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A mechanism for posttranslational modifications of proteins by yeast protein farnesyltransferase

(Ras/prenyl transfer/farnesyl diphosphate)

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ABSTRACT **Protein farnesyltransferase catalyzes the alkylation of cysteine in C-terminal CaaX sequences of a variety of proteins, including Ras, nuclear lamins, large G proteins, and phosphodiesterases, by farnesyl diphosphate (FPP). These modifications enhance the ability of the proteins to associate with membranes and are essential for their respective functions. The enzyme-catalyzed reaction was studied by using a series of substrate analogs for FPP to distinguish between electrophilic and nucleophilic mechanisms for prenyl transfer. FPP analogs containing hydrogen, fluoromethyl, and trifluoromethyl substituents in place of the methyl at carbon 3 were evaluated as alternative substrates for alkylation of the sulfhydryl moiety in the peptide dansyl-GCVIA. The analogs were alternative substrates for the prenylation reaction and were competitive inhibitors against FPP. A comparison of k_{cat} for FPP and the analogs with k_{solv}, the rate constants for solvolysis of related *p*-methoxybenzenesulfonate derivatives, indicated that protein prenylation occurred by an electrophilic mechanism.**

Approximately 30% of human cancers are associated with mutations in Ras proteins. These include pancreatic adenocarcinomas (90%), colon adenocarcinomas and adenomas (50%), thyroid carcinomas and adenomas (50%), lung adenocarcinomas (30%), myeloid leukemias (30%), and melanomas (20%) (1, 2). Ras proteins are major components in the signal transduction pathway leading to cell division. They bind GDP when inactive and, upon stimulation, exchange GDP for GTP. An activating protein (GAP) stimulates Ras to hydrolyze GTP to GDP, which terminates the signal. Mutant Ras proteins bind GTP but do not hydrolyze it efficiently. The resulting stimulation of downstream protein kinases leads to uncontrolled cell growth. Both normal and mutant forms of Ras must associate with the inner surface of the outer membrane to participate in signal transduction (3). The discovery that farnesylation is required for mutant forms of Ras to manifest their transforming activity has promoted widespread interest in protein prenylation (4, 5).

A large group of proteins has been identified that are posttranslationally modified by the attachment of a prenyl group to a cysteine residue near the C terminus through a thioether bond. Although many are "true" Ras proteins or other members of the Ras superfamily (Rab, Ral, Rac, and Rap), some non-Ras proteins such as some nuclear lamins, large G proteins, and phosphodiesterases are also prenylated (4). Addition of a hydrophobic prenyl group increases the protein's affinity for a membrane and assists in membrane recognition (4). Although prenylated proteins selectively associate with different membranes in a cell, there is currently no conclusive evidence for a highly selective receptor-mediated affiliation. For some proteins such as rhodopsin kinase, prenyl modification may be necessary for the cycling of proteins between membranes and the cytosol (6).

Protein farnesyltransferase (PFTase) and protein geranylgeranyltransferase I prenylate proteins containing C-terminal CaaX consensus sequences, where the a is an aliphatic amino acid and X is one of several amino acids, as illustrated in Fig. 1. The enzymes are heterodimers that share a common α subunit but differ in their β subunits (7). Whether the protein substrate is modified by a farnesyl or geranylgeranyl group is determined by the X residue. If X is Ser, Ala, or Met, the protein is farnesylated; if X is Leu, the protein is geranylgeranylated (8, 9). Two other prenylation-recognition motifs have been identified. In the Rab GTP-binding proteins, both cysteines of C-terminal -CC or -CXC sequences are geranylgeranylated by protein geranylgeranyltransferase II (10).

