ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS
Vol. 356, No. 2, August 15, pp. 167–176, 1998
Article No. BB980768

Role of the Carboxyterminal Residue in Peptide Binding to Protein Farnesyltransferase and Protein Geranylgeranyltransferase

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Received February 26, 1998, and in revised form May 18, 1998

Protein farnesyltransferase and protein geranylgeranyltransferase-I catalyze the prenylation of a cysteinyl group located four residues upstream of the carboxyl terminus. The identity of the carboxyterminal residue plays a significant role in determining the ability of compounds to bind to each enzyme and to serve as substrate. We compared the binding and substrate specificities of peptides with carboxyterminal substitutions to determine which residues promote selectivity and which residues promote recognition by both enzymes. Using tetrapeptide inhibitors with the general structure L-penicillamine–valine–isoleucine–X and substrates with the structure Lys–Lys–Ser–Ser–Cys–Val–Ile–X, we measured their respective K_i, K_m, and k_{cat} values for both recombinant rat protein farnesyltransferase and recombinant rat protein geranylgeranyltransferase-I. We studied the roles of carboxyterminal branched residues (leucine, isoleucine, valine, and penicillamine) and linear residues (methionine, cysteine, homocysteine, alanine, aminobutyrate, and aminohexanoate) in promoting interaction with the enzymes. For protein geranylgeranyltransferase-I, peptide substrates with carboxyterminal branched or linear residues had K_m values that were 5- to 15-fold greater than the K_i values of the corresponding peptide inhibitors. For protein farnesyltransferase, peptide substrates with carboxyterminal branched residues, proline, or homoserine had K_m values that were 7- to 200-fold greater than the K_i values of the corresponding peptide inhibitors. For protein farnesyltransferase the K_m and K_i values for peptides ending with linear residues were in general agreement. Our studies indicate that the substrate and inhibitor binding specificities of protein geranylgeranyltransferase was much more restricted than those of protein farnesyltransferase. © 1998 Academic Press

Key Words: **protein farnesyltransferase; protein geranylgeranyltransferase; prenylation; CaaX box; Ras; substrate specificity; peptides; coded and noncoded amino acids; competitive inhibitors; binding energy.**

The joining of the 15-carbon farnesyl group or the 20-carbon geranylgeranyl group to protein cysteines is a pivotal posttranslational protein modification (1). Protein farnesyltransferase (FTase)[2] and protein geranylgeranyltransferase-I (GGTase-I) catalyze the prenylation of substrates with a carboxyterminal tetrapeptide sequence called a CaaX box, where C refers to cysteine, a refers to an aliphatic residue, and X typically refers to methionine, serine, or glutamine (FTase) or leucine (GGTase-I). Protein geranylgeranyltransferase-II (GGTase-II), or Rab geranylgeranyltransferase, catalyzes the geranylgeranylation of substrates that terminate in Cys–Cys or Cys–X–Cys sequences (2). Substrates for FTase include H-Ras, K-RasA, K-RasB, N-Ras, lamin A, lamin B, and the α-subunit of retinal transducin. Substrates for GGTase-I include a variety of G proteins such as Rap1A, Rac1, and the γ-subunit of heterotrimeric ($\alpha\beta\gamma$) G proteins such as G_s, G_i, and G_o (1, 3). Substrates for GGTase-II include G proteins in the Rab family (1). Significantly, substrates for the prenyltransferases re-

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[2] Abbreviations used: Ahx, aminohexanoate (norleucine); Ape, aminopentanoate (norvaline); FTase, protein farnesyltransferase; GGTase, protein geranylgeranyltransferase; Hcy, homocysteine; Hse, homoserine; PA, prenyl acceptor; Pen, L-penicillamine; Abu; aminobutyrate.

0003-9861/98 $25.00

quire the attachment of these lipid groups to become functional. FTase and GGTase-I catalyze the prenylation of peptides containing appropriate CaaX box sequences (1).

The three prenyltransferases are heterodimers consisting of an α- and a β-subunit. The α-subunits of FTase and GGTase-I are identical, and the β-subunits are homologous (2). The β-subunit of FTase functions in the binding of the prenyl donor and in the Zn^{2+}-dependent binding of the peptide or protein substrate (4, 5). X-ray diffraction studies of FTase by Park *et al.* (6) show that zinc occurs at a junction between a hydrophilic surface groove near the subunit interface (peptide binding site) and a deep lipophilic cleft in the β-subunit lined with aromatic residues (farnesyl diphosphate binding site). However, the X-ray diffraction studies of FTase do not define the binding site for classical CaaX box substrates with carboxyterminal methionine, serine, or glutamine (6).

