Isoprenoid Diphosphate Utilization by Recombinant Human Farnesyl:Protein Transferase: Interactive Binding between Substrates and a Preferred Kinetic Pathway

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ABSTRACT: The catalytic utilization of dimethylallyl, geranyl, farnesyl, and geranylgeranyl diphosphates in the reaction catalyzed by recombinant human farnesyl:protein transferase (hFPTase) has been examined in the presence of three different protein substrates, Ras-CVLS, Ras-CVIM, and Ras-CAIL. hFPTase catalyzed both farnesylation and geranylation of Ras-CVLS and of Ras-CVIM but not of Ras-CAIL. Geranylgeranylation was observed, but only when Ras-CVIM was the acceptor substrate. Steady-state initial velocity and dead-end inhibitor studies indicate that hFPTase-catalyzed geranylation, like bovine FPTase-catalyzed farnesylation, proceeds through a random order, sequential mechanism. Surprisingly, however, Michaelis constants for a given protein acceptor substrate varied depending upon which isoprenoid diphosphate was used as the donor substrate, showing that these substrates do not bind independently to the enzyme (under catalytic conditions). In addition, at very high concentrations of Ras-CVIM, substrate inhibition was observed in the presence of both FPP and GPP. Isotope partitioning studies showed that, at high concentrations of Ras-CVIM, more than 80% of the bound farnesyl diphosphate (FPP) can be trapped as product, suggesting that the binary complex is catalytically competent and that the ternary complex proceeds to product faster than it releases FPP. The release rate of FPP from the binary complex was calculated to be 0.05 s^{-1}, which is only about eight times greater than k_{cat}. Thus, the binding of FPP to the enzyme in the presence of the protein substrate is not an equilibrium situation. The isotope partitioning studies together with the steady-state kinetic data lead us to conclude that the mechanism is random in principle (since both substrates appear to bind independently to free enzyme) but ordered in practice, since the preferred catalytic pathway is through the FPTase·FPP binary complex. Computer simulations of a steady-state, random order sequential mechanism using experimentally determined kinetic constants as constraints provide a model which describes the observed behavior.

Posttranslational prenylation is required for the proper biological function of a diverse group of cellular proteins (Glomset et al., 1990; Maltese, 1990; Rine & Kim, 1990; Der & Cox, 1991; Sinensky & Lutz, 1992). Thus far, only farnesylated (C15) and geranylgeranylated (C20) proteins have been observed *in vivo*. The addition of the prenyl groups to proteins containing the C-terminal consensus sequence C*aa*X[1] (C, cysteine; *a*, a usually aliphatic amino acid, X, another amino acid) is mediated by at least two different enzymes. Farnesyl:protein transferase (FPTase) catalyzes the transfer of the farnesyl moiety of farnesyl diphosphate (FPP) to the thiol of the conserved cysteine residue of these protein substrates, forming a thioether-linked adduct and releasing inorganic diphosphate (Scheme I). Proteins with a C-terminal methionine, serine, cysteine, alanine, or glutamine residue are good substrates for FPTase (Reiss et al., 1990; Schaber et al., 1990; Moores et al., 1991). However, when the C-terminal residue of the protein substrate is leucine, the protein is a much better substrate for geranylgeranyl:protein transferase (GGPTase-I) (Casey et al., 1991; Kinsella et al., 1991; Moores et al., 1991; Seabra et al., 1991; Yokoyama et al., 1991), which catalyzes the analogous reaction using geranylgeranyl diphosphate as the donor substrate. Proteins terminating with a different motif, XXCC, are modified by a third enzyme, another geranylgeranyl:protein transferase (GGPTase-II) (Kinsella & Maltese, 1991; Moores et al., 1991). Enzyme-catalyzed geranylgeranylation of substrates with a C-terminal sequence XCXC has also been described (Horiguchi et al., 1991; Seabra et al., 1992).

Interest has focused on FPTase because farnesylation is required for membrane association and biological function of *ras*-encoded proteins (Willumsen et al., 1984; Hancock et al., 1989; Jackson et al., 1990), mutant forms of which play a biological role in over 20% of all human cancers and in greater than 50% of pancreatic and colon tumors (Bos, 1990). Inhibition of FPTase represents a possible method for preventing relocation of mutant Ras from the cytosol to the membrane, thereby blocking its cell transforming function (Gibbs, 1991). The design of potent FPTase inhibitors, however, requires knowledge of the mechanistic details of the enzyme-catalyzed reaction.

The reaction catalyzed by bovine FPTase proceeds through a random order, sequential mechanism, as shown by steady-

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[1] Abbreviations: DMAP, dimethylallyl diphosphate; GPP, geranyl diphosphate; FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate; C*aa*X, Cys (C), aliphatic amino acid (*a*), any amino acid (X); hFPTase, recombinant human farnesyl:protein transferase; GGPTase, geranylgeranyl:protein transferase; Ras-C*aa*X, [Leu68]RAS1(term. 185)-C*aa*X.