PFTase was purified from bovine brains (11), rat brains (12), and yeast (13). Recombinant human (14) and yeast (15) PFTases with C-terminal Glu-Glu-Phe (EEF) epitopes in the β subunit were overproduced in *Escherichia coli* and purified by immunoaffinity chromatography. Short peptides conforming to the CaaX rule were competitive inhibitors of PFTase, acting either as alternative substrates or true dead-end inhibitors (usually when the second aliphatic amino acid is Phe, Tyr, or Trp) (16). Detailed kinetic analyses of the PFTase reaction were performed for the purified bovine and human enzymes. Bovine PFTase appears to add substrates by a random sequential mechanism (17), and the binding mechanism for the human enzyme is apparently an ordered sequential process (18) with farnesyl diphosphate (FPP) binding first. Preliminary experiments showed that the yeast PFTase also prefers to bind FPP first by an ordered sequential mechanism (13). Although inhibition studies with substrate analogs and alternative substrates were performed for PFTase in an intense search for potent inhibitors of Ras farnesylation, to our knowledge, there are no reports that bear on the chemical mechanism of farnesyl transfer to the cysteine residue. The design of potent PFTase inhibitors, especially transition-state analogs, should be facilitated by understanding the mechanism of the reaction.

MATERIALS AND METHODS

Materials. Diphosphates **1–4** (see Fig. 2) were synthesized from their corresponding alcohols by the method of Davisson *et al.* (19). The syntheses of alcohols **2–4** are available from the authors upon request. Dansyl-GCVIA was synthesized by solid-phase peptide methods.

Prenyl Transfer Assays. Recombinant yeast PFTase was purified by immunoaffinity chromatography as described (15). Catalytic rate constants (k_{cat}) were measured by using a fluorescence assay that continuously monitored farnesylation of dansylated pentapeptide dansyl-GCVIA (20, 21) with a Spex FluoroMax model spectrofluorimeter with λ_{ex} = 340 nm

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Abbreviations: FPP, farnesyl diphosphate; PFTase, protein farnesyltransferase; TFA, trifluoroacetic acid; FPPSase, FPP synthase.

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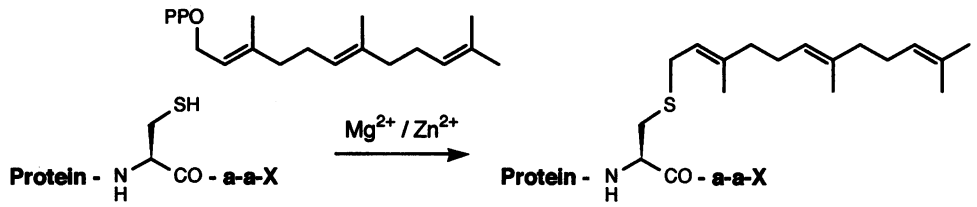
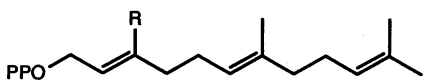


FIG. 1. Reaction catalyzed by PFTase.

(slit width = 5.1 or 8.5 nm) and λ_{em} = 486 nm (slit width = 5.1 or 8.5 nm) and 3-mm square cuvettes. Assays (250 μl) were conducted at 30°C in 50 mM Tris·HCl/10 mM $MgCl_2$/10 μM $ZnCl_2$/5 mM dithiothreitol/0.04% *n*-dodecyl β-D-maltoside, pH 7.0. Diphosphate compounds (**1–4**) were present at saturating concentrations (10–20 μM). Due to substrate inhibition (21), a saturating concentration of the peptide substrate was not used, and the concentration that gave a maximal rate (2.4 μM) was chosen instead. PFTase (1–47 nM) was used to initiate the reactions. Initial rates were measured from the linear region of each experiment, and all measurements were made in duplicate. Rates were measured in cps/s and converted to units of s^{-1} by using a conversion factor calculated from the slope of a line generated in a plot of concentration of synthetic dansyl-G(*S*-farnesyl)CVIA (dansyl-GC^{F}VIA) (where ^{F} is farnesyl) vs. fluorescence intensity. For each alternative substrate, compounds **2–4**, the S-farnesylated dansylated product was synthesized from the corresponding fluorinated or desmethyl alcohol and dansyl-GCVIA and used to generate calibration curves. Values for each conversion factor did not appreciably differ.