The stoichiometries of the prenylation reactions are shown in the following chemical equations:

$$\text{farnesyl-PP}_i + \text{HS-acceptor}_f \rightarrow \text{farnesyl-S-acceptor}_f + \text{PP}_i$$

$$\text{geranylgeranyl} - \text{PP}_i + \text{HS-acceptor}_{gg} \rightarrow \text{geranylgeranyl-S-acceptor}_{gg} + \text{PP}_i.$$

The isoprenoid groups become linked to a polypeptidic cysteine through a thioether (C–S–C) bond. Following prenylation of physiological substrates, the terminal three residues (aaX) are subsequently removed by proteolysis, and the carboxyl group of the terminal cysteine is methyl esterified (1). Mutants of *ras* occur in 20–30% of all human cancer cells (7), and inhibition of Ras prenylation represents a strategy for the treatment of cancer (8). Moreover, compounds based upon the structure of peptide substrates have been used to design inhibitors for FTase and GGTase-I (9–13).

Some proteins can be modified in reactions catalyzed by both FTase and GGTase-I (14–19). For example, K-RasB, which has a Cys–Val–Ile–Met CaaX box and an upstream polybasic sequence, can serve as a substrate for both FTase and GGTase-I (15, 17–19), and inhibition of GGTase-I represents a potential cancer therapy (20). For FTase and GGTase, we compared the binding and substrate specificities of peptides with carboxyterminal substitutions to determine which residues promote selectivity and which residues promote recognition by both enzymes.

Goldstein *et al.* (21) reported that tetrapeptides with L-penicillamine (Pen), or 3,3-dimethylcysteine, substituted for cysteine served as inhibitors and not substrates for FTase, and we measured the affinity (K_i) of tetrapeptide inhibitors with the sequence Pen–Val–Ile–X. We examined the role of genetically encoded and noncoded amino acid residues in promoting binding of inhibitory peptides (not alternative substrates) to FTase and GGTase-I, and we determined the ability of these residues in peptides to promote enzymatic prenylation. We found that the K_i value of several inhibitors was lower than the K_m value of the corresponding substrates.

MATERIALS AND METHODS

Materials. Recombinant rat FTase (22) and GGTase-I (23), which were purified from Sf9 insect cells using baculovirus expression systems in culture, were the generous gift of Drs. Patrick Casey and John Moomaw (Duke University). Peptides were synthesized on a Milligen 9050 synthesizer using 9-fluorenylmethyloxycarbonyl chemistry by the Louisiana State University Medical Center Core Laboratories. The peptides were purified by high-performance liquid chromatography, and their identity was confirmed by fast atom bombardment mass spectrometry. Stock solutions of the hydrophilic substrate peptides (10–100 mM) were made in water containing 10 mM dithiothreitol, and stock solutions of the hydrophobic penicillamine tetrapeptides were made in dimethyl sulfoxide; solutions were stored at −20°C for up to 3 months without adverse effects. Appropriate dilutions were made before each experiment. Tritiated farnesyl diphosphate and geranylgeranyl diphosphate were obtained from Amersham Corporation (Arlington Heights, IL). The sources of other materials were noted previously (24).

Determination of the steady-state kinetic constants for FTase. The transfer of the [^{3}H]farnesyl group from [^{3}H]farnesyl diphosphate to an acceptor peptide was determined as previously described (24). The final reaction mixture (30 μL) contained 50 mM Tris–HCl (pH 7.5), 20 μM $ZnCl_2$, 20 mM KCl, 1 mM dithiothreitol, 0.33% octylmethylglucoside, acceptor peptide, 0.4 μM [^{3}H]farnesyl diphosphate (≈15,000 dpm/pmol), and 0.25 ng (90 pM) of recombinant rat enzyme. The range of concentrations of the acceptor peptide for K_m and V_{max} determinations was established experimentally. Following a 15-min incubation at 37°C, portions (25 μL) were applied to numbered 1 × 1-cm Whatman P81 phosphocellulose paper strips. The strips were immersed and processed in ethanol/phosphoric acid (24). To determine the K_i value of inhibitory peptides for FTase, five fixed concentrations of inhibitor were used with 25, 50, 100, 150, and 250 μM concentrations of Lys–Arg–Lys–Cys–Val–Leu–Ser as acceptor peptide; the K_m of this peptide was about 180 μM. No-enzyme blank values were ≈100 counts/min with an input of ≈60,000 counts/min. At 90 pM FTase and millimolar acceptor peptide concentrations, substrates with second-order rate constants of 0.7 M^{-1} s^{-1} could be observed, assuming that the detection limits of the assay were twice (>200 counts/min) the background value.