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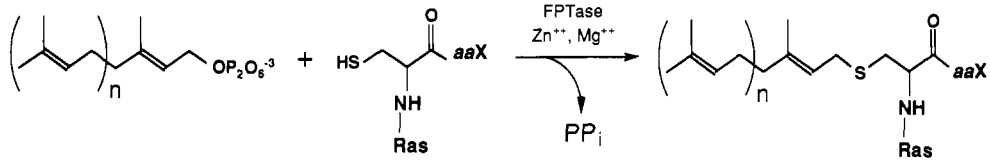
Scheme I: Reactions Catalyzed by FPTase



state initial velocity experiments and dead-end substrate and product inhibitor studies (Pompliano et al., 1992). Both Mg^{2+} and Zn^{2+} are required for catalysis: Mg^{2+} apparently is necessary to activate the diphosphate leaving group, and Zn^{2+} is involved in the interaction of enzyme with protein substrate (Reiss et al., 1990, 1992). The protein substrate specificity has been closely examined and well characterized by measuring the ability of short peptides with varying sequences to compete in the enzyme-catalyzed reaction using FPP and Ras-CVLS or Ras-CVIM as substrates (Reiss et al., 1990, 1991; Schaber et al., 1990; Goldstein et al., 1991; Moores et al., 1991; Pompliano et al., 1992) as well as by making Ras-C*aa*X mutants and assaying for their ability to act as FPTase substrates in the presence of FPP (Reiss et al., 1990; Schaber et al., 1990; Moores et al., 1991). However, interactions of the isoprenoid diphosphate substrate with FPTase have not been thoroughly considered. We previously found that diphosphate intermediates of the mevalonate pathway other than FPP (dimethylallyl, geranyl, and geranylgeranyl) were competitors of Ras farnesylation (Schaber et al., 1990). Surprisingly, although geranylgeranyl diphosphate (GGPP) was not a FPTase substrate in the presence of Ras-CVLS, GGPP was able to compete on a par with FPP in the reaction when [^{3}H]FPP and Ras-CVLS were substrates (Schaber et al., 1990; Goldstein et al., 1991; Moores et al., 1991; Yokoyama et al., 1991; Reiss et al., 1992). To explore further the interactions between FPTase and isoprenoid diphosphate, we have examined the steady-state catalytic utilization of four isoprenoid diphosphates in the presence of three different Ras-C*aa*X substrates.

EXPERIMENTAL PROCEDURES

Materials and Methods. Human recombinant farnesyl: protein transferase (hFPTase) was expressed in *Escherichia coli* and purified to homogeneity using antibody affinity and ion exchange chromatography (Omer et al., 1993). Ras-C*aa*X proteins were expressed in *E. coli* and purified as previously described (Moores et al., 1991). [^{3}H]Farnesyl diphosphate (20 Ci/mmol) was purchased from DuPont-NEN (Boston, MA). [^{3}H]Dimethylallyl (15 Ci/mmol), [^{3}H]geranyl (15 Ci/mmol), and [^{3}H]geranylgeranyl (15 Ci/mmol) diphosphates were obtained from American Radiolabeled Chemicals (St. Louis, MO). All other chemicals and reagents were from Sigma (St. Louis, MO), Aldrich (Milwaukee, WI), Pierce (Rockford, IL), or Fisher (Springfield, NJ) and were used without further purification. General biochemical procedures, FPTase steady-state kinetic assays, and data analyses were performed as described previously (Pompliano et al., 1992). Steady-state kinetic assay mixtures contained 0.5–1 nM of hFPTase. Nonsaturating substrate assay conditions (for IC_{50} determinations) were 30 nM FPP and 350 nM Ras-CVLS or 100 nM Ras-CVIM. The equilibrium constant (K_D) for FPP binding to FPTase was determined using the spin column assay previously described (Omer et al., 1993). In short, aliquots (100 μL) containing hFPTase (30 nM) and varying concentrations of [^{3}H]FPP (5–1000 nM) were fractionated using G-50 Fine Sephadex spin chromatography. Bound [^{3}H]-FPP was quantitated by scintillation counting. Binding constants (K_D) were calculated from a nonlinear least-squares fit of the data to the following equation: [bound FPP] = [Et][free FPP]/(K_D + [free FPP]). The concentration of hFPTase was determined using the Bradford assay (Bradford, 1976).

Isotope trapping experiments were carried out essentially as described (Rose et al., 1974; Rose, 1980; Cleland, 1990) using hFPTase and radiolabeled isoprenoid diphosphates. In a typical experiment, pulse solution containing hFPTase (400 nM) and [^{3}H]FPP (400 nM) or [^{3}H]GPP (8000 nM) was preincubated at 30 °C in assay buffer [50 mM HEPES, pH 7.5, 5 mM $MgCl_2$, 10 μM $ZnCl_2$, 5 mM DTT, and 0.2% (w/v) PEG 20000] for 10 min. Reaction solutions (1 mL) containing unlabeled FPP (50 μM) and variable concentrations of Ras-C*aa*X in assay buffer were separately preincubated for 10 min at 30 °C. Pulse solution (20 μL, containing 8 pmol of hFPTase) was added to rapidly stirring reaction mixtures (1 mL), followed 3 s later by addition of concentrated HCl (100 μL) to quench the reaction. The extent of reaction was quantitated by measuring the amount of precipitated product farnesylated Ras-C*aa*X (P^*) (Pompliano et al., 1992).

