$, and the wild type, $(WT)_2$ (Figure 1).

In a similar manner, another expression plasmid pR98E/F220A yields a mixture of a hybrid type of FPP synthase, R98E/F220A, as well as the homodimers $(R98E)_2$ and $(F220A)_2$.

Purification of Hybrid-Type Heterodimers of FPP Synthases. The SDS−PAGE analysis of the whole protein showed that the *E. coli* cells harboring pwt/R98E that had been treated with IPTG for induction gave a major protein band at 32.5 kDa, which was similar to that of the wild-type enzyme. When the cell-free homogenate of the *E. coli* cells was subjected to Mono Q chromatography under conditions similar to those for the wild-type enzyme (*7*), the major protein fraction was eluted as a single peak showing a moderate FPP synthase activity (data not shown). To obtain a pure form of the hybrid-type enzyme, we subjected the "FPP synthase" fraction to Mono-P chromatography. As a result, three distinct protein peaks were obtained around pH values of 4.9−5.3 (Figure 2C), which could be tentatively assigned as the wild type, $(WT)_2$ (peak 1), WT/R98E (peak 2), and $(R98E)_2$ (peak 3). The first two peaks exhibited distinct enzymatic activities as prenyltransferases, but the third peak exhibited very little activity, although the amount of this protein that was produced predominated over the former two peaks.

Native PAGE of the three protein peaks clearly indicated that peak 1 and peak 3 exactly coincided with those of $(WT)_2$ and $(R98E)_2$, respectively, both of which had been obtained as homodimers from each of the *E. coli* cells harboring the expression plasmids pEX11 and pR98E, respectively (Figure 3). All of the protein bands exhibited similar movement close to the wild type in the native PAGE gel. This fact indicates that the mutant enzymes are really dimers and not higher-order oligomers, which may move more slowly in the polyprenyl amide gel. Hence, it is reasonable to assign peak 2 to the hybrid-type heterodimer, WT/R98E, because it

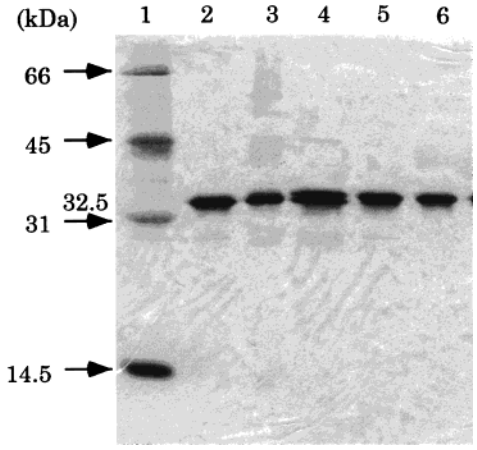

FIGURE 4: SDS−PAGE of purified homo- and heterodimeric FPP synthases: lane 1, molecular mass standards; lane 2, $(WT)_2$ (fraction 21 in Figure 2A); lane 3, peak 1 of Figure 2C [$(WT)_2$, fraction 30]; lane 4, peak 2 of Figure 2C (WT/R98E, fraction 35); lane 5, peak 3 of Figure 2C [$(R98E)_2$, fraction 40]; and lane 6, $(R98E)_2$ (fraction 32 of Figure 2B). %T = 14 (silver-stained). Because of a delay of timing on silver staining, some diffusion of the protein bands resulted in digitizations.

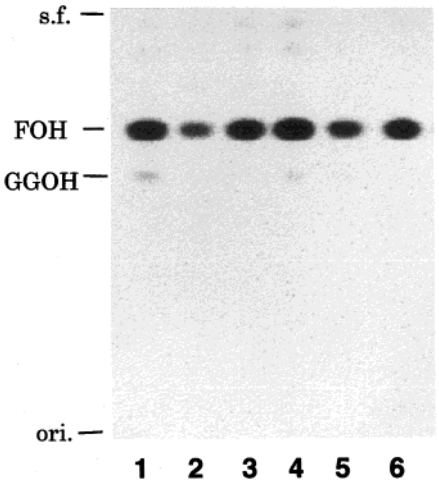

FIGURE 5: TLC autoradiogram of the alcohols derived from the enzymatic reaction products of various homo- and heterodimeric FPP synthases. The prenyl alcohols from the incubations of [1-^{14}C]-IPP and DMAPP with indicated amounts of FPP synthases followed by acid phosphatase treatment were analyzed by reversed phase TLC as described in Experimental Procedures. Spots of authentic standard prenyl alcohols were as follows: FOH, (*E*,*E*)-farnesol, GGOH, (*E*,*E*,*E*)-geranylgeraniol; ori., origin; and s.f., solvent front. Lanes 1 and 3, $(WT)_2$ [fraction 21 of Figure 2A and fraction 30 of Figure 2C, respectively (50 ng of enzyme, incubation time of 5 min)]; lanes 2 and 5, $(R98E)_2$ [fraction 32 of Figure 2B and fraction 40 of Figure 2C, respectively (20 mg of enzyme, incubation time of 60 min)]; lane 4, WT/R98E [fraction 35 of Figure 2C (50 ng of enzyme, incubation time of 10 min)]; and lane 6, R98E/F220A [fraction 36 of Figure 2D (1.5 mg of enzyme, incubation time of 50 min)].

exhibits an intermediate p*I* value between those of $(WT)_2$ and $(R98E)_2$, although the pH values at which the three peaks eluted during Mono P chromatography were somewhat unsettled depending on the conditions of the pH gradients formed during each chromatographic run. As shown in Figure 4, all of the three proteins exhibited essentially the same mobility in the SDS−PAGE gel, indicating that all the fractions are homodimers of 32.5 kDa proteins.