Determination of the steady-state kinetic constants for GGTase-I. The transfer of the [^{3}H]geranylgeranyl group from [^{3}H]geranylgeranyl diphosphate to acceptor peptide for GGTase-I was measured as described above for FTase except for 0.2 μM [^{3}H]geranylgeranyl diphosphate as prenyl donor, 0.5 mM Zwittergent additional detergent, and 1.25 ng (450 pM) of purified recombinant GGTase-I. For K_i value determinations, 25, 50, 100, 250, and 500 μM concentrations of Lys–Thr–Lys–Cys–Ala–Ile–Leu as acceptor substrate were used; the K_m of this peptide was about 180 μM. No-enzyme blank values were ≈150 counts/min with an input of ≈35,000 counts/min. At 450 pM GGTase-I and millimolar concentrations of acceptor peptide, substrates with second-order rate constants of 0.2 M^{-1} s^{-1} could be observed, assuming that the detection limits of the assay were twice (>300 counts/min) the background value. The K_i, K_m, and V_{max} values for GGTase-I and FTase were determined by the algorithms of Cleland (25) on a personal com-

puter using a BASIC program obtained from Dr. Ronald E. Viola (University of Akron). The means of duplicate determinations are reported, and the values agreed within 20%.

Pompliano *et al.* (26) and Yokoyama *et al.* (27) found that peptide or protein substrates of FTase and GGTase-I, respectively, are inhibitory at high concentrations. We also observed such inhibition at low concentrations of the prenyl donor. Because we used near saturating concentrations of farnesyl diphosphate and geranylgeranyl diphosphate, inhibition by peptides was avoided.

Calculation of the difference of the standard free energy difference of the binding of inhibitors and of the binding of substrates to the enzyme. For the dissociation of the enzyme–inhibitor complex, EI $\rightleftharpoons$ E + I, the equilibrium constant for this process is given by K_{eq} = [E][I]/[EI]; when [E] = [EI] the concentration of free [I] represents the K_i value. The standard free energy corresponding to the equilibrium constant for binding of the inhibitor to the enzyme is given by $\Delta G° = -RT\ln(1/K_i)$ or $\Delta G° = +RT\ln K_i$, where R is the temperature–energy coefficient, or gas constant (1.98 kcal K^{-1} mol^{-1}), and T is the temperature (310 K). The difference of the difference in standard free energy changes for the binding of the parent inhibitor (I_P) and the derivative inhibitor (I_D) is $\Delta\Delta G°_B = RT\ln K_D - RT\ln K_P = RT\ln K_D/K_P$, where K_D and K_P are K_i values. A negative value for $\Delta\Delta G°_B$ indicates that the derived compound binds with greater affinity than the parent compound, allowing quantification of the effect of a particular substituent (28).

Enzymes accelerate the rate of a chemical reaction by decreasing the standard free energy of activation of the process. When an enzyme catalyzes related reactions such as the farnesylation of Lys–Lys–Ser–Ser–Cys–Val–Ile–Gly (parent, or P) or Lys–Lys–Ser–Ser–Cys–Val–Ile–Ala (derivative, or D), the difference of the difference in binding energies is given by the expression $\Delta\Delta G°_B = -RT\ln (k_{cat}/K_m)_D/(k_{cat}/K_m)_P$ (28). This equation provides a minimum estimate for the differences in intrinsic binding energy (29).

RESULTS

Inhibition of FTase and GGTase-I by peptides. The time course of product formation for both FTase and GGTase-I was linear for about 40 min with or without the penicillamine- or phenylalanine-containing inhibitors, and inhibition was competitive with respect to the peptide substrate. The K_i values of inhibitory peptides were independent of the peptide acceptor substrate. For example, the respective K_i values of inhibitory peptides were independent of the peptide acceptor substrate. For example, the K_i value of Pen–Val–Ile–Met or Cys–Val–Phe–Met for FTase was the same, within experimental error, with two different acceptor peptides (Lys–Arg–Lys–Cys–Val–Leu–Ser or Lys–Lys–Ser–Ser–Cys–Val–Ile–Met). On the basis of the ordered nature of this enzyme-catalyzed reaction with farnesyl diphosphate as leading substrate (26, 30), we surmise that our K_i values for FTase reflect the binding of inhibitory peptides to an enzyme–farnesyl diphosphate complex. Moreover, the respective K_i value for GGTase-I was the same for Pen–Val–Ile–Leu or Cys–Val–Phe–Leu with two different acceptor peptides (Lys–Thr–Lys–Cys–Ala–Ile–Leu or Lys–Lys–Ser–Ser–Cys–Val–Ile–Leu). On the basis of the binding of geranylgeranyl diphosphate to GGTase-I as leading substrate (27), we surmise that our K_i values reflect binding to an enzyme–geranylgeranyl diphosphate complex.