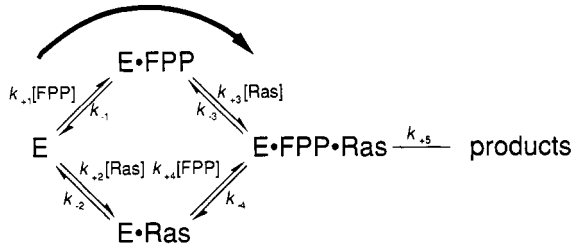
To trap product when the hFPTase was preincubated with Ras-C*aa*X, the reaction solutions contained cold cognate peptide (50 μM) and various concentrations of *labeled* isoprenoid diphosphate. Product was quantified as described above. Note that farnesylated peptide product is not retained by the glass fiber filters used to quantitate product and therefore is not measured as farnesylated *protein* product. The trapping parameters $K_{1/2}$ and appP^*_{max} (Rose et al., 1974; Rose, 1980; Cleland, 1990) were calculated from a nonlinear least-squares fit of the [s]–P^* data to the Michaelis–Menten equation.

The kinetic behavior of FPTase was simulated using the equation describing the *steady-state* random order, sequential pathway for a two-substrate system in which initial velocity (i.e., [product] = 0) as a function of one substrate concentration ([a]) in the presence of fixed cosubstrate concentration ([b] = constant) is given by (Dixon & Webb, 1979)

$$v_f = \frac{(i[a]^2 + j[a])[e]}{k + l[a]^2 + m[a]} \quad (1)$$

where a = substrate 1 (isoprenoid diphosphate); b = substrate 2 (Ras-C*aa*X); e = enzyme; $i = k_{+1}k_{+3}k_{+4}k_{+5}[b]$; $j = (k_{+1}k_{-2}k_{+3}k_{+5} + k_{-1}k_{+2}k_{+4}k_{+5})[b] + k_{+2}k_{+3}k_{+4}k_{+5}[b]^2$; $k = (k_{-1}k_{-2}k_{-3} + k_{-1}k_{-2}k_{-4} + k_{-1}k_{-2}k_{+5}) + (k_{-1}k_{+2}k_{-3} + k_{-1}k_{+2}k_{-4} + k_{-1}k_{+2}k_{+5} + k_{-2}k_{+3}k_{-4} + k_{-2}k_{+3}k_{+5})[b] + (k_{+2}k_{+3}k_{-4} + k_{+2}k_{+3}k_{+5})[b]^2$; $l = k_{+1}k_{-3}k_{+4} + k_{+1}k_{+4}k_{+5} + k_{+1}k_{+3}k_{+4}[b]$; $m = k_{+1}k_{-2}k_{-3} + k_{+1}k_{-2}k_{-4} + k_{+1}k_{-2}k_{+5} + k_{-1}k_{-3}k_{+4} + k_{-1}k_{+4}k_{+5} + (k_{+1}k_{-2}k_{+3} + k_{-1}k_{+2}k_{+4} + k_{+1}k_{+3}k_{-4} + k_{+2}k_{-3}k_{+4} + k_{+3}k_{+4}k_{+5})[b] + k_{+2}k_{+3}k_{+4}[b]^2$.

Scheme II: Kinetic Pathway for the Reaction Catalyzed by FPTase



The step with which each rate constant is associated is given in Scheme II. Experimentally determined rate constants (k_{cat}, K_M, K_D, k_{-1}, k_{-4}) were fixed, and parameter space was searched to find the best fit of the [s]–v data (e.g., Figure 1). Calculations were performed using a spreadsheet program (Excel 4.0) running on a Macintosh IIci computer.

RESULTS

To examine the catalytic competence of geranyl diphosphate (GPP), FPP, and GGPP, each of them was screened for its ability to act as substrate for FPTase in the presence of three different Ras-C*aa*X substrates: the known farnesylation substrates Ras-CVLS (Harvey Ras C-terminal sequence) and Ras-CVIM (Kirsten Ras C-terminal sequence) as well as the preferred geranylgeranylation substrate Ras-CAIL (γ-6 C-terminal sequence).[2] FPTase assays were initially performed at arbitrary substrate concentrations of 100 and 3000 nM for isoprenoid diphosphate and Ras-C*aa*X, respectively. Recombinant human FPTase (hFPTase), which has been expressed in *E. coli* and can be purified to homogeneity by affinity chromatography (Omer et al., 1993), was used in all of the experiments described in this paper. The results (not shown) revealed several features. First, hFPTase can catalyze prenylation of Ras-C*aa*X substrates using isprenoid diphosphates other than FPP. Second, the efficiency of an isoprenoid diphosphate to serve as a donor substrate varies with Ras-C*aa*X substrate, as measured by the initial velocities. For example, FPP was the best substrate under these assay conditions with Ras-CVLS and Ras-CAIL, whereas GPP was the preferred substrate with Ras-CVIM. Although, in the presence of Ras-CVIM, GGPP was a kinetically competent substrate, GGPP appeared to be nonreactive with Ras-CVLS and Ras-CAIL, as reported previously for the rat and bovine enzymes (Goldstein et al., 1991; Moores et al., 1991; Yokoyama et al., 1991; Reiss et al., 1992). DMAP was not a substrate in the presence of any of the three Ras-C*aa*X substrates under these assay conditions with hFPTase. Similar results were obtained using homogeneous FPTase purified from bovine brain (not shown).

