Steady-State Kinetic Mechanism of Ras Farnesyl:Protein Transferase

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Received October 3, 1991; Revised Manuscript Received January 16, 1992

ABSTRACT: The steady-state kinetic mechanism of bovine brain farnesyl:protein transferase (FPTase) has been determined using a series of initial velocity studies, including both dead-end substrate and product inhibitor experiments. Reciprocal plots of the initial velocity data intersected on the 1/[s] axis, indicating that a ternary complex forms (sequential mechanism) and suggesting that the binding of one substrate does not affect the binding of the other. The order of substrate addition was probed by determining the patterns of dead-end substrate and product inhibition. Two nonhydrolyzable analogues of farnesyl diphosphate, (α-hydroxyfarnesyl)phosphonic acid (**1**) and [[(farnesylmethyl)hydroxyphosphinyl]methyl]phosphonic acid (**2**), were both shown to be competitive inhibitors of farnesyl diphosphate and noncompetitive inhibitors of Ras-CVLS. Four nonsubstrate tetrapeptides, CV[D-L]S, CVLS-NH_2, *N*-acetyl-L-penicillamine-VIM, and CIFM, were all shown to be noncompetitive inhibitors of farnesyl diphosphate and competitive inhibitors of Ras-CVLS. These data are consistent with random order of substrate addition. Product inhibition patterns corroborated the results found with the dead-end substrate inhibitors. We conclude that bovine brain FPTase proceeds through a random order sequential mechanism. Determination of steady-state parameters for several physiological Ras-CaaX variants showed that amino acid changes affected the values of K_M, but not those of k_{cat}, suggesting that the catalytic efficiencies (k_{cat}/K_M) of Ras-CaaX substrates depend largely upon their relative binding affinity for FPTase.

Site-specific farnesylation is the first step in a series of posttranslational modifications leading to the biological activation of a variety of cellular polypeptides [reviewed in Glomset et al. (1990) and Maltese (1990)]. In a reaction catalyzed by farnesyl:protein transferase (FPTase),[1] the farnesyl moiety of the cholesterol biosynthetic intermediate, farnesyl diphosphate, is linked through a thioether bond to a conserved cysteine residue positioned four amino acids from the C-terminus of the protein acceptor substrate (Scheme I). Following farnesylation, the three C-terminal amino acids are proteolytically removed, and the newly formed farnesylated cysteine residue is methyl esterified. Proteins that can be farnesylated (or otherwise prenylated by other transferases) have a consensus C-terminal amino acid sequence Ca_1a_2X.[1] The acronym codes for a cysteine residue (C), followed (generally) by two aliphatic amino acid residues (a_1a_2), and ending with another amino acid (X). Exceptions to this Ca_1a_2X mnemonic abound, however. Although the cysteine residue is essential, many proteins with non-glycine residues in the a_1 position are farnesylation substrates. On the other hand, not all proteins with Ca_1a_2X-like sequences are prenylated (e.g., the $G_i\alpha$ subunit has the Ca_1a_2X sequence CGLF but is not modified; Mumby et al., 1990). Fungal mating factors, nuclear lamins, the γ subunit of retinal transducin, and the cell-transforming GTPase protein Ras all must be farnesylated prior to membrane association, which, in turn, is required for in vivo protein activity (Fukada et al., 1990; Glomset et al., 1990; Goldstein & Brown, 1990; Lai et al., 1990; Maltese, 1990; Mumby et al., 1990; Rine & Kim, 1990; Gibbs, 1991). That Ras requires this modification is of particular interest, since activated forms of Ras are found in 20% of human cancers and >50% of colon and pancreatic carcinomas (Bos, 1990). Interference of Ras membrane localization by inhibition of the enzyme-mediated farnesylation reaction is thus a possible anticancer strategy (Goldstein & Brown, 1990; Gibbs, 1991).

FPTase purified to homogeneity from either rat (Reiss et al., 1990) or bovine brain (Moores et al., 1991) is an α/β heterodimer, with its subunits having molecular masses of 47 kDa (α) and 45 kDa (β). A cDNA encoding the β subunit from rat brain (Chen et al., 1991) has been cloned and found to be homologous to the yeast *RAM1* gene. A partial cDNA encoding the C-terminal 329 amino acid residues of the α subunit cloned from bovine brain is homologous to the yeast *RAM2* gene (Kohl et al., 1991). Transfection of the rat β subunit cDNA together with a cDNA encoding part of the rat α subunit yields FPTase activity in human kidney cells (Chen et al., 1991). Since mutations in either *RAM1* or *RAM2* disrupt FPTase activity in yeast, it now appears that the β and α subunits are the mammalian counterparts of the yeast *RAM1* and *RAM2* genes, respectively (Kohl et al., 1991).

The enzymology of FPTase has focused on its isoprenyl diphosphate and protein substrate specificity (Reiss et al., 1990, 1991a,b; Schaber et al. 1990; Moores et al., 1991). FPTase is particular about its isoprenoid substrate, but is quite promiscuous with regard to its protein substrate. Although geranylgeranyl diphosphate and farnesyl diphosphate bind to FPTase with roughly the same affinity (Schaber et al. 1990),

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[1] Abbreviations: Ca_1a_2X, Cys (C), an aliphatic amino acid (a), and any amino acid (X); FPTase, farnesyl:protein transferase; GGPTase, geranylgeranyl:protein transferase; Ras-CaaX, [Leu-68]RAS1(term. 185)-CaaX; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography.