Specificity of the carboxyterminal residue in peptide binding and catalysis by FTase. Methionine is a preferred amino acid in determining the specificity of FTase, and methionine promoted binding of Pen–Val–Ile–Met compared with Pen–Val–Ile–Gly. The difference in the difference of the standard free energy change ($\Delta\Delta G°_B$) of binding of Pen–Val–Ile–Gly (parent) and Pen–Val–Ile–Met (derivative) was −2.0 kcal/mol, and this difference reflects greater binding (a smaller dissociation constant) of the methionine-containing tetrapeptide to the enzyme (Table I). Similarly, the $\Delta\Delta G°_B$ of the substrate Lys–Lys–Ser–Ser–Cys–Val–Ile–Gly (parent) compared with Lys–Lys–Ser–Ser–Cys–Val–Ile–Met (derivative), based upon the k_{cat}/K_m values, was −1.9 kcal/mol. Carboxyterminal cysteine, which occurs in the X position of yeast Ras, yielded an inhibitor and a substrate with binding characteristics comparable to those of methionine. Carboxyterminal homocysteine, a noncoded amino acid, was considerably less effective than methionine or cysteine in promoting peptide binding or catalysis. These data indicate that the methyl group of methionine, which is lacking in homocysteine, was important in promoting favorable interactions of peptides with FTase. These data also indicate that the difference in the chain lengths of cysteine and homocysteine was critical. Methionine contains a linear R group with sulfur as heteroatom (31), and the favorable effect of sulfur on catalysis can be seen when the methionine-containing substrate is compared with that of aminohexanoate.

The branched-chain aliphatic amino acid residues (leucine, valine, isoleucine, and penicillamine) were not as effective in promoting binding to FTase as were the linear aliphatic residues. Among the branched-chain residues, valine yielded the compound that had the greatest affinity for FTase and yielded the best substrate (Table I). FTase preferred linear residues in the X position of the CaaX box over branched residues (the opposite of GGTase-I). The K_m values of the peptides with branched carboxyterminal residues or proline were 7- to 22-fold greater than the K_i values of the corresponding inhibitors.

The α-subunit of rat retinal cGMP phosphodiesterase (3) and the α-subunit of rat phosphorylase kinase (32) contain glutamine as the carboxyterminal residue of their CaaX boxes. Glutamine yielded both a good peptide substrate and the peptide inhibitor in this study with the highest affinity for FTase (Table I). That asparagine failed to yield peptides with similar characteristics indicates that the chain length of the carboxyterminal residue is critical. Phenylalanine yielded an inhibitory peptide with good binding properties. However, peptides containing aromatic residues

TABLE I

Steady-State Kinetic Values of Peptide Inhibitors and Substrates for FTase[a]

Inhibitor	K_i (μM)	$\Delta\Delta G°_B$[b] (kcal/mol)	Substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	$\Delta\Delta G°_B$[c] (kcal/mol)
Pen–Val–Ile–Gly	36	0	KKSSCVI–Gly	140	1.3	9,200	0
Pen–Val–Ile	110	0.69	KKSSCVI	NR[d]			
Pen–Val–Ile–Met	1.1	−2.0	KKSSCVI–Met	0.61	0.12	200,000	−1.9
Pen–Val–Ile–Cys	0.82	−2.3	KKSSCVI–Cys	3.3	0.73	220,000	−2.0
Pen–Val–Ile–Hcy	20	−0.36	KKSSCVI-Hcy	9.6	0.094	9,800	−0.038
Pen–Val–Ile–Ala	5.3	−1.2	KKSSCVI–Ala	19	1.8	95,000	−1.4
Pen–Val–Ile–Abu	9.9	−0.81	KKSSCVI–Abu	14	0.41	29,000	−0.71
Pen–Val–Ile–Ape	17	−0.48	KKSSCVI–Ape	4.3	0.15	34,000	−0.79
Pen–Val–Ile–Ahx	3.4	−1.5	KKSSCVI–Ahx	13	0.088	6,800	0.19
Pen–Val–Ile–Leu	35	−0.022	KKSSCVI–Leu	630	1.9	3,000	0.69
Pen–Val–Ile–Val	11	−0.76	KKSSCVI–Val	77	0.67	8,700	0.038
Pen–Val–Ile–Ile	30	−0.12	KKSSCVI–Ils	280	0.39	1,400	1.2
Pen–Val–Ile–Pen	41	0.064	KKSSCVI–Pen	910	0.84	930	1.4
Pen–Val–IUle–Pro	330	1.4	KKSSCVI–Pro	3300	0.50	150	2.6
Pen–Val–Ile–Ser	20	−0.38	KKSSCVI–Ser	3.1	0.62	200,000	−1.9
Pen–Val–Ile–Thr	38	0.018	KKSSCVI–Thr	57	1.4	24,000	−0.60
Pen–Val–Ile–Hse	1.7	−1.9	KKSSCVI–Hse	430	0.23	540	1.7
Pen–Val–Ile–Asn	34	−0.043	KKSSCVI–Asn	63	0.14	22,000	−0.53
Pen–Val–ILe–Gln	0.26	−3.6	KKSSCVI–Gln	1.2	0.11	90,000	−1.4
Pen–Val–Ile–Phe	4.5	−1.3	KKSSCVI–Phe	12	0.14	12,000	−0.14
Pen–Val–Ile–Tyr	250	1.2	KKSSCVI–Try	72	0.24	3,400	0.62
Pen–Val–Ile–Trp	130	0.79	KKSSCVI–Trp	140	0.21	1,500	1.1
Pen–Val–Ile–His	310	1.3	KKSSCVI–His	100	1.7	17,000	−0.36
Pen–VAlnIle–Glu	240	1.1	KKSSCVI–Glu	130	0.078	600	1.7
Pen–Val–Ile–Lys	310	1.3	KKSSCVI–Lys	710	0.12	170	2.4

[a] Steady-state kinetic values were determined as described under Materials and Methods.
[b] Relative to Pen–Val–Ile–Gly.
[c] Relative to KKSSCVI–Gly.
[d] NR, not reactive.

were much less effective as substrates than were those containing methionine, serine, or glutamine (Table I).