To compare quantitatively the relationships among the isoprenoid diphosphates and Ras-C*aa*X substrates, values of $^{app}K_M$ for each of the Ras-C*aa*X substrates were determined at a saturating concentration of isoprenoid diphosphate (Table I). For a random order sequential mechanism operating at equilibrium, the value of $^{app}K_M$(Ras-C*aa*X) determined at saturating concentrations of isoprenoid diphosphate is equal to the equilibrium constant for dissociation of the protein from the ternary FPTase·isoprenoid diphosphate·protein complex. Notice that the value of $^{app}K_M$(Ras-CVIM) varies from 60 to 480 to 1100 nM in the presence of FPP, GPP, and GGPP, respectively. This observation indicates that binding of at least two of the isoprenoid diphosphate substrates must alter the affinity of the enzyme for Ras-CVIM. Figure 1 shows double-reciprocal plots of the [s]–v data for the reaction catalyzed by FPTase using GPP and Ras-CVIM as substrates which *can* be fit such that the lines intersect within experimental error on the 1/[s] axis, if one ignores the data at very high Ras-CVIM concentrations (this fit is *not* shown). A common intersection on the 1/[s] axis for a sequential two-substrate reaction indicates that binding of one substrate to the enzyme does not affect the affinity of the enzyme for the other substrate. Furthermore, steady-state inhibition patterns with dead-end substrate inhibitors were consistent with random order: an isoprenoid diphosphate mimic was competitive against GPP [(α-hydroxyfarnesyl)phosphonic acid (Pompliano et al., 1992)] and noncompetitive against Ras-CVIM, and a Ras mimic (peptide CIFM) was just the reverse (Table II). Other data together with a study of the FPTase-catalyzed reaction of FPP and Ras-CVIM (see below), however, are most consistent with a fit of the [s]–v data at low Ras-CVIM

Table I: Steady-State Kinetic Constants of Ras-C*aa*X and Isoprenoid Diphosphate Substrates in the Reaction Catalyzed by hFPTase[a]

Ras-C*aa*X	isoprenoid diphosphate	apparent K_M(Ras-C*aa*X) (nM)	apparent k_{cat} (s^{-1})
CVLS	GPP	6200 ± 380	0.100 ± 0.003
	FPP	390 ± 20[b]	0.010 ● 0.005[b]
	GGPP	ND[c]	ND[c]
CVIM	GPP	480 ± 60	0.093 ± 0.009
	FPP	60 ± 10	0.006 ± 0.0003
	GGPP	1100 ± 80	0.015 ± 0.002
CAIL	GPP	67000 ± 20000	0.005 ± 0.0005
	FPP	4900 ± 500	0.012 ± 0.001
	GGPP	ND[c]	0.008 ± 0.003

[a] Assays were conducted at 30 °C. [b] Values taken from Omer et al. (1993). [c] ND, not determined.

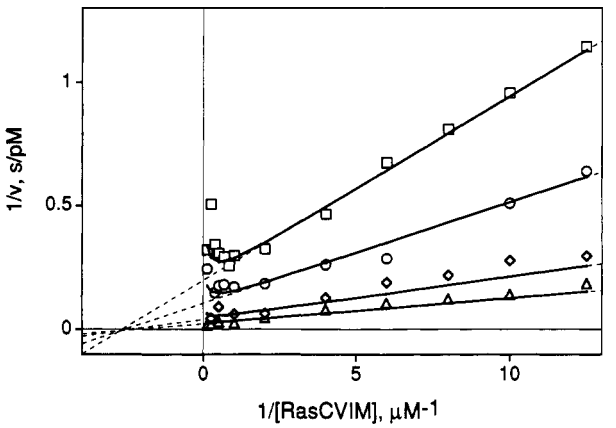


FIGURE 1: Double-reciprocal plot of initial velocity versus Ras-CVIM concentration in the presence of different fixed geranyl diphosphate (GPP) concentrations. The fixed concentrations of GPP were 0.05, 0.10, 0.33, and 1.0 μM. The dotted and solid lines represent data generated from a computer simulation of the steady-state rate equation using the rate constants in Table III. The dotted lines represent fits of the simulated data at lower Ras-CVIM concentrations (before substrate inhibition sets in).