Synthesis of Farnesylated Products. The farnesylated products were synthesized essentially as described by Pompliano *et al.* (20). Alcohols **2** and **3** were converted to their corresponding bromides by using CBr_4 and PPh_3. The bromides (1.2 equivalents) were allowed to react with dansyl-GCVIA in the presence of diisopropylethylamine (3.0 equivalents) in dimethylformamide. The trifluoromethyl compound was synthesized in a similar manner except that alcohol **4** was converted to the corresponding tosylate with tosyl chloride and dimethylaminopyridine. After 1–3 hr, the reactions were quenched with water, and the resulting mixture was frozen and lyophilized. The residue solids were dissolved in 1:1 (vol/vol) acetic acid/acetonitrile (CH_3CN) and purified by reversed-phase HPLC (Vydac C_{18} protein and peptide column). A gradient of 5% solvent B to 100% B over 35 min was used, where solvent A was H_2O/0.1% trifluoroacetic acid (TFA) and solvent B was CH_3CN/0.1% TFA. The farnesylated products were eluted between 90% and 95% B and detected by UV absorbance at 214 nm.

Analysis of Enzymatic Reactions by HPLC. Reaction mixtures were analyzed on a Bondasil SP C_{18} column with a linear gradient from 5% CH_3CN/0.1% TFA to 100% CH_3CN/0.1% TFA over 25 min at 1.0 ml/min, and products eluted were detected at 214 nm. Individual reaction mixtures consisted of the FPP analog (10 nmol), dansyl-GCVIA (10 nmol), and PFTase (2 μg) in 50 mM Tris·HCl/10 mM $MgCl_2$/10 μM $ZnCl_2$/0.04% *n*-dodecyl β-D-maltoside, pH 7.0, incubated at 30°C for 5 hr. Coinjections consisted of the reaction mixture plus 5 nmol of the corresponding synthetic standard.



1 R = CH_3, **FPP**
2 R = CH_2F
3 R = H
4 R = CF_3

FIG. 2. Structures of FPP and related analogs.

RESULTS

Kinetic Measurements. Kinetic constants for alkylation of the cysteine in dansyl-GCVIA by diphosphates **1–4** (Fig. 2) were determined from initial velocity measurements by using the fluorescence assay of Pompliano *et al.* (20) and a buffer specifically optimized for yeast PFTase (21). The results are given in Table 1. Substitution of the methyl group at carbon 3 of FPP by CH_2F (**2**), H (**3**), and CF_3 (**4**) led to a progressive decrease in k_{cat}, with the CF_3 analog (**4**) being 770-fold less reactive than FPP. K_m values for the allylic substrate also decreased progressively from 1.3 μM for FPP to 36 nM for compound **3**. We were unable to measure the K_m for compound **4**. Yeast PFTase was saturated by the trifluoromethyl diphosphate down to a concentration of 20 nM. The fluorescence assay was not sufficiently sensitive to accurately measure initial rates at lower substrate concentrations.

Analogs **2–4** were competitive inhibitors of FPP and noncompetitive inhibitors of dansyl-GCVIA (Table 2). These patterns are consistent with a mechanism where the analogs exert their influence by binding selectively to the FPP site (22). Trifluoromethyl derivative **4** (K_i = 11 nM) is one of the most potent inhibitors yet reported for a protein prenyltransferase.

Product Studies. To confirm that FPP analogs **2–4** were alternative substrates for yeast PFTase, the respective products expected for alkylation of dansyl-GCVIA were synthesized (20). The alcohols corresponding to diphosphates **2** and **3** were converted to bromides, which in turn were used to alkylate the cysteine moiety in dansyl-GCVIA. A similar procedure was used for the trifluoromethyl analog, except the alcohol was converted to a tosylate derivative for the alkylation step. The alkylated pentapeptides were purified by reversed-phase HPLC, and each gave a fast atom bombardment/mass spectrum with a characteristic molecular ion at M+1.