FTase did not tolerate charged residues in the X position. That a peptide containing aspartate in the X position is bound in the crystal of FTase represents an unusual situation, and the relationship of this finding to the binding of traditional CaaX box peptides remains to be established (6). We found that Pen–Val–Ile–Asp had a K_i value of 350 μM, and this value confirms the effect of a charge on decreasing binding to FTase. Although the tripeptide lacking an X residue (Pen–Val–Ile) bound to FTase (albeit with low affinity), the corresponding heptapeptide failed to serve as a substrate (Table I). This result emphasizes the importance of the X residue of the CaaX box in promoting the enzyme-catalyzed reaction.

Specificity of the carboxyterminal residue in peptide binding and catalysis by GGTase-I. CaaX boxes with carboxyterminal leucine are the predominant substrates for GGTase-I. However, bovine brain G25K protein, which contains carboxyterminal phenylalanine, is geranylgeranylated *in vivo* (33). Leucine in the X position of inhibitory peptides yielded the compound with the greatest affinity for GGTase-I and the best substrate (Table II). However, the k_{cat}/K_m value for the best substrate for GGTase-I of this class of peptides was only one-tenth that of the best substrates for FTase. Phenylalanine yielded an inhibitory tetrapeptide with intermediate affinity and an octapeptide that was a passable substrate for GGTase-I compared with leucine (Table II). Unexpectedly, the substrate with carboxyterminal phenylalanine had a larger k_{cat}/K_m value for FTase than for GGTase-I (Tables I and II).

Valine, isoleucine, and penicillamine are branched at the β-position, and leucine is branched at the γ-position. A comparison of the affinities indicates that γ-branching promoted peptide binding to GGTase-I and catalysis more effectively than β-branching. This pattern of recognition reflects the role of leucine in the X position of physiological substrates of GGTase-I. Of the linear aliphatic residues, aminohexanoate yielded an inhibitory peptide with an affinity that compared favorably with the peptide containing isoleucine.

Methionine and leucine in the X position are key residues that provide selectivity for FTase and GGTase-I, respectively, and these residues occur in the X position of physiological substrates. Pen–Val–Ile–Met bound 32-fold more tightly to FTase than to GGTase-I.

TABLE II

Steady-State Kinetic Values of Peptide Inhibitors and Substrates for GGTase[a]

Inhibitor	K_i (μM)	$\Delta\Delta G°_B$[b] (kcal/mol)	Substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	$\Delta\Delta G°_B$[c] (kcal/mol)
Pen–Val–Ile–Gly	1100	0	KKSSCVI–Gly	4300	0.019	4.4	0
Pen–Val–Ile–	2100	0.41	KKSSCVI	NR[d]			
Pen–Val–Ile–Met	44	−1.9	KKSSCVI–Met	250	0.16	630	−3.1
Pen–Val–Ile–Cys	23	−2.4	KKSSCVI–Cys	160	0.024	150	−2.2
Pen–Val–Ile–Hcy	34	−2.2	KKSSCVI-Hcy	350	0.22	640	−3.1
Pen–Val–Ile–Ala	170	−1.1	KKSSCVI–Ala	1300	0.029	22	−1.0
Pen–Val–Ile–Abu	890	−0.11	KKSSCVI–Abu	2300	0.25	110	−1.9
Pen–Val–Ile–Ape	72	−1.7	KKSSCVI–Ape	85	0.15	1,800	−3.6
Pen–Val–Ile–Ahx	3.0	−3.6	KKSSCVI–Ahx	47	0.094	2,000	−3.8
Pen–Val–Ile–Leu	0.27	−5.0	KKSSCVI–Leu	5.1	0.11	22,000	−5.3
Pen–Val–Ile–Val	12	−2.6	KKSSCVI–Val	90	0.13	1,400	−3.1
Pen–Val–Ile–Ile	3.6	−3.6	KKSSCVI–Ile	250	0.15	600	−3.6
Pen–Val–Ile–Pen	38	−2.1	KKSSCVI–Pen	430	0.060	140	−2.1
Pen–Val–Ile–Pro	1500	0.20	KKSSCVI–Pro	NR			
Pen–Val–Ile–Ser	7500	1.2	KKSSCVI–Ser	6700	0.025	3.7	0.11
Pen–Val–Ile–Thr	1600	0.24	KKSSCVI–Thr	1100	0.081	74	−1.7
Pen–Val–Ile–Hse	560	−0.41	KKSSCVI–Hse	2000	0.22	110	−2.0
Pen–Val–Ile–Asn	2800	0.60	KKSSCVI–Asn	9300	0.30	32	−1.2
Pen–Val–Ile–Gln	1500	0.20	KKSSCVI–Gln	NR			
Pen–Val–Ile–Phe	19	−2.4	KKSSCVI–Phe	250	0.15	600	−3.1
Pen–Val–Ile–Tyr	2400	0.50	KKSSCVI–Try	570	0.042	73	−1.7
Pen–Val–Ile–Trp	2000	0.38	KKSSCVI–Trp	1200	0.026	22	−1.0
Pen–Val–Ile–His	130	−1.3	KKSSCVI–His	1900	0.15	81	−1.8
Pen–Val–Ile–Glu	2000	0.38	KKSSCVI–Glu	2700	0.024	8.9	−0.43
Pen–Val–Ile–Lys	6400	1.1	KKSSCVI–Lys	NR			