[2] Our interest in the catalytic utilization of different isoprenoid diphosphates by FPTase was piqued by our earlier observation that, using FPP and Ras-CVLS as substrates (at saturating concentrations), DMAP (IC_{50} = 325 μM), GPP (IC_{50} = 67 μM), and GGPP (IC_{50} = 1.4 μM) were all competitors of the reaction catalyzed by bovine FPTase (Schaber et al., 1990), suggesting that these isoprenoid diphosphates could interact with the bovine enzyme. FPP and GGPP each are reported to form a binary complex with rat brain FPTase (Reiss et al., 1991, 1992). When the isoprenoid diphosphate competition experiment was repeated with the recombinant human enzyme in the presence of nonsaturating concentrations of FPP and Ras-CVLS, DMAP (IC_{50} = 22 μM), GPP (IC_{50} = 9 μM), and GGPP (IC_{50} = 0.12 μM) were again found to be inhibitors of the farnesylation reaction.

Table II: Inhibition Patterns and Constants for Substrate Analog Inhibitors of FPTase

	with respect to GPP		with respect to Ras-CVIM	
inhibitor	type of inhibition	K_i (nM)	type of inhibition	K_i (nM)
(α-hydroxyfarnesyl) phosphonic acid	competitive	<1	noncompetitive	<1
CIFM	noncompetitive	11 ± 1	competitive	10 ± 2

concentrations to the dotted lines in Figure 1. These lines intersect at a point below the 1/[s] axis consistent with a value of K_D(Ras-CVIM) = 435 nM. Comparison of this estimate of the value of K_D(Ras-CVIM) with the values for the K_M-(Ras-CVIM) (480 nM; Table I) suggests that GPP (and GGPP) decreases, whereas FPP increases, the affinity of the enzyme for Ras-CVIM.

For a random order mechanism, where the concentrations of the enzyme·substrate complexes of Scheme II are close to their equilibrium values, the value of K_M(GPP) determined at saturating protein substrate concentrations should be equal to the dissociation constant of GPP from the ternary E·GGPP·Ras complex. Since isoprenoid diphosphate binding to FPTase alters the interactions between enzyme and protein substrate, binding of protein substrate to FPTase should reciprocally affect the association of enzyme and isoprenoid diphosphate substrate. The effect of protein substrate structure of $^{app}K_M$(GPP) was not characterized, however, since the use of saturating protein substrate concentrations was precluded by the onset of substrate inhibition (Figure 1; note upward curvature of the double-reciprocal plots at high Ras-CVIM concentrations), which would have confounded the analysis. Note, however, that the substrate inhibition decreases as the GPP concentration increases. Substrate inhibition at high Ras-CVIM concentrations (above 1 μM) was also observed when FPP was cosubstrate. Using a spin column assay, it was determined that K_D(FPP) = 12 ± 2 nM, which is very close to the value of K_M(FPP) = 9 ± 2 nM (Omer et al., 1993).

To investigate further the interactions of the isoprenoid diphosphate with hFPTase, we turned to the isotope partitioning technique of Rose and his colleagues (Rose et al., 1974; Rose, 1980; Cleland, 1990). In this method, the partitioning of a binary E·FPP or E·Ras complex between product formation and substrate release is measured during a single enzyme turnover. From the resulting data, the rate of dissociation of a substrate from the binary complex compared with the catalytic rate (k_{cat}) can be calculated. [^{3}H]-FPP and hFPTase were allowed to form a binary complex by incubating saturating concentrations of [^{3}H]FPP with equivalent amounts of the enzyme. The trapping experiment was initiated by diluting (by at least 20-fold) an aliquot of this complex containing 8.0 ± 0.2 pmol of hFPTase (E_t) into rapidly stirring reaction mixtures containing a 100-fold molar excess of unlabeled FPP and varying amounts of Ras-CVIM. After 3 s, the reaction was stopped by the addition of acid, and the farnesylated Ras-CVIM product (P^*) was quantitated. From a nonlinear least-squares fit of the [s]-P^* data, the trapping coefficient, $K_{1/2}$, was calculated to be 0.48 ± 0.03 μM, and the maximum amount of FPP trapped, appP^*, was determined to be 7.0 ± 0.5 pmol. Similar trapping efficiencies were seen when Ras-CVLS was used as the trapping substrate. Approximately 80% of the FPP was trapped as product (appP^* = 0.87E_t) in the reaction, indicating that FPP partitions forward to product about 2.5 times faster than it dissociates from the ternary complex ($k_{+5} > k_{-4}$ of Scheme II). Thus