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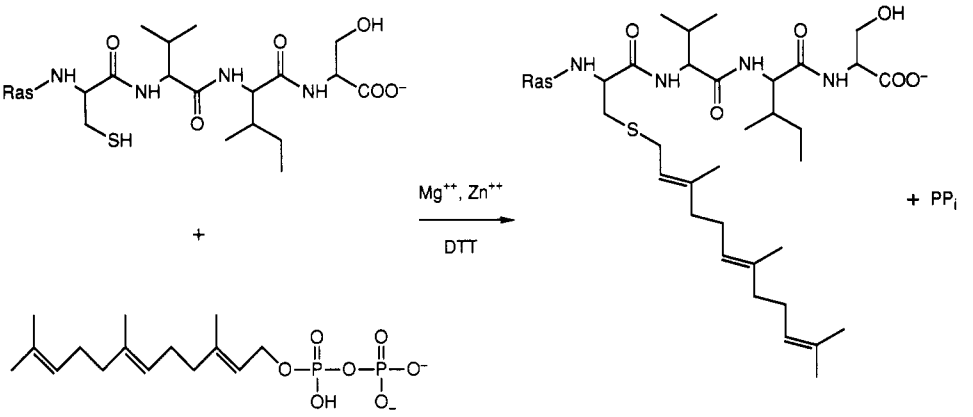
Scheme I: Reaction Catalyzed by Farnesyl:Protein Transferase



only farnesyl diphosphate is a kinetically competent substrate in the reaction, while geranylgeranyl diphosphate behaves as a nonreactive inhibitor, using Harvey-Ras as the acceptor protein (Yamane et al., 1990; Moores et al., 1991; Seabra et al., 1991). There are other classes of prenyltransferases (GGPTases) that utilize geranylgeranyl diphosphate as the donor substrate (Casey et al., 1991; Horiguchi et al., 1991; Joly et al., 1991; Moores et al., 1991; Seabra et al., 1991; Yokoyama et al., 1991). Interaction between FPTase and its protein substrates appears to depend only upon the amino acid sequence of the Ca_1a_2X box, on the basis of the observation that cognate tetrapeptides can competitively inhibit the binding of protein substrates (Reiss et al., 1990, 1991a; Schaber et al., 1990; Moores et al., 1991). Within the Ca_1a_2X sequence, substitutions are better tolerated in the a_1 position than in the a_2 position (Stimmel et al., 1990). The best farnesylation substrates contain aliphatic or aromatic residues in the a_2 position. Changes in the X position, however, severely affect the efficiency of farnesylation. In fact, it appears that the C-terminal residue of Ca_1a_2X proteins determines to which type of transferase the protein will bind, with Ala, Cys, Gln, Ser, and Met favoring binding to FPTase and Leu preferring binding to GGTase-I (Moores et al., 1991; Reiss et al., 1991a).

While an understanding of the substrate specificity is a useful first step, the design of novel compounds that will inhibit the action of FPTase requires a detailed knowledge of the chemical mechanism through which the enzyme-catalyzed reaction proceeds. Thus far, few mechanistic details of the reaction catalyzed by FPTase have been reported. With a two substrate/two product (Bi Bi) system, the logical first question is whether a ternary complex, consisting of FPTase, farnesyl diphosphate, and protein, is formed (sequential mechanism), or whether the first product leaves the active site before the second substrate is bound (ping-pong mechanism). In this paper, steady-state enzyme kinetics, including nonreactive substrate and product inhibitor studies, were used to distinguish between these two mechanistic possibilities and to determine the relative binding order of the two substrates.

Experimental Procedures

Materials

FPTase was purified to homogeneity from bovine brain as described in Moores et al. (1991). The *ras* construct [Leu-68]RAS1(term.)-SLKCVLS, whose gene product is referred to as Ras-CVLS, has been described previously (Temeles et al., 1985; Gibbs et al., 1989). Ras-CaaX proteins were purified as described previously (Moores et al., 1991). [^{3}H]Farnesyl diphosphate (20 Ci/mmol) was purchased from Du Pont-NEN (Boston, MA). The farnesyl diphosphate analogue, [[(farnesylmethyl)hydroxyphosphinyl]methyl]phosphonic acid (**2**; McClard et al., 1987; Biller et al., 1988), was synthesized by T. J. Lee and W. J. Holtz (Merck Research Laboratories). Peptides and peptide derivatives were synthesized by V. M. Garsky (Merck Research Laboratories) using an Applied Biosystems Model 430A synthesizer, purified by reverse-phase HPLC, and characterized by amino acid analysis and fast atom bombardment mass spectrometry. All other chemicals and reagents were from Sigma, Aldrich, or Fisher and were used without further purification.

Methods

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was carried out as described by Laemmli (1970). Gels were stained either with Coomassie Blue or with silver (Daiichi Silver Stain, Emprotech, Hyde Park, MA). Protein concentrations were determined using the Bradford (1976) method or were estimated by densitometry on gels. Bovine serum albumin (affinity purified, Pierce, Rockford, IL) was used as the protein standard. Protein solutions were concentrated by ultrafiltration with Amicon PM-30 membranes or by centrifugation in an Amicon Centricon-30 ultrafiltration apparatus.

Transferase Assays. FPTase activity was assayed in the biosynthetically forward direction at 30 °C. Reactions were never allowed to proceed to more than 10% completion based on the limiting substrate. Each velocity data point shown is the average of at least three determinations. For calculations, the molecular mass of the transferase was assumed to be 90 kDa and that of the Ras-CVLS to be 21 kDa. A typical reaction contained the following: [^{3}H]farnesyl diphosphate (10–500 nM), Ras-CVLS (0.2–8 μM), 50 mM HEPES, pH 7.5, 5 mM $MgCl_2$, 20 μM $ZnCl_2$, 5 mM dithiothreitol, 0.1% PEG 20000, and pure FPTase (0.2–2 nM). After thermally preequilibrating the assay mixture in the absence of enzyme, reaction was initiated by adding the transferase. Aliquots (250–500 μL) were withdrawn and diluted into 10% HCl in ethanol (1–2 mL) at timed intervals. The quenched reactions were allowed to stand at room temperature for 15 min (to hydrolyze unreacted farnesyl diphosphate). After adding 100% ethanol (2 mL), the reactions were vacuum-filtered through Whatman GF/C filters using a Brandel cell harvester (Model MB-24L, Gaithersburg, MD). Filters were washed four times with 100% ethanol (2-mL aliquots), mixed with scintillation fluid (10 mL, ReadiSafe, Beckman), and then counted in a Beckman LS3801 scintillation counter.