[a] Steady-state kinetic values were determined as described under Materials and Methods.
[b] Relative to Pen–Val–Ile–Gly.
[c] Relative to KKSSCVI–Gly.
[d] NR, not reactive.

In contrast, Pen–Val–Ile–Met bound with 160-fold less affinity to GGTase-I than did Pen–Val–Ile–Leu. Methionine-containing peptides yielded good substrates for FTase and poor substrates for GGTase-I, and leucine behaved reciprocally. The $\Delta\Delta G°_B$ of Pen–Val–Ile–Leu (K_i = 0.27 μM) and Pen–Val–Ile–Met (44 μM) for GGTase-I was about 3.1 kcal/mol. These values are near the expected maximum for the structural differences of these two nonpolar residues (28).

Phenylalanine in the X position of peptides promoted binding and catalysis for both FTase and GGTase-I. Phenylalanine occurs in a bovine protein (G25a) that is geranylgeranylated (33). However, our peptide with a carboxyterminal phenylalanine was a better substrate for FTase than for GGTase-I. Peptides with a carboxyterminal tyrosine or tryptophan yielded poor inhibitors and poor substrates for GGTase-I and for FTase. These residues may be too large to occupy the subsite in the prenyltransferases that accommodates the X residue.

In contrast to FTase, GGTase-I could not tolerate polar residues such as serine, homoserine, cysteine, or glutamine in the carboxyterminal position. Therefore, GGTase-I was much more selective for residues in the X position of the CaaX box than was FTase. Kohl and co-workers (9) designed a tetrapeptide inhibitor of FTase that contained homoserine as a replacement for serine in the X position. They chose this residue because of its similarity to serine and their goal of preparing a lactone that would more readily cross biological membranes. Our studies suggest that homoserine promoted exceptional selectivity for FTase over GGTase-I (Tables I and II). Neither enzyme had great affinity for compounds with charged carboxyterminal residues such as histidine, glutamate, or lysine, and neither enzyme could catalyze the prenylation of the heptapeptide lacking the X residue of the CaaX box.

Binding of inhibitory tetrapeptides to FTase and GGTase-I. Brown and co-workers (34) reported that CaaX box tetrapeptides with an aromatic residue such as phenylalanine next to X are inhibitors and not substrates for FTase, and Zhang *et al.* (23) reported that Cys–Val–Phe–Leu is a competitive inhibitor and not a substrate of GGTase-I. We compared the influence of residues in the X position on the binding of inhibitory peptides containing an aminoterminal penicillamine or a penultimate phenylalanine to FTase and GGTase.

For FTase, we found that methionine quantitatively increased the affinity of these two series of inhibitors to the same extent. For Cys–Val–Phe (parent) and Cys–

TABLE III

Peptide Inhibition Constants for FTase

Peptide	K_i (μM)	$\Delta\Delta G°_B$[a] (kcal/mol)	Peptide	K_i (μM)	$\Delta\Delta G°_B$[b] (kcal/mol)
Cys–Val–Phe	9.5	0	Pen–Val–Ile	110	0
Cys–Val–Phe–Met	0.088	−2.9	Pen–Val–Ile–Met	1.1	−2.9
Cys–Val–Phe–Cys	2.9	−0.74	Pen–Val–Ile–Cys	0.82	−3.1
Cys–Val–Phe–Ser	2.1	−0.93	Pen–Val–Ile–Ser	20	−1.1
Cys–Val–Phe–Ahx	5.9	−0.29	Pen–Val–Ile–Ahx	4.6	−2.0
Cys–Val–Phe–Leu	5.1	−0.38	Pen–Val–Ile–Leu	35	−0.72
Cys–Val–Phe–Val	4.3	−0.48	Pen–Val–Ile–Val	11	−1.5
Cys–Val–Phe–Ile	5.3	−0.38	Pen–Val–Ile–Ile	30	−0.79
Cys–Val–Phe–Pen	12	0.13	Pen–Val–Ile–Pen	41	−0.65
Cys–Val–Phe–Phe	2.8	−0.76	Pen–Val–Ile–Phe	4.5	−1.3

[a] Relative to Cys–Val–Phe.
[b] Relative to Pen–Val–Ile.