Table III: Calculated Rate Constants for the Reaction Catalyzed by FPTase

	substrates	
parameter	GPP + Ras-CVIM	FPP + Ras-CVIM
k_{+1} (M^{-1} s^{-1})	2.2×10^6	4.0×10^6
k_{-1} (s^{-1})	1.1	4.8×10^{-2}
k_{+2} (M^{-1} s^{-1})	2.3×10^8	2.3×10^8
k_{-2} (s^{-1})	1.0×10^2	1.0×10^2
k_{+3} (M^{-1} s^{-1})	8.3×10^8	8.3×10^8
k_{-3} (s^{-1})	4.0×10^2	5.0×10^1
k_{+4} (M^{-1} s^{-1})	6.7×10^4	1.4×10^6
k_{-4} (s^{-1})	3.7×10^{-2}	2.4×10^{-3}
k_{+5} (s^{-1})	9.3×10^{-2}	6.0×10^{-3}
[enzyme] (nM)	0.75	0.5

FPP qualifies as a very "sticky" substrate (Viola et al., 1982; Cleland, 1990), which has been defined (by W. W. Cleland) as one that dissociates from the enzyme at a rate equal to or less than k_{cat}. The rate constant for the dissociation of the binary hFPTase·FPP complex is calculated from the equation k_{-1}(FPP) = ($K_{1/2}/K_M$)k_{cat} (Rose et al., 1974; Rose, 1980; Cleland, 1990). Using the values of k_{cat} and K_M for Ras-CVIM listed in Table I, we calculate k_{-1}(FPP) = 0.048 s^{-1} and k_{-4}(FPP) = 0.0024 s^{-1} from the partitioning data with Ras-CVIM as trapping substrate. The GPP·hFPTase complex was also trapped with Ras-CVIM with values of $K_{1/2}$ = 6.5 ± 0.8 μM and appP^* = 5.7 ± 1 pmol. Thus, in the presence of GPP and Ras-CVIM, k_{-1}(GPP) = 1.1 s^{-1} and k_{-4}(GPP) = 0.037 s^{-1}. We were unable to trap the E·GGPP complex, even in the presence of extremely high concentrations of GGPP and Ras-CVIM.

Since the isotope partitioning data suggested that FPTase may not be operating under equilibrium conditions over the entire range of substrate concentrations examined, we wondered whether the exact equation for a two-substrate, steady-state, random order sequential mechanism could account for our observations, including inhibition by the protein substrate. Though it is usually assumed that this mechanism gives rise to nonlinear reciprocal plots (which we have not observed for FPTase, at least in the low Ras-CVIM concentration range), it has been shown that certain conditions and collections of rate constants will give rise to linear reciprocal plots (Pettersson, 1969, 1970). Using experimentally observed values (E_t, K_M, k_{-1}, k_{-4}, and k_{cat}, where $k_{cat} = k_{+5}$) together with estimates of binary and ternary complex dissociation rates based on isotope partitioning data, the kinetic behavior of the steady-state random order mechanism was simulated. The catalytic step with which a given rate constant is associated is shown in Scheme II. The kinetic parameters that best fit the data for GPP or FPP and Ras-CVIM as substrates in the FPTase reaction are presented in Table III. The solid lines in Figure 1 show the double-reciprocal plots determined from eq 1 using the rate constants from Table III superimposed on experimental data collected with GPP and Ras-CVIM as substrates. Note the excellent agreement between simulated and experimentally obtained data, including the prediction of substrate inhibition. The data from our earlier report (using bovine FPTase enzyme with FPP and Ras-CVLS as substrates) also fit the steady-state model well.

DISCUSSION

The results of the isoprenoid diphosphate utilization experiments presented in this study somewhat alter our understanding of the mechanism through which FPTase proceeds. We earlier reported that the steady-state mechanism was sequential and that the order of substrate addition was

random (Pompliano et al., 1992). At first glance, the results of Table I and of the isotope trapping experiments might be construed as evidence for an ordered mechanism. That is, under the conditions used to determine the kinetic parameters, the reaction may proceed via a kinetic pathway wherein the isoprenoid diphosphate forms a binary complex with FPTase followed by binding of the protein substrate and in which the affinity of the protein substrate for the binary complex is modulated by the bound isoprenoid diphosphate. However, we will suggest that, in some sense, both interpretations are true and that designating this mechanism as random or ordered is really one of semantics.

Steady-state inhibition patterns with dead-end substrate and product inhibitors first established a random order of substrate addition: FPP mimics were competitive against FPP and noncompetitive against Ras, and Ras mimics were just the reverse (Pompliano et al., 1992). The same results were obtained when GPP and Ras-CVIM were substrates (Table II). Apart from the initial velocity and inhibitor studies, there are two other observations in support of a random mechanism. First, FPTase can be purified using an affinity column functionalized with a short peptide containing the C-terminal sequence of an FPTase substrate (CVIM) (Reiss et al., 1990). Presumably, the active site of FPTase interacts productively with the immobilized peptide substrate column (in the absence of FPP). Second, Ras can be cross-linked to FPTase in the absence of FPP. The cross-linking is specifically inhibited by cognate peptide (Reiss et al., 1991, 1992). While there has been no direct measurement of the binding affinity of Ras for FPTase, these two different experiments clearly indicate that Ras does bind to free enzyme. Our inability to trap any of the hFPTase·Ras-CVIM complex with FPP means only that the rate of dissociation from the binary complex is fast compared to k_{cat}. Since an ordered mechanism (for a two-substrate reaction) is defined as one in which the second substrate is unable to bind to free enzyme, the experimental evidence is securely on the side of a (formally) random mechanism.