FPTase was also assayed by quantitating the amount of farnesylated Ras-CVLS product isolated directly from polyacrylamide gels cross-linked with *N,N'*-diallyltartardiamide (DATD, Bio-Rad; Anker, 1970). Transferase kinetic assays

were performed as described above, except that reaction mixtures (120 μL) were quenched by the addition of 5× sample loading buffer (20 μL; 100 mM Tris-HCl, pH 6.8, containing 50% glycerol, 10% SDS, 200 μM DTT, 50 mM EDTA, and 10 μg/μL Ras-CVLS). Ras-CVLS was included in the sample loading buffer to aid in visualizing the product band in the gel after staining. Polyacrylamide (15%) gels were cast using DATD mole for mole in the place of methylenebis(acrylamide) as the cross-linking agent. Following separation of the quenched samples by electrophoresis, the gel was stained for protein with Coomassie Blue. Product [^{3}H]-farnesyl-Ras-CVLS (21-kDa band) was cut out of the gel and then incubated with 2% periodic acid in 0.1% H_3PO_4 (2 mL) at 60 °C for 3 h to dissolve the gel. Scintillation fluid (10 mL) was added, and the samples were counted.

When peptide CVLS was used as the protein acceptor substrate, product [^{3}H]farnesyl-CVLS was isolated from the reaction mixture by reverse-phase HPLC and then counted. Solvent A was 30% CH_3CN containing 10 mM sodium phosphate, pH 6.8, and solvent B was 60% CH_3CN containing 10 mM sodium phosphate, pH 6.8. Samples (100 μL) were injected onto an analytical Vydac C4 column (150 mm) equilibrated in 0% solvent B at a flow rate of 1.5 mL/min. Product farnesyl-CVLS was eluted by running a linear CH_3CN gradient from 0% to 100% solvent B over 7 min. Total analysis time for each sample was 12 min. Farnesyl-CVLS eluted with a retention time of 7 min. Synthetic farnesyl-CVLS (Schaber et al., 1990) was used to calibrate the column before radioactive samples were injected.

For inhibition studies, assays were run as described above, except inhibitors (peptides or farnesyl diphosphate analogues) were included at various concentrations. Product was quantitated using the acidic ethanol precipitation assay.

Steady-state kinetic parameters were determined either from reciprocal plots or from a nonlinear least squares fit to the relevant rate equation of initial velocity data using the Marquardt algorithm (Marquardt, 1963). For the inhibitor studies, the reciprocal plots were drawn to show the general inhibition pattern, but the values of K_i in Table II were calculated from nonlinear least squares fits to the appropriate inhibition model.

(R,S)-Dimethyl [1-Hydroxy-(E,E)-3,7,11-trimethyl-2,6,10-dodecatrienyl]phosphonate (**3**). To a stirred solution of farnesal (245 mg, 1.11 mmol) in acetonitrile (1.1 mL) under argon at room temperature were added triethylamine (0.31 mL, 2.22 mmol) and dimethyl phosphite (0.153 mL, 1.67 mmol), and the resulting mixture was stirred at room temperature for 24 h. After the reaction mixture was concentrated in vacuo, the residue was chromatographed over silica gel eluted with ethyl acetate to afford a colorless oil (141 mg, 38% yield): ^{1}H NMR ($CDCl_3$) δ 1.60 (6 H, s), 1.68 (3 H, s), 1.71 (3 H, d, J = 3.1 Hz), 1.80–2.30 (9 H, m), 3.80 (6 H, m), 4.69 (1 H, dt, J = 9 and 5.4 Hz), 5.00–5.20 (2 H, m), 5.34 (1 H, m).

(R,S)-[1-Hydroxy-(E,E)-3,7,11-trimethyl-2,6,10-dodecatrienyl]phosphonic Acid (**1**). Trimethylsilyl bromide (0.107 mL, 0.81 mmol) was added to a stirred solution of **3** (67 mg, 0.203 mmol) and 2,4,6-collidine (0.107 mL, 0.81 mmol) in dichloromethane (3 mL) under argon at 0 °C, and the mixture was stirred at 0 °C for 30 min and then at room temperature for 5 h. After first diluting the white suspension with toluene (10 mL) and then evaporating the solvent in vacuo, the resulting white solid was dissolved in ethyl acetate and water and the pH was adjusted to pH 3 by the addition of 1 M HCl. The organic layer was separated, dried (over $MgSO_4$), and concentrated in vacuo to afford a pale yellow solid. The solid was washed with dichloromethane (2 × 5 mL) and then dried in vacuo to yield a white solid (40 mg, 65% yield): ^{1}H NMR (DMSO) δ 1.55 (3 H, s), 1.56 (3 H, s), 1.60 (3 H, d, J = 2.0 Hz), 1.80–2.20 (9 H, m), 4.24 (1 H, dd, J = 9.5 and 10.5 Hz), 5.00–5.30 (3 H, m). Anal. Calcd for $C_{15}H_{27}O_4P \cdot 0.25H_2O$: C, 58.71; H, 9.03. Found: C, 58.72; H, 8.94.

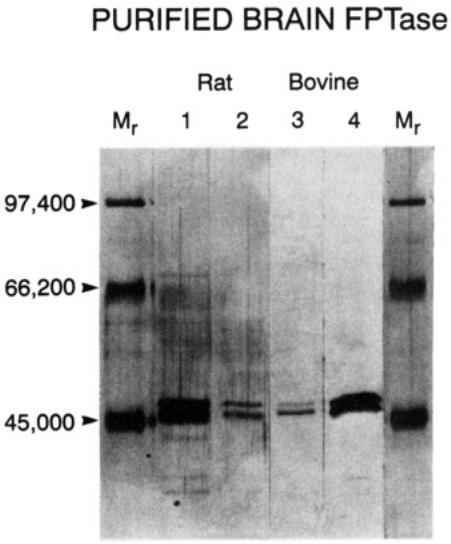


FIGURE 1: SDS–polyacrylamide gel electrophoresis of FPTase isolated from rat and bovine brain. Lanes 1 and 2: 20- and 2-ng samples of purified rat brain FPTase, respectively. Lanes 3 and 4: 2- and 20-ng samples of purified bovine brain FPTase, respectively. Electrophoresis through 7.5% acrylamide gels (160 × 180 × 0.75 mm) was carried out at constant current (40 mA) for 1.5 h. Protein was visualized after silver staining. The protein molecular weight standards flank the FPTase lanes.