In preparative-scale experiments, 10 nmol of each diphosphate and 10 nmol of dansyl-GCVIA were incubated with 2 μg of PFTase. The reaction mixtures were analyzed by HPLC, and each analog gave a new peak with a retention time characteristic for the alkylated pentapeptides. The identity of each peak was confirmed by coinjection with an authentic sample of alkylated dansyl-GCVIA. The results for compound **2** are illustrated in Fig. 3 and are typical of those for compounds **3** and **4** as well.

DISCUSSION

PFTase is only one member of the much larger family of prenyltransferase enzymes that catalyze alkylation of electron-

Table 1. Kinetic constants for condensation of dansyl-GCVIA with diphosphates **1**, **2**, **3**, and **4**

Diphosphate	K_m, nM	k_{cat}, s^{-1}	k_{cat}^{rel}
1	1300	2.30	1
2	96	3.5×10^{-1}	1.5×10^{-1}
3	36	4.7×10^{-2}	2.1×10^{-2}
4	ND	3.1×10^{-3}	1.3×10^{-3}

ND, not determined.

Table 2. Inhibition constants for **2**, **3**, and **4**

Inhibitor	FPP	Dansyl-GCVIA	
	K_i, nM	K_{is}, nM	K_{ii}, nM
2	140 ± 11	333 ± 25	290 ± 16
3	36 ± 2	723 ± 747	83 ± 28
4	11 ± 2	120 ± 40	51 ± 19

To determine FPP K_i values, the concentration of dansyl-GCVIA was held constant at 2.4 μM; all analogues showed a competitive inhibition pattern. To determine dansyl-GCVIA K_{is} (slope) and K_{ii} (intercept) values, the concentration of FPP was held constant at 2.0 μM. All analogs showed a noncompetitive inhibition pattern.

rich acceptors by allylic isoprenyl diphosphates (23). Although some prenyl acceptors, such as the sulfhydryl moiety alkylated by protein prenyltransferases, are potent nucleophiles, most are weakly nucleophilic. In the only two instances where mechanisms were established—the alkylation of the carbon–carbon double bond in isopentenyl diphosphate catalyzed by FPP synthase (FPPSase) and alkylation of the indole ring in tryptophan by dimethylallyltryptophan synthase—the weakly nucleophilic prenyl acceptors were alkylated by electrophilic carbocations produced from the allylic substrates (24, 25) by the general mechanism shown in Fig. 4. Strong support for the electrophilic mechanism was obtained from linear free energy correlations by using fluorinated analogs of the allylic diphosphates as alternative substrates for the enzymes. The distinction between electrophilic alkylations and nucleophilic displacements is dramatic. When hydrogen atoms in the allylic substrates were substituted by strongly electron-withdrawing fluorines, rates of electrophilic alkylations, including prenyltransferase, were retarded in proportion to the number of hydrogens replaced, as expected for reactions proceeding through transition states that developed positive charge (25). Similar substitutions exerted a slight accelerating effect on direct nucleophilic displacements (26).