Because the double-reciprocal plots intersected at a common point that was within experimental error of being on the $1/[s]$ axis, we concluded previously that there was no interaction between FPP and Ras-CVLS with bovine FPTase as catalyst (Pompliano et al., 1992). Thus the results in Table I were surprising in another context. The variation of the Michaelis constants for a given protein substrate in the presence of different isoprenoid diphosphate substrates (Table I) argues persuasively that the substrates are interacting in the case of hFPTase and suggests a modest interaction between FPP and Ras-CVLS that cannot be ruled out from the double-reciprocal plots in our previous study with bovine FPTase (Pompliano et al., 1992). Such variation in the Michaelis constants with changing cosubstrate has been observed before in other two-enzyme systems [hexokinase (Viola et al., 1982), 5-enolpyruvylshikimate-3-phosphate synthase (Gruys et al., 1992), and glycerokinase (Knight & Cleland, 1989)] and has been explained in terms of synergistic substrate binding (*vide infra*).

Further experimentation using the isotope partitioning method demonstrated that $k_{+5} > k_{-4}$ (Scheme II) in the reaction catalyzed by hFPTase using FPP and Ras-CVIM (or Ras-CVLS) as substrates. Measurements of the amount of FPP trapped as a function of the Ras-CVIM concentration allowed us to calculate the rate constant for dissociation of FPP from the E·FPP binary complex ($k_{-1} = 0.048\ s^{-1}$) and from the ternary complex ($k_{-4} = 0.0024$). Since k_{-1} is only eight times greater than $k_{cat}(k_{+5})$ and k_{-4} is actually smaller than k_{+5}, the reaction cannot be operating under rapid equilibrium substrate binding conditions. A further complication came in the form of substrate inhibition at high concentrations of Ras-CVIM. In principle, a random order reaction operating at equilibrium should not show substrate inhibition (though substrate inhibition is often observed in two-substrate systems and is usually attributed to binding of the inhibiting substrate at a second site). So the partitioning experiments along with the implications of substrate inhibition and the dependence of the protein substrate K_M value on the structure of the isoprenoid diphosphate suggested that, although the FPTase·Ras binary complex can form, formation of the ternary complex proceeds preferentially from the FPTase·FPP complex. Thus, we set out to develop a more quantitative mechanistic understanding that embodied all of the observed kinetic behavior.

Using the exact equation for a *steady-state* random order, sequential mechanism (Dixon & Webb, 1979), we searched parameter space with the following experimental observations as constraints: (1) experimentally determined rate constants are fixed [K_M, k_{-1}, k_{-4}, k_{+5}, and k_{cat}], (2) double-reciprocal plots of varied concentration of one substrate at different fixed concentrations of the other substrate (in the concentration range where there is no substrate inhibition) are linear and intersect at a common point close to the $1/[s]$ axis, (3) the value of appK_M[Ras-C*aa*X] varies with the structure of the isoprenoid diphosphate, and (4) Ras-CVIM shows substrate inhibition in the presence of either GPP or FPP. Our model (Table III) fits the [s]–v data using GPP and Ras-CVIM surprisingly well (Figure 1, solid lines). These plots are linear at the lower Ras-CVIM concentrations, as opposed to what might have been expected for steady-state, random order mechanism. However, the model does predict that the point of intersection, in the absence of substrate inhibition, would *not* be on the $1/[s]$ axis, indicating that there is interaction between the two substrates. Thus, different isoprenoid diphosphate substrates will interact differently with a given Ras-C*aa*X, leading to a measurable difference in the value of the Ras-C*aa*X Michaelis constants (e.g., Table I). More interestingly, the model predicts quantitatively the observed substrate inhibition by Ras-CVIM at low GPP concentration (Figure 1) and qualitatively fits the substrate inhibition by Ras-CVIM at low FPP concentration (not shown).

In cases (such as the present one) where $k_{+5} > k_{-4}$ and $k_{-3} \gg k_{+5}$ (Scheme II), substrate inhibition should occur when the concentration of protein substrate ([Ras]) is sufficiently high so that k_{+3}[Ras] $> k_{-1}$ and [Ras] $> k_{-2}/k_{+2}$. Under these conditions, the rate of formation of E·FPP (or of E·GPP) becomes the rate-determining step for formation of the ternary E·FPP·Ras complex via the upper pathway (Scheme II), and addition of Ras results in the formation of E·Ras with concomitant depletion of the free enzyme (E) that is available for reaction with the isoprenoid diphosphate (FPP). As the concentration of Ras is increased further, substrate inhibition ensues because the rate of formation of E·FPP eventually becomes so slow that either (1) the rate of formation of E·FPP becomes the rate-determining step for product formation or (2) the reaction is forced to proceed via the lower, slower reaction pathway (through the E·Ras binary complex) wherein conversion of E·Ras to E·FPP·Ras is the rate-determining step for product formation. Here we have not assumed the existence of an alternate or second binding site to account for the substrate inhibition. Conceivably the substrate inhibition often observed in two-substrate enzymically catalyzed reactions similarly reflects the forced formation of a binary