Results

FPTase Activity Assay. A filter binding assay was developed to improve the sample to sample reproducibility, lower the background signal, and allow the process to be semiautomated (Moores et al., 1991). By quenching the reaction with acidic ethanol, which precipitates the radioactive product farnesyl-Ras-CVLS and hydrolyzes the remaining substrate farnesyl diphosphate, and by using ethanol to wash the filter, only two solutions were required, which made automating the procedure simple (using a cell harvester to do the washes). Washing the filter with ethanol helped to lower the observed background radioactivity, probably by better solubilizing the hydrolyzed, unreacted radioactive portion of the substrate ([^{3}H]farnesol). With this assay, we were able to collect statistically relevant initial rate data. Except where noted, all of the results presented below were collected using this acidic ethanol precipitation assay.

Steady-State Kinetics. Pure enzyme, free from competing phosphatase or other prenyltransferase activities, is essential for measuring true initial rates. Like the enzyme from rat brain (Reiss et al., 1990), FPTase isolated from bovine brain (Moores et al., 1991) is an α/β heterodimer, comprised of subunits having molecular masses of approximately 47 (α) and 45 (β) kDa. Figure 1 shows a silver-stained denaturing polyacrylamide gel comparing the rat and bovine brain enzymes. Both enzymes have similar specific activities, 40 and 200 nmol min^{-1} mg^{-1} for the rat (Reiss et al., 1990) and the bovine brain enzymes, respectively, using Harvey-Ras as protein substrate. Bovine brain FPTase of the purity represented in Figure 1 or better was used exclusively in the steady-state kinetic studies.

A double-reciprocal plot of initial velocity against the concentration of farnesyl diphosphate at a series of fixed concentrations of Ras-CVLS is presented in Figure 2. The

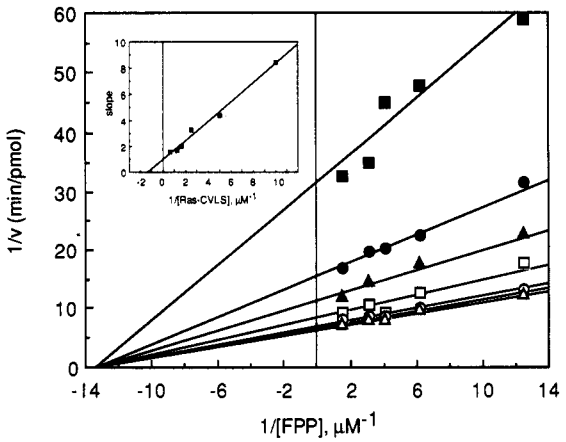


FIGURE 2: Double-reciprocal plot of initial velocity versus farnesyl diphosphate (FPP) concentration at variable fixed Ras-CVLS concentrations. The concentrations of Ras-CVLS were as follows: (■) 0.1, (●) 0.2, (▲) 0.4, (□) 0.6, (○) 0.8, and (△) 1.6 μM. Inset: Secondary plot of the slopes against the reciprocals of Ras-CVLS concentration.

Table I: Comparison of Steady-State Kinetic Parameters for Different Substrates

substrate	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M (M^{-1} s^{-1})	rel k_{cat}/K_M
farnesyl diphosphate	0.04 ± 0.01	0.09	2×10^6	
Ras-CVLS	0.63 ± 0.05	0.093	1.5×10^5	1.0
Ras-CVIM	0.13 ± 0.03	0.023	1.8×10^5	1.2
Ras-CAIM	0.13 ± 0.03	0.028	2.2×10^5	1.5
Ras-CAIL	20 ± 3	0.028	0.014×10^5	0.0096
Ras-CNIQ	0.20 ± 0.02	0.018	1.1×10^5	0.75

intersecting pattern is consistent with a sequential mechanism, in which both substrates must add to the enzyme before either product is released. The inverse reciprocal plot—initial velocity against the concentration of Ras-CVLS at fixed concentrations of farnesyl diphosphate—was also intersecting and, at the crossover point, gave the same value of $1/v$ (not shown). The intersecting pattern was observed again when initial velocity measurements were determined by quantitating the product farnesyl-Ras-CVLS isolated from polyacrylamide gels (not shown; see Experimental Procedures). Kinetic parameters were determined from slope and intercept replots of the primary reciprocal plots (for a sample slope replot, see Figure 2, inset) as well as from a nonlinear least squares fit of the data to the appropriate rate equation. The values of the kinetic parameters are tabulated in Table I. Note that the value of K_M for farnesyl diphosphate (0.04 μM) varied from 0.02 to 0.07 μM, due to difficulties inherent in the measurement of reaction velocities at low farnesyl diphosphate concentrations. Since the point of intersection of the reciprocal plots is on the 1/[s] axis, the value of the Michaelis constant (K_M) equals the value of the dissociation constant (K_i) for each substrate.