In a similar manner, the other hybrid-type heterodimer, R98E/F220A, was purified from the mixtures of $(R98E)_2$, R98E/F220A, and $(F220A)_2$, which had been overexpressed in the *E. coli* cells harboring pR98E/F220A. As shown in Figure 2D, formation of the hybrid-type enzyme corresponding to peak 2 predominated. As the $(F220A)_2$ exhibited a p*I* value coinciding with that of the $(WT)_2$ as expected, peak 2 was reasonably assigned to the heteromeric enzyme, R98E/F220A. It is interesting that the R98E/F220A hybrid enzyme (Figure 3, lanes 11, 12, and 14) exhibits a slightly faster mobility than the other hybrid, WT/R98E, in the native PAGE gel (Figure 3, lane 13).

Product Analysis. Figure 5 shows the TLC autoradiogram of the alcohols derived from the enzymatic reaction products of various homo- or heterodimeric FPP synthases. Although the specific activities of the enzymes differ significantly, all of the homo- or heterodimeric FPP synthases were shown to produce FPP as the exclusive reaction product.

Kinetic Analysis of the FPP Synthase Hybrid Mutants. Kinetic parameters of the purified hybrid-type mutants of FPP synthase as well as the mutant homodimers are listed in Table 1. The mutagenesis of Arg-98 and Phe-220 in the homodimer enzyme resulted in approximately 10^4- and 10^5-fold decreases in catalytic activity as mentioned above and in the previous communication (*22*), respectively, indicating further that both of the conserved residues in regions II and VI are critical for efficient enzymatic function. Hence, it was necessary to substantially increase both of the enzyme concentrations and incubation times so that reliable values of reaction rates for these least reactive mutant enzymes could be measured. To our surprise, the hybrid-type mutant WT/R98E exhibited 78% of the activity of the wild type, $(WT)_2$. Furthermore, even the hybrid, R98E/F220A, exhibited distinct catalytic activity, showing activities 17.7- and 395-fold higher than those for $(R98E)_2$ and $(F220A)_2$, respectively.

On the other hand, all the K_m values for either the allylic (GPP and DMAPP) or homoallylic substrate (IPP) of the mutant FPP synthases resulted in no drastic changes, exhibiting values comparable to or a few times larger than (within 3-fold) that of the wild-type enzyme.

DISCUSSION

Substrate binding experiments with avian liver FPP synthase revealed that each subunit of the liver enzyme has a single allylic binding site accommodating DMAPP, GPP, and FPP, and one binding site for IPP (*24*). Furthermore, X-ray structural analysis of the avian enzyme has confirmed the allylic substrate binding site (*25*). The diphosphate groups of the allylic compounds were found to be binding to the aspartate carbonyl oxygens through magnesium bridges formed by the first aspartate-rich motif (region II), showing that two divalent metal ions and one GPP or FPP molecule bound per monomer of the FPP synthase. These findings verified the existence of the substrate binding sites for each of the allylic and homoallylic substrates in one subunit of the enzyme. Hence, there are two sets of binding sites in one molecule of the FPP synthase having a tightly coupled homodimer structure. However, it is still difficult to elucidate the feature of a dynamic "catalytic site(s)" which directly participates in the prenyltransferase reaction during catalysis by the homodimeric structure of the enzyme.

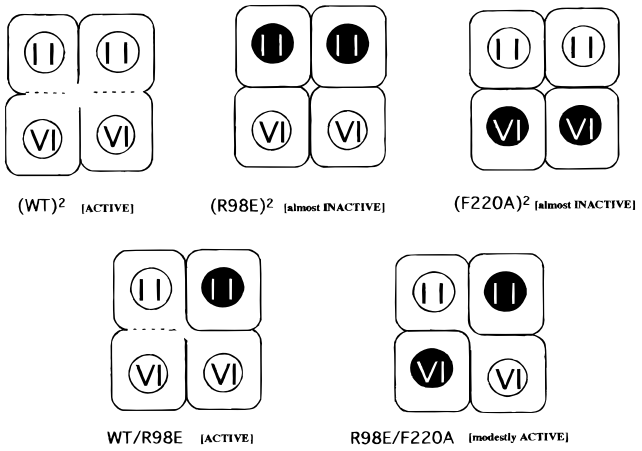

FIGURE 6: Schematic illustrations of the homo- and heterodimeric FPP synthases. Each subunit consists of two parts having catalytically critical regions II and VI. Intact regions II and VI are depicted as white circles. Mutated subunits having inactive regions are depicted as black circles.