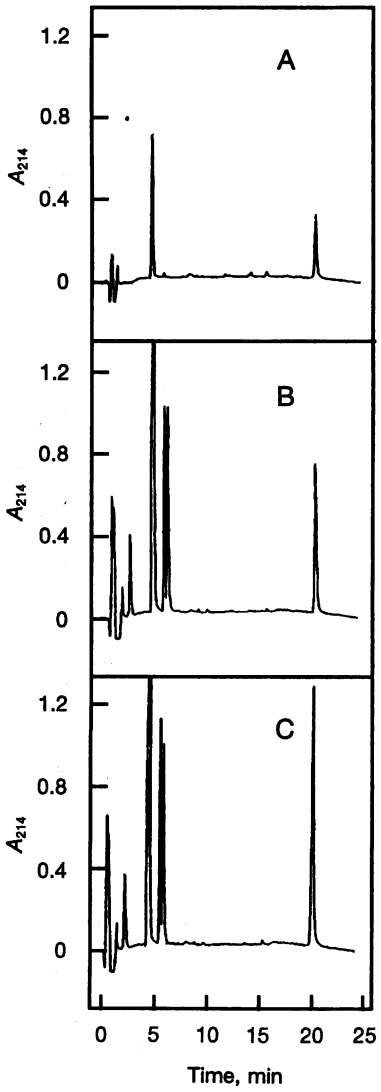


FIG. 3. HPLC profiles of products from the PFTase-catalyzed condensation of dansyl-GCVIA with compound **2** and of an authentic sample of synthetic dansyl-G(S-fluoromethylfarnesyl)CVIA on a Bondasil SP C_{18} reversed-phase column. Elution buffer was a linear gradient from 0.1% TFA/5% CH_3CN/95% water (vol/vol) to 0.1% TFA in CH_3CN. Detection was at 214 nM. (*A*) Synthetic dansyl-G(*S*-fluoromethylfarnesyl)CVIA (3 nmol). (*B*) Products from incubation of dansyl-GCVIA (10 nmol) and compound **2** (10 nmol) with 2 μg of PFTase. (*C*) Coinjection of products shown in *B* with the synthetic sample shown in *A*.

Although protein prenylation can be rationalized by an electrophilic mechanism, it would at best be tenuous to extrapolate results for other prenyltransferases to this reaction. As mentioned previously, the sulfhydryl, or perhaps thiolate, moiety in cysteine is a potent nucleophile for a direct displacement through a transition state that does not develop positive charge. Furthermore, protein prenyltransferases lack the aspartate-rich regions normally associated with binding and catalysis of many other prenyltransferases such as FPPSase, and they do not show any other discernable similarities in their amino acid sequences to those of other prenyltransferases (27).

The effect of substituting the methyl on carbon 3 in FPP with hydrogen, fluoromethyl, and trifluoromethyl on the rate of the protein prenyl-transfer reaction supports an electrophilic alkylation of the cysteine sulfur. As illustrated in Table 3, the rates of solvolysis reactions for dimethylallyl models of FPP and analogs **2–4** were strongly depressed by substitutions that destabilized the corresponding allylic cations (28). A similar trend was reported for the prenyl-transfer reaction catalyzed by FPPSase, where the electron-rich acceptor was a nonnucleophilic carbon–carbon double bond (24).

Although the overall trends seen in rates of prenyl transfer by FPTase and FPPSase are similar, the magnitude of the effect was smaller for alkylation of sulfur than a carbon–carbon double bond. It is interesting to note that the kinetic behavior of the alternative substrates for FPPSase was more closely modeled by k_{solv}, the solvolysis rate constant, for the allylic derivatives in aqueous solvents, while the behavior of the alternative substrates for PFTase was more closely modeled by k_{nuc}, the nucleophilic component for solvolysis of the compounds in aqueous solvents containing azide (28). The compression of substituent effects by azide in the model studies resulted from an acceleration of the rates of the less reactive analogs, presumably through an enforced solvolysis mechanism (29). The similar trends seen for alkylation of cysteine by PFTase and reaction with azide during solvolysis are consistent with an electrophilic reaction involving a potent nucleophile.

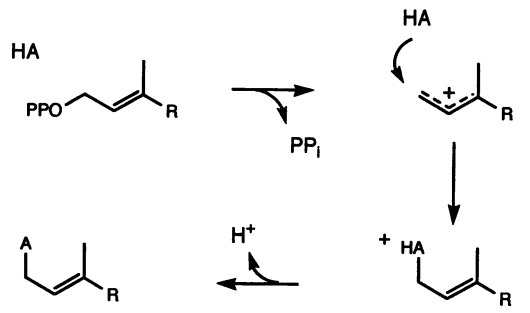


FIG. 4. Electrophilic alkylation mechanism for prenyl transfer.