We previously reported the value of K_M(Ras-CVLS) to be 3.5 μM (Moores et al., 1991), whereas the value obtained in this work is 0.63 μM. One possibility for this discrepancy is that the unmodified recombinant Ras-CVLS protein substrate, which was expressed in and isolated from *Escherichia coli*, was exposed to adventitious carboxypeptidases that degraded the C-terminus of the Ras-CVLS substrate during the growth and purification procedures. Since truncated versions of Ras-CVLS are not substrates (nor are they particularly good inhibitors), the presence of these nonsubstrate Ras-CVLS proteins would lead to overestimation of the concentration of substrate-quality Ras-CVLS in the preparation. In turn, the values of parameters which depend on the accurate determination of substrate concentration, such as the value of K_M, would likewise be overestimated. We looked for an independent method to determine the value of K_M for the protein substrate. Since only the four C-terminal residues of Ras-CVLS appear to interact with the transferase (Moores et al., 1991; Reiss et al., 1991a), we reasoned that the cognate tetrapeptide CVLS should have a value of K_M very similar to that for the actual protein substrate. Using an HPLC-based assay to measure FPTase activity with tetrapeptide CVLS as the varied substrate in the presence of saturating farnesyl diphosphate, the value of K_M(CVLS) was determined to be 0.77 μM, which agrees closely with the value of K_M for the protein substrate. It thus appears that the current value of 0.63 μM for the value of K_M(Ras-CVLS) is the most reliable figure to date.

With pure FPTase, it was possible to extend the findings on the sequence dependence of prenylation (Moores et al., 1991), since the values of both k_{cat} and K_M for each protein substrate could be determined. The second-order rate constant, k_{cat}/K_M, and not K_M alone, is the true measure of the specificity of a substrate for an enzyme. To evaluate the specificity of certain Ras-CaaX substrates for FPTase and to highlight further the importance of the C-terminal residue in determining prenylation specificity, the values of k_{cat}/K_M were determined for five Ras-CaaX variants and are presented in Table I. Four of the five naturally occurring sequences (CVLS, Ha-Ras; CVIM, Ki-Ras; CAIM, nuclear lamin B; CNIQ, Rap2) were approximately equally specific substrates for FPTase. The Ras-CAIL substrate (γ-6 subunit), which is a known geranylgeranylation substrate (Mumby et al., 1990; Yamane et al., 1990), had a k_{cat}/K_M value 100–200-fold lower than the other protein substrates, indicating that it was a considerably poorer substrate. The value of K_M for farnesyl diphosphate was the same in the presence of all the protein substrates (0.04 μM, data not shown), suggesting that there is no interaction between the farnesyl diphosphate and protein binding sites. The values of k_{cat} for these protein substrates were all roughly equal, making the K_M term, which reflects the relative binding affinities, dominant in the measure of catalytic efficiency. Thus, though we previously only classified the suitability of Ras-CaaX variants to act as FPTase substrates on the basis of their relative K_M values (Moores et al., 1991), that ranking also likely reflects the relative catalytic efficiencies (k_{cat}/K_M values) of the various protein substrates.

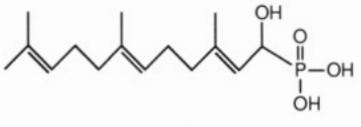
Dead-End Substrate and Product Inhibitor Studies. While the initial velocity studies described above show that both substrates must be on the enzyme at the same time, they cannot be used to determine whether there is an obligatory order of addition or release of reactants, or whether binding in random order occurs. Dead-end substrate and/or product inhibition studies can be used to distinguish between a random order and an obligatory order sequential mechanism (Fromm, 1975). The limitation of the substrate analogue inhibition studies is that a nonsubstrate competitive inhibitor of each substrate (i.e., of farnesyl diphosphate and of Ras-CVLS) must be found. Two nonhydrolyzable analogues of farnesyl diphosphate that cannot participate in the transferase reaction were synthesized (Chart I): (α-hydroxyfarnesyl)phosphonic acid (**1**, hydroxy phosphonic acid), a novel compound, and [[(farnesylmethyl)hydroxyphosphinyl]methyl]phosphonic acid (**2**, farnesyl PMP; McClard et al., 1987; Biller et al., 1988), a known inhibitor of other farnesyl diphosphate handling enzymes, squalene synthetase, and farnesyl diphosphate synthase. Both of these molecules were expected to be competitive inhibitors of farnesyl diphosphate binding. To find nonsubstrate

Table II: Inhibition Patterns and Constants for Substrate Analogue and Product Inhibitors of FPTase[a]

inhibitor	with respect to FPP		with respect to Ras-CVLS	
	type of inhibition	K_i (μM)	type of inhibition	K_i (μM)
hydroxy phosphonate (**1**)	competitive	0.0052 ± 0.0007	noncompetitive	0.0047 ± 0.0005
farnesyl PMP (**2**)	competitive	0.83 ± 0.08	noncompetitive	1.3 ± 0.2
CVLS-NH_2	noncompetitive	32 ± 4	competitive	11 ± 2
Ac-L-penicillamine-VIM	noncompetitive	4.3 ± 0.6	competitive	4.0 ± 1
CV[D-L]S	noncompetitive	8.1 ± 0.5	competitive	13 ± 2
CIFM	noncompetitive	0.034 ± 0.004	competitive	0.0079 ± 0.0007
farnesyl-CVLS	competitive	5.3 ± 1	competitive	8.2 ± 4
PP_i	noncompetitive	1400 ± 300	competitive	1400 ± 400

[a] The K_i value is derived from a nonlinear least squares fit of the initial velocity data to either a purely competitive or purely noncompetitive inhibition model. FPP, farnesyl diphosphate; PP_i, inorganic pyrophosphate; Ac, *N*-acetyl.

Chart I: Structures of Farnesyl Diphosphate Analogues



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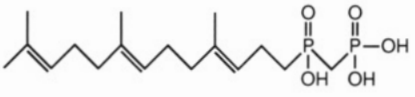

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inhibitors of Ras-CVLS, tetrapeptides were incubated with [^{3}H]farnesyl diphosphate in the presence of FPTase, and the reactions were quenched and then resolved by thin-layer chromatography (Figure 3). In the control reaction (no peptide), only unreacted starting material, farnesyl diphosphate, could be seen, indicating that the purified enzyme preparation does not catalyze the hydrolysis of farnesyl diphosphate. For the positive control reaction, known tetrapeptide substrates (Moores et al., 1991; Reiss et al., 1991a) CVLS and CVIM were used and, as expected, showed radioactive product spots with the predicted R_f values. A negative control peptide having a Cys to Ser substitution was not a substrate. Conversion of the carboxyl terminus to an amide (CVLS-NH_2) abolished the ability of the parent compound CVLS to act as a substrate, but a change of the amino terminus to the *N*-acetyl derivative (Ac-CVLS) had no effect. Inversion of stereochemistry at a single center (CV[D-L]S) or substitution of Phe in the a_2 position rendered the parent compound CVLS inactive as a substrate. Recently, Goldstein et al. (1991) have also identified nonsubstrate inhibitor tetrapeptides of FPTase.