Val–Phe–Met (derivative) and Pen–Val–Phe (parent) and Pen–Val–Phe–Met (derivative) the $\Delta\Delta G°_B$ was about −2.9 kcal/mol (Table III). Moreover, serine, leucine, isoleucine, penicillamine, and phenylalanine had about equal effects on these two classes of inhibitory peptides. Other amino acid residues, however, differed in their ability to increase binding in the two series of peptides. Cysteine, aminohexanoate, and valine, for example, promoted greater binding with the Pen–Val–Ile family than with the Cys–Val–Phe family. Moreover, we found that the parent peptides (Cys–Val–Phe and Pen–Val–Ile) bound to FTase with much greater affinity than to GGTase-I (Tables III and IV).

For GGTase-I, leucine was an order of magnitude more effective than the other residues in mediating inhibitor binding (Table IV). We also tested 4,5-dehydroleucine (peptide K_i = 9.7 μM) and 3,3-dimethyl-2-aminobutyrate, or tertiary leucine (peptide K_i = 58 μM), in the X position of the penicillamine-containing scaffold, and we found that they were less effective than leucine (peptide K_i = 0.27 μM) in promoting peptide binding to GGTase-I. Except for methionine and serine (which provide specificity for FTase), the other amino acids increased binding to GGTase-I more in the Pen–Val–Ile family of inhibitors than in the Cys–Val–Phe family of inhibitors.

The data in Tables III and IV indicate that the effect of a substituent in the X position depended upon the scaffold to which the residue is attached. The K_i values of Cys–Val–Phe–Ile for FTase and GGTase-I were nearly identical, but those for Pen–Val–Ile–Ile for FTase and GGTase-I differed by a factor of 8. The effects of serine on the two classes of inhibitors were disparate. Serine promoted peptide binding of the phenylalanine-containing inhibitor, but serine repressed binding of the penicillamine-containing inhibitor. Thus, the efficacy of residues will have to be determined for each family of peptide mimetic inhibitors of both FTase and GGTase.

TABLE IV

Peptide Inhibition Constants for GGTase

Peptide	K_i (μM)[a]	$\Delta\Delta G°_B$[b] (kcal/mol)	Peptide	K_i (μM)	$\Delta\Delta G°_B$[b] (kcal/mol)
Cys–Val–Phe	210	0	Pen–Val–Ile	2100	0
Cys–Val–Phe–Met	35	−2.9	Pen–Val–Ile–Met	44	−2.4
Cys–Val–Phe–Cys	14	−1.7	Pen–Val–Ile–Cys	23	−2.9
Cys–Val–Phe–Ser	94	−0.50	Pen–Val–Ile–Ser	7500	0.79
Cys–Val–Phe–Ahx	8.8	−2.0	Pen–Val–Ile–Ahx	14	−3.1
Cys–Val–Phe–Leu	0.89	−3.3	Pen–Val–Ile–Leu	0.27	−5.5
Cys–Val–Phe–Val	15	−1.6	Pen–Val–Ile–Val	2.6	−4.1
Cys–Val–Phe–Ile	7.5	−2.1	Pen–Val–Ile–Ile	3.6	−3.8
Cys–Val–Phe–Pen	33	−1.1	Pen–Val–Ile–Pen	38	−2.4
Cys–Val–Phe–Phe	8.8	−2.0	Pen–Val–Ile–Phe	19	−2.9

[a] Relative to Cys–Val–Phe.
[b] Relative to Pen–Val–Ile.

TABLE V

Steady-State Kinetic Values of Peptide Inhibitors and Substrates for FTase[a]

Inhibitor	K_i (μM)	$\Delta\Delta G°_B$[b] (kcal/mol)	Substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	$\Delta\Delta G°_B$[c] (kcal/mol)
Pen–Val–Leu–Ser	60	0	KKSSC–Val–Leu–Ser	37	0.74	20,000	0
Pen–Val–Val–Ser	36	−0.33	KKSSC–Val–Val–Ser	7.6	1.1	140,000	−1.2
Pen–Val–Leu–Met	1.5	−2.3	KKSSC–Val–Leu–Met	1.5	0.26	170,000	−1.3
Pen–Val–Val–Met	0.82	−2.6	KKSSC–Val–Val–Met	2.2	0.31	140,000	−1.2
Pen–Ile–Val–Met	3.4	−1.8	KKSSC–Ile–Val–Met	0.12	0.06	500,000	−2.0
Pen–Ile–Ile–Met	0.52	−2.9	KKSSC–Ile–Ile–Met	0.30	0.09	300,000	−1.7
Pen–Val–Ile–Ser	20	−0.67	KKSSC–Val–Ile–Ser	3.1	0.62	200,000	−1.4
Pen–Val–Ile–Met	1.2	−2.4	KKSSC–Val–Ile–Met	0.61	0.12	200,000	−1.4

[a] Steady-state kinetic values were determined as described under Materials and Methods.
[b] Relative to Pen–Val–Leu–Ser.
[c] Relative to KKSSC–Val–Leu–Ser.