Table 3. Kinetic constants of analogs for solvolysis of dimethylallyl *p*-methoxybenzenesulfonate, 1′–4 condensation of geranyl diphosphate with isopentenyl diphosphate, and protein prenylation of dansyl-GCVIA by FPP

Substituent at carbon 3	Solvolysis		k_{cat}^{rel} for FPPSase	k_{cat}^{rel} for PFTase
	k_{solv}^{rel}	k_{nuc}^{rel}		
CH_3	1	1	1	1
H	1.5×10^{-3}	1.0×10^{-1}	—	2.1×10^{-2}
CH_2F	9.1×10^{-4}	6.1×10^{-2}	1.8×10^{-2}	1.5×10^{-1}
CHF_2	7.4×10^{-6}	1.2×10^{-2}	1.9×10^{-6}	—
CF_3	6.7×10^{-7}	6.5×10^{-3}	3.6×10^{-7}	1.3×10^{-3}

Solvolysis of dimethylallyl *p*-methoxybenzenesulfonates was in aqueous dioxane or mixtures of sodium azide and aqueous dioxane (28). Condensation of geranyl diphosphate and related analogs with isopentenyl diphosphate is catalyzed by avian FPPSase (24). Condensation of FPP and related analogs with dansyl-GCVIA is catalyzed by yeast PFTase.

For an ordered addition of substrates by the mechanism shown in Eq. **1**,

$$E \underset{k_{-1}}{\overset{k_1[FPP]}{\rightleftharpoons}} E{\cdot}FPP \underset{k_{-2}}{\overset{k_2[dansyl\text{-}GCVIA]}{\rightleftharpoons}} E{\cdot}FPP{\cdot}dansyl\text{-}GCVIA \xrightarrow{k_3} E{\cdot}PP_i{\cdot}dansyl\text{-}GC^FVIA \xrightarrow{k_4} E + PP_i + dansyl\text{-}GC^FVIA \quad [1]$$

$V_{max} = k_3k_4[E_\tau]/(k_3 + k_4)$, where E_τ is total enzyme, and $K_m^{FPP} = k_3k_4/k_1(k_3 + k_4)$. We do not know whether the step represented by k_3 (condensation) or k_4 (release of products) is rate limiting when FPP is the substrate. However, k_3 will become progressively smaller as the allylic diphosphates are rendered less reactive by the substituents at carbon 3. The correlation we found between the rates for prenyl transfer and solvolysis are consistent with k_3 becoming rate limiting for the analogs. The alternative explanation, that the rate constants for release of farnesylated peptides become progressively smaller in a manner that parallels the decrease in chemical reactivity of the analogs, seems unlikely. In addition, there is no evidence from our inhibition studies to suggest large differences in the affinity of PFTase for the hydrocarbon moieties in analogs **2–4** that might lead to a decrease in the rate of release of products.

Under the conditions where $k_3 << k_4$, $k_3 \approx k_{cat}$, and $K_m \approx k_3/k_1 \approx k_{cat}/k_1$. If we assume that the substitutions in analogs **2–4** do not significantly alter the on-rate for their binding to PFTase, K_m for the analogs should decrease as they become less reactive. We found that the Michaelis constants for the allylic substrates decreased from 1.3 μM for FPP to <20 nM for compound **4**. In addition, for an ordered binding mechanism where the allylic diphosphates add first, $K_d^{allylic} \approx K_i^{allylic} = k_{-1}/k_1$ (30). Thus, $K_i^{allylic}/K_m^{allylic} \approx k_{-1}/k_{cat}$ or $k^{-1} = k_{cat}K_i/K_m$. The condition $k_3 << k_4$ should hold for analog **3** where the prenyl-transfer reaction is ≈50 times slower than for FPP. By using the values given in Tables 1 and 2, $k_{cat}^3 \approx k_{-1}^3 \approx 0.05\ s^{-1}$ and $k_1 \approx 1 \times 10^6\ s^{-1}{\cdot}M^{-1}$. The estimated value of the second-order rate constant for addition of the allylic analog is substantially below those normally associated with a diffusion-controlled addition of substrate (31) and suggests that the binding step might be associated with a conformational change in the enzyme.

In summary, a kinetic study of the prenylation of dansyl-GCVIA by recombinant yeast PFTase with analogs of FPP indicates that the reaction is an electrophilic alkylation. In view of the high degree of similarity among protein prenyltransferases, our results for yeast PFTase should be generally applicable to type I and type II protein geranylgeranyltransferases as well.

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