The effects of the two classes of inhibitors on the rates of the enzyme-catalyzed reaction were examined with respect to both their cognate and noncognate substrates under nonsaturating concentrations of the fixed substrate (Table II). Reciprocal plots of initial velocities with respect to the concentration of farnesyl diphosphate in the presence of nonsaturating levels of Ras-CVLS at different fixed concentrations of **1** intersected on the $1/v$ axis, suggesting that **1** behaves as a competitive inhibitor with respect to farnesyl diphosphate. Reciprocal plots of initial velocities with respect to the concentration of Ras-CVLS in the presence of nonsaturating levels of farnesyl diphosphate at different fixed levels of **1** intersected on the 1/[s] axis, indicating that **1** is a noncompetitive inhibitor of Ras-CVLS. Compound **2** showed the same competitive and noncompetitive behavior against farnesyl diphosphate and Ras-CVLS, respectively. The reciprocal plot pattern for the inhibition of the protein substrate mimic CV[D-L]S showed noncompetitive inhibition with respect to the noncognate

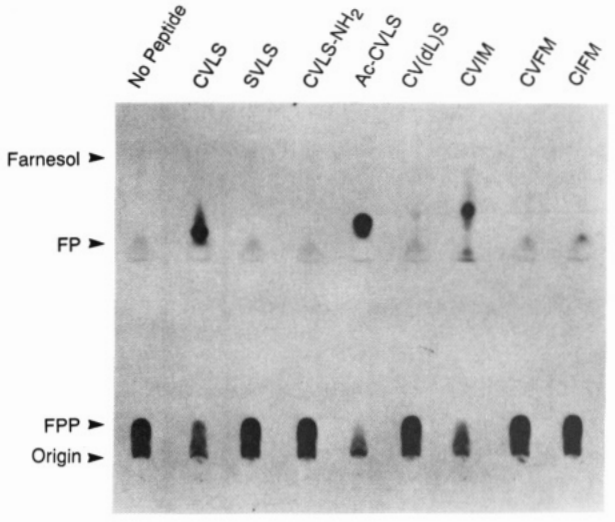


FIGURE 3: Tetrapeptide farnesylation. The indicated peptides (100 μM) were incubated in the presence of pure bovine FPTase (1.2 nM) and farnesyl diphosphate (0.5 μM) for 4 h at 30 °C. The TLC plate was eluted with 1-propanol/water (7:3) and visualized by autoradiography after fluorographic enhancement. Exposure time at −70 °C was 48 h. FPP, farnesyl diphosphate; FP, farnesyl monophosphate.

substrate farnesyl diphosphate and showed competitive inhibition with respect to the cognate substrate Ras-CVLS. In addition, at least three other nonsubstrate competitive inhibitors of Ras-CVLS, CVLS-NH_2, *N*-acetyl-L-penicillamine-VIM, and CIFM, showed the same noncompetitive behavior with respect to farnesyl diphosphate. The collection of patterns shown for the dead-end substrate competitive inhibitors is consistent only with the random order sequential mechanism. The inhibitor constants corresponding to the type of inhibition observed for each inhibitor against each substrate are given in Table II.

To confirm the random order of substrate addition, the inhibitory effects of product on the reaction catalyzed by FPTase were next examined. Products should always show inhibition, since they are certainly able to bind to the enzyme. Unfortunately, one of the natural products of the reaction, farnesyl-Ras-CVLS, cannot be readily prepared by synthetic or enzymatic methods. Therefore, we synthesized a truncated (by 182 residues) form of the actual product, the modified peptide farnesyl-CVLS. Since the enzyme recognizes only the four C-terminal amino acid residues of Ras-CVLS, we reasoned that farnesyl-CVLS would act as a product and would bind to the same site as farnesyl-Ras-CVLS. Moreover, because farnesyl-CVLS is made by synthetic chemical methods, its purity and quantity are easily ascertained. When farnesyl-CVLS was examined as an inhibitor of FPTase with respect to each substrate, it was found to be a competitive inhibitor with the same K_i values with respect to each substrate (within

error, Table II). This inhibition pattern is expected for the random order mechanism when no abortive ternary complexes are formed (Fromm, 1975). This pattern is also consistent with farnesyl-CVLS acting as a bisubstrate analogue, blocking access to both the isoprenoid and protein binding sites. At high farnesyl-CVLS concentrations, we observed an inhibition pattern against farnesyl diphosphate that more closely resembled noncompetitive behavior. When the other product of the reaction, inorganic pyrophosphate, was tested for inhibition of FPTase, it was found to be noncompetitive with respect to farnesyl diphosphate and competitive with respect to Ras-CVLS. At concentrations of inorganic pyrophosphate below 1 mM, however, FPTase activity was actually slightly stimulated (up to roughly 2-fold). Squalene synthetase, another farnesyl diphosphate handling enzyme, showed the same (inexplicable) stimulation–inhibition pattern in the presence of increasing concentrations of product inorganic pyrophosphate (Poulter et al., 1989). The patterns and associated constants for product inhibition are summarized in Table II. This series of product inhibition patterns, on its own, is consistent with a random order sequential mechanism and corroborates the results found with the dead-end substrate inhibitors.