The high catalytic activity observed for the heteromeric enzyme WT/R98E, in which one of the subunits is the wild type, implies the presence of an independent catalytic site in each of the homodimeric subunits. However, the relatively high recovery of the catalytic activities in the heterodimer, R98E/F220A, which consists of two heavily inactivated subunits, suggests the formation of a shared active site at the interface between the two subunits, in other words, evidence of intersubunit participation in catalysis as illustrated in Figure 6.

Among many kinds of prenyl chain-elongating enzymes, medium-chain (*E*)-prenyl diphosphate synthases, which we have classified as prenyltransferase II (*1*), are exceptional because of their heterodimeric structures. These enzymes catalyze the condensation of IPP with FPP to form medium-chain (*E*)-prenyl diphosphates with prenyl chain length of C_{30} or C_{35}, which are responsible for the biosynthesis of the side chain of menaquinones or ubiquinones of some microorganisms. These enzymes comprise two essential protein components; one has highly conserved regions characteristic of (*E*)-prenyl diphosphate synthases, including two aspartate-rich regions, while the other has no such similar regions. These two components exist as separate forms that may be readily dissociated chromatographically. Although neither component has prenyltransferase activity alone, the enzyme activity is restored when they are recombined (*11*, *12*).

Our recent reports on the dynamic interaction between the two dissociable heteromeric components of heptaprenyl diphosphate (HepPP) synthase from *Bacillus subtilis* have indicated that the two components, I and II, allylic substrate FPP, and Mg^{2+} form a catalytically active complex which represents an intersubunit participation between the heteromeric components in catalysis (*13*, *26*). These results on the dynamic feature of the medium-chain prenyl diphosphate synthase forming a catalytically active ternary complex will provide some hints for explaining the findings on the intersubunit participation in the catalytic function of the homodimeric FPP synthase presented here. In the relationship between the two heteromeric components of the HepPP synthase, neither exhibits catalytic activity by itself but both exhibit catalytic activity via the intersubunit participation between the two. One can make a plausible explanation of a combination of two intact regions that have not been mutated which may participate in the creation of a catalytically functional site of the heteromeric FPP synthase, R98E/F220A.

It is likely that a combination between regions II and VI, which have the first and second DDXXD motifs, respectively, from the counterpart subunit may form the "real catalytic site" for undergoing the sequential condensation of IPP with allylic diphosphate.

Although the smaller component of the HepPP synthase, component I, does not have any similar regions typical of (*E*)-prenyl chain-elongating enzymes, including the larger component of this enzyme, component II (*30*), we have recently found that there are three conserved regions, regions A−C, within the primary structures for component I, the equivalent component I′ of *B. stearothermophilus* HepPP synthase (*31*), and component A of *Micrococcus luteus* B-P 26 hexaprenyl diphosphate synthase (*32*). We have further determined that some of the residues in region B of component I of *B. subtilis* HepPP synthase are involved in the binding of the allylic substrate FPP as well as in determining the prenyl chain length of the enzymatic reaction product (*26*). Photoaffinity labeling experiments with a benzophenone-containing allylic substrate homologue also revealed the specific binding of the alkyl group of FPP to the component I (*13*).

According to the X-ray structural analysis of avian FPP synthase (*14*, *25*), region II which contains the first DDXXD motif plays the role of the allylic substrate binding site, and region VI which contains the second DDXXD motif is the putative homoallylic substrate binding site. Hence, it is probable that within the "active" R98E/F220A mutant enzyme, the subunit of F220A, which has a fatal mutation in region VI (*22*), may provide intact region II as the allylic binding site, and that the other subunit of R98E, which has a mutation in region II, may provide intact region VI as the IPP binding site. The real active site must be formed by a mutual combination of several critical portions of the conserved regions to construct a shared active site between the two subunits. A schematic illustration of the homo- and heterodimeric FPP synthases used in this study is presented in Figure 6.

Although the wild-type enzyme $(WT)_2$ has two sets of intact pairs of active sites in the homodimer structure of the whole enzyme molecule, the nearly 80% recovery of the catalytic activity of the hybrid-type heterodimer WT/R98E implies that the wild-type enzyme does not exhibit its full catalytic function as a dimeric enzyme with the two independently functional subunits, which have been implied to function independently with no relationship with each other. It is possible that one catalytic site can exhibit its full activity only when the other site is resting, or that one of the two catalytic sites is only functional in the homodimeric enzyme. As Reed and Rilling have reported, with regard to the precise number of substrates bound to avian FPP synthase, two sites for allylic and four sites for IPP in the presence of divalent metal ions (*24*), the former possibility seems more probable.

Although all the discussion presented above is essentially based on the whole structure of avian FPP synthase, which has been determined by the X-ray crystallographic analysis to 2.6 Å resolution (*14*, *25*), it is reasonable to apply it to

the bacterial FPP synthase because there is a considerable degree of structural similarity with respect to the secondary structures between the avian and *B. stearothermophilus* FPP synthases in addition to the presence of seven highly conserved regions (*33*). X-ray diffraction studies with the FPP synthase of *B. stearothermophilus* are now in progress (*34*).

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