E·substrate complex that does not readily form the ternary complex. In the presence of less extreme substrate concentrations, however, the model predicts that the kinetically preferred pathway to product formation (a consequence of the rate constants for enzyme-associated steps) is through the E·FPP binary complex (upper pathway in Scheme II). Yet the enzyme is not constrained to operate through this pathway. That is, the mechanism is intrinsically random, because both binary complexes can form, but is functionally ordered since (1) the path of least resistance to product involves FPP binding to the enzyme first and (2) conversion of E·Ras-CVIM to the ternary complex is a slow reaction. Although the rate constants listed in Table III exemplify the ability of eq 1 to account for our observations with GPP and FPP, our kinetic data do not uniquely establish the values of each of these rate constants (i.e., the set of rate constants in Table III is one of a constellation of those that could fit our data).

Whether an enzyme proceeds through an ordered or random mechanism is often a difficult point to resolve, especially when there are data in support of both reaction pathways. Our model (with few assumptions) has implied a "favored pathway" description of the reaction catalyzed by FPTase. The apparently conflicting data surrounding the hexokinase mechanism is analogous to the present FPTase conundrum. Steady-state kinetic data and inhibitor studies (Hohnadel & Cooper, 1973; Danenberg & Danenberg, 1977) suggested that glucose bound to hexokinase first, followed by MgATP. Yet MgATP could bind to free enzyme (Kaji & Colowick, 1965), an activity that is not consistent with a (strictly) ordered mechanism. Interestingly, the affinity of MgATP for hexokinase (as measured by its K_M value) varied dramatically (over 100-fold) depending upon which sugar was used as the other substrate in the reaction (Viola et al., 1982). This enhancement of substrate binding that occurs in the presence of another substrate ($K_i/K_M > 1$) is referred to as synergism and can make a reaction appear ordered. When a good (or high affinity) substrate (say glucose in the case of hexokinase) binds to enzyme, it forms a binary complex that has a higher affinity for the second substrate (MgATP in the hexokinase case) than the free enzyme does (Viola et al., 1982). Hence, the second substrate (MgATP) will prefer to bind to the binary complex, and the reaction will appear to be ordered. Isotope partitioning studies showed that glucose dissociated from the binary complex at a rate equal to or less than k_{cat} (Rose et al., 1974). This is consistent with glucose binding first and being tightly bound and also provides a link between the binding data and the isotope trapping results. Generally, the best substrates show the greatest synergism and are the most sticky (Viola et al., 1982). Thus, like FPTase, there is a kinetically preferred reaction pathway for hexokinase—a consequence of the rate constants for enzyme-associated steps—that proceeds with glucose binding first and MgATP binding second. However, the enzyme is not obligated to function in this way. Synergistic substrate binding has been observed in the kinetic mechanism for other two-substrate enzyme systems, glycerokinase (Knight & Cleland, 1989), and 5-enolpyruvolyshikimate-3-phosphate (EPSP) synthase (Gruys et al., 1992). In both of these cases, replacement of the sticky first substrate with a poorly bound (nonsynergistic), slower alternative substrate changed the apparent order of substrate addition. For example, when shikimate 3-phosphate (S3P) and phosphoenolpyruvate (PEP) are substrates, EPSP synthase binds S3P first, but, when shikimate (a much slower substrate) is substituted for PEP, EPSP synthase binds PEP first (Gruys et al., 1992). While the mechanism is formally random with synergistic binding between substrates in these systems, substrate binding can appear to be ordered depending upon the choice of substrates.

Given the high affinity (and stickiness) of FPP, it is likely that FPTase is found in the FPP-bound state under physiological conditions (assuming that the intracellular FPP concentration is at least 10 nM). It is unlikely that GGPP in the cell is either a serious competitor for or alternative substrate to FPP in the reaction catalyzed by FPTase. Thus far, normal farnesylation substrates (X = Met, Ser, Ala, Cys, or Gln) have not been reported to be genranylgeranylated (by either FPTase or GGPTase) *in vivo*, nor do we expect them to be found. The only natural farnesylation substrate (which we have examined) that might be geranylgeranylated by FPTase is Ras-CVIM. However, given that the intracellular concentration of GGPP is probably much lower than that of FPP (Reiss et al., 1992) together with the facts that the specificity constant (k_{cat}/K_M) of GGPP is, at most, 10% of the value for FPP in the presence of Ras-CVIM and that K_M(GGPP) is at least 20-fold greater than K_M(FPP), the net rate of FPTase-catalyzed geranylgeranylation under normal cellular conditions is probably extremely slow.

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