Discussion

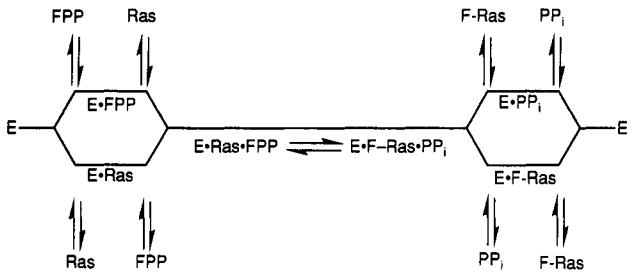
FPTase catalyzes the transfer of a farnesyl group from farnesyl diphosphate to the thiol of a specific C-terminal Cys residue of a protein substrate, forming a thioether bond and displacing inorganic pyrophosphate in the process (Scheme I). This posttranslational modification is critical for the full expression of biological activity for many cellular polypeptides. Apart from the great interest of the enzyme itself, attention has focused on FPTase recently because one of its natural substrates, the protein Ras, is a key regulator of cellular proliferation and a potential factor in the development of human cancer. Since Ras must be farnesylated before it can perform its normal cellular tasks, inhibition of the enzyme-mediated farnesyl transfer reaction might be both a useful probe of Ras function in vivo as well as an attractive approach to anticancer therapy. The search for a potent, selective inhibitor of FPTase led us first to consider the mechanism through which the enzyme-catalyzed reaction proceeds.

Testing mechanistic possibilities using steady-state kinetics requires a reliable measure of initial rates of reaction. Several different assays of FPTase activity were evaluated for their accuracy and reproducibility. Since none of the substrates or products of the reaction catalyzed by FPTase possess a unique chromophore or fluorophore, the continuous progress of the reaction cannot be easily or directly monitored spectrophotometrically. While we considered using a coupled assay that linked the formation of inorganic pyrophosphate to the oxidation of NADH to NAD^+ through fructose-6-phosphate kinase, aldolase, triosephosphate isomerase, and α-glycerophosphate dehydrogenase (O'Brien, 1976), the assay simply was not sensitive enough to detect turnover at low (V/K) substrate concentrations. The immunoprecipitation assay we used in our initial report of FPTase activity was very sensitive (Schaber et al., 1990), but the response became nonlinear as the Ras-CVLS concentration was increased because the anti-Ras antibody was unable to immunoprecipitate quantitatively the product farnesyl-Ras-CVLS. Another possible method was to separate (by gel electrophoresis, spin-column gel filtration, or HPLC) and directly quantitate substrate and/or product. While this approach was generally accurate and reproducible, it proved to be too labor-intensive to generate the large number of data points needed for initial rate measurements. The filter binding assay reported by others (Reiss et al., 1990) proved too cumbersome (four different quench and wash solutions) and tended to give high background signals in our hands. We developed a new filter binding assay amenable to automation. The assay we devised requires only two solutions: acidic ethanol to quench the reactions and ethanol to wash the filters. With a cell harvester and an automatic scintillation fluid dispenser, up to 300–400 assays per person per day can be run, processing 24 filters at a time.

Once the validity of the assay was established, we proceeded with the steady-state mechanistic studies of pure FPTase. For a two-substrate/two-product reaction like the one catalyzed by FPTase, there are only two possible kinetic mechanisms: sequential, in which a ternary complex is formed, or ping-pong, which requires two distinct steps and usually involves a modified enzyme intermediate. Enzymes that handle farnesyl diphosphate can use either mechanism. Initial velocity studies of the squalene synthetase reaction point to a ping-pong mechanism involving a farnesyl-enzyme intermediate (Beytia et al., 1973). On the other hand, farnesyl diphosphate synthetase proceeds through an ordered sequential mechanism (Laskovics et al., 1979). When FPTase was considered, initial velocity measurements as a function of either farnesyl diphosphate or Ras concentration in the presence of a series of different fixed concentrations of the nonvaried substrate resulted in reciprocal plots that showed an intersecting pattern representative of a sequential mechanism (Figure 2). From these initial velocity data, the various steady-state kinetic parameters were determined (Table I). The intersection point in the reciprocal plot was on the $1/[s]$ axis, suggesting that one substrate had no effect on the binding of the other. Hence the values of the K_M and the K_i (a pure binding constant) were the same for each substrate. The specificity constant, k_{cat}/K_M, was determined for several naturally occurring Ras-CaaX proteins (Table I). The values of k_{cat} were roughly the same for all of the protein variants tested, indicating that, for FPTase, specificity is determined predominantly by the substrate's binding affinity, and not by any discrimination in the catalytic step(s). Compared to a diffusion-limited, evolutionarily perfected enzyme like triosephosphate isomerase ($k_{cat}/K_M = 4 \times 10^8\ M^{-1}\ s^{-1}$; Putman et al., 1972), FPTase is a very slow enzyme ($k_{cat}/K_M = 1.3 \times 10^5\ M^{-1}\ s^{-1}$), although it is similar to another transferase which posttranslationally modifies a protein substrate, myristoyl CoA:protein transferase ($k_{cat}/K_M = 1.3 \times 10^5\ M^{-1}\ s^{-1}$; Rudnick et al., 1990). We have considered the possibility that allosteric activators of FPTase activity might affect its activity in vivo.

To determine whether the ternary complex is formed by successive ordered addition or by random combination of the two substrates, dead-end competitive substrate and product inhibition studies were carried out. Dead-end inhibitors of substrates show distinctive patterns of inhibition with respect to the two substrates depending on whether the binding of substrates is random or ordered. After identifying nonsubstrate competitive inhibitors of each substrate (Figure 3, Table II), the patterns of inhibition against the noncognate substrate were determined. The farnesyl diphosphate analogue (**1**) and the protein-CaaX mimic (CV[dL]S) were noncompetitive inhibitors of Ras-CVLS and farnesyl diphosphate, respectively (Table II). The competitive–noncompetitive pattern shown for each inhibitor against the cognate and noncognate substrate is representative of a random order sequential mechanism. These studies were confirmed with at least one other farnesyl diphosphate mimic and three other protein mimics (Table II). If the addition of substrate to the enzyme had been ordered,

Scheme II: Proposed Kinetic Mechanism of Farnesyltransferase



one of the inhibition patterns would have been *un*competitive instead of *non*competitive (Fromm, 1975).

The patterns of product inhibition in a two-substrate reaction can also be diagnostic for the order of binding in a sequential mechanism. A random order mechanism will show competitive inhibition with both products against both substrates, assuming no abortive ternary complexes are formed. If abortive ternary complexes do result (which is common for two substrate systems), then the expected pattern is competitive–noncompetitive with respect to the two substrates for each inhibitor. An ordered mechanism has a pattern of inhibition in which one product is a noncompetitive inhibitor of *both* substrates (the other product is a competitive and noncompetitive inhibitor of the two substrates). The patterns observed for product inhibition of FPTase are consistent with the random order mechanism (Table II). Note that the farnesyl-CVLS product showed competitive inhibition with respect to both farnesyl diphosphate and Ras-CVLS. This is expected for a product that spans the binding sites of the two substrates, since it can deny active site access (by steric hindrance) to either substrate. Binding studies have shown that farnesyl diphosphate binds reversibly to free enzyme in the absence of Ras protein substrate (Reiss et al., 1991b). Moreover, Ras can be cross-linked to the enzyme without farnesyl diphosphate present (Reiss et al., 1991b), suggesting that Ras can bind to the enzyme without a farnesyl diphosphate-induced conformational change. Both of these observations are consistent with a random order sequential mechanism described in the current study.

Given that FPTase proceeds through a ternary complex (Scheme II), the simplest chemical mechanism to propose is an S_N2 displacement of inorganic pyrophosphate from farnesyl diphosphate by the thiol of the protein substrate's conserved Cys residue. Alternatively, like several of the enzymes of the isoprenoid biosynthetic pathway (Poulter, 1990), the reaction might follow a more S_N1-like course, with formation of a farnesyl cation–pyrophosphate ion pair intermediate, followed by alkylation of the Cys thiol by the farnesyl cation. Inhibition studies with trifluorinated farnesyl derivatives or ammonium analogues of the farnesyl cation pioneered by Poulter with squalene synthetase (Poulter & Satterwhite, 1977; Poulter et al., 1978, 1981) and farnesyl diphosphate synthetase (Sandifer et al., 1982; Poulter et al., 1989) might distinguish between these two mechanistic possibilities. FPTase requires two divalent metals, Mg^{2+} and Zn^{2+} (Reiss et al., 1990). The Mg^{2+} probably activates the diphosphate leaving group of farnesyl diphosphate. The Zn^{2+} could be acting catalytically, also by activating the diphosphate leaving group, or structurally, perhaps by stabilizing the heterodimeric form of the enzyme.

The most important finding of this work is that the reaction catalyzed by bovine brain FPTase follows a random order sequential reaction pathway (Scheme II). This conclusion is based on steady-state initial velocity, dead-end substrate, and product inhibitor studies. Knowledge of the mechanism should help guide the search for rationally designed inhibitors of FPTase.

ACKNOWLEDGMENTS

We are grateful to J. A. Shafer for helpful enzyme kinetics discussions, to N. E. Kohl and to C. A. Omer for their continuing intellectual and experimental contributions, and finally to A. I. Oliff for his support.

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Stopped-Flow Kinetic Analysis of the Bacterial Luciferase Reaction†

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Received July 24, 1991; Revised Manuscript Received February 4, 1992

ABSTRACT: The kinetics of the reaction catalyzed by bacterial luciferase have been measured by stopped-flow spectrophotometry at pH 7 and 25 °C. Luciferase catalyzes the formation of visible light, FMN, and a carboxylic acid from $FMNH_2$, O_2, and the corresponding aldehyde. The time courses for the formation and decay of the various intermediates have been followed by monitoring the absorbance changes at 380 and 445 nm along with the emission of visible light using *n*-decanal as the alkyl aldehyde. The synthesis of the 4a-hydroperoxyflavin intermediate (FMNOOH) was monitored at 380 nm after various concentrations of luciferase, O_2, and $FMNH_2$ were mixed. The second-order rate constant for the formation of FMNOOH from the luciferase–$FMNH_2$ complex was found to be 2.4×10^6 M^{-1} s^{-1}. In the absence of *n*-decanal, this complex decays to FMN and H_2O_2 with a rate constant of 0.10 s^{-1}. The enzyme–$FMNH_2$ complex was found to isomerize prior to reaction with oxygen. The production of visible light reaches a maximum intensity within 1 s and then decays exponentially over the next 10 s. The formation of FMN from the intermediate pseudobase (FMNOH) was monitored at 445 nm. This step of the reaction mechanism was inhibited by high levels of *n*-decanal which indicated that a dead-end luciferase–FMNOH–decanal could form. The time courses for the these optical changes have been incorporated into a comprehensive kinetic model. Estimates for 15 individual rate constants have been obtained for this model by numeric simulations of the various time courses.

Bacterial luciferase is a flavin hydroxylase which catalyzes the reaction of $FMNH_2$, O_2, and an aliphatic aldehyde to yield the carboxylic acid, FMN, and blue-green light (λ_{max} = 490 nm). The enzyme is a heterodimer consisting of homologous subunits, α and β, with molecular weights of 40 108 (Cohn et al., 1985) and 36 349 (Johnston et al., 1986), respectively. The active center resides primarily, if not exclusively, on the α subunit; the role of the β subunit remains unclear, but it is required for the efficient generation of light (Baldwin & Ziegler, 1991). The enzyme is commonly assayed by monitoring light emission following injection of $FMNH_2$ into a vial containing enzyme, an aliphatic aldehyde (e.g., *n*-decanal), and O_2 dissolved in a buffered solution (Hastings et al., 1978). The light intensity rises to a maximum, which is proportional to the amount of enzyme, and then decays exponentially with a rate constant characteristic of the enzyme and the alkyl chain length of the aldehyde (Hastings et al., 1966). This format comprises a single-turnover assay since free (excess) $FMNH_2$ is quickly depleted by the nonenzymatic autoxidation pathway

† This work was supported by the National Institutes of Health (GM 33894) and the National Science Foundation (DMB 87-16262).
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