Substrate characteristics of peptides with Ras CaaX box sequences. H-Ras has a CaaX box sequence of Cys–Val–Leu–Ser, and K-RasB has the sequence Cys–Val–Ile–Met. We found that peptides containing these sequences exhibited different steady-state kinetic values, and we performed studies with synthetic peptides to address the basis of the differences. We found that Pen–Val–Leu–Ser had a relatively high dissociation constant (60 μM) for FTase compared with Pen–Val–Ile–Met (1.2 μM). Pen–Val–Leu–Ser had a larger dissociation constant (lower binding affinity) for FTase than did two other peptides with carboxyterminal serine (Pen–Val–Val–Ser and Pen–Val–Ile–Ser) (Table V). Thus, leucine in the penultimate position of the CaaX box peptides adversely influenced binding. Peptides with carboxyterminal methionine bound to FTase more avidly than the peptides with carboxyterminal serine. K-RasA has a CaaX box with a Cys–Val–Val–Met sequence, and N-Ras has Cys–Ile–Ile–Met for its sequence. The inhibitory peptides bound these sequences with high affinity, and the steady-state kinetic constants were much more favorable than those for Cys–Val–Leu–Ser.

Because K-RasB, but not H-Ras, is a substrate for GGTase-I *in vitro* (15, 17, 18), we determined the binding affinity and substrate characteristics of these and related peptides for GGTase-I. Pen–Val–Leu–Ser bound to GGTase-I with low affinity, and the steady-state kinetic characteristics of the corresponding substrate peptide were poor (Table VI). The Cys–Val–Ile–Met peptides had better characteristics than Cys–Val–Leu–Ser peptides, but not surprisingly they were not as good as peptides with carboxyterminal leucine. The Cys–Ile–Ile–Met sequence that occurs in K-RasA and the Cys–Val–Val–Met sequence that occurs in N-Ras had relatively favorable steady-state kinetic properties when present in peptide substrates. Three of the inhibitory peptides given in Table VI were not linear competitive inhibitors, and the reason for their anomalous behavior is unclear.

DISCUSSION

The relative ability of prenyl acceptors (PA1 and PA2) to serve as substrates for FTase and GGTase-I is described in terms of the ratio of the k_{cat}/K_m values, or

TABLE VI

Steady-State Kinetic Values of Peptide Inhibitors and Substrates for GGTase

Inhibitor	K_i (μM)	$\Delta\Delta G°_B$[a] (kcal/mol)	Substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	$\Delta\Delta G°_B$[b] (kcal/mol)
Pen–Val–Leu–Ser	3800	0	KKSSC–Val–Leu–Ser	4900	0.0059	1.2	0
Pen–Val–Val–Ser	ND[c]		KKSSC–Val–Val–Ser	NR[d]			
Pen–Val–Leu–Met	48	−2.6	KKSSC–Val–Leu–Met	590	0.10	170	−3.1
Pen–Val–Val–Met	ND		KKSSC–Val–Val–Met	1800	0.079	44	−2.2
Pen–Ile–Val–Met	ND		KKSSC–Ile–Val–Met	3700	2.2	590	−3.8
Pen–Ile–Ile–Met	330	−1.5	KKSSC–Ile–Ile–Met	82	0.11	1400	−4.3
Pen–Val–Ile–Ser	7500	0.43	KKSSC–Val–Ile–Ser	6700	0.025	3.7	−0.69
Pen–Val–Ile–Met	55	−2.6	KKSSC–Val–Ile–Met	250	0.16	640	−3.8

[a] Relative to Pen–Val–Leu–Ser.
[b] Relative to KKSSC–Val–Ile–Gly.
[c] ND, not determined because of anomalous behavior.
[d] NR, not reactive.

specificity constants, according to the following equation (35):

$$v_{PA1}/v_{PA2} = (k_{cat}/K_m)_{PA1}/(k_{cat}/K_m)_{PA2} \times [PA1]/[PA2].$$

This equation relates the ratio of the velocity of the two reactions (v_{PA1}/v_{PA2}) to the ratio of k_{cat}/K_m values and the ratio of the concentrations of the prenyl acceptors (PA1 and PA2), where k_{cat} is the turnover number for the corresponding prenyl acceptor and K_m is its Michaelis constant. This equation applies to FTase, GGTase-I, or both enzymes. For FTase, the specificity constant for the farnesylation of peptide–Cys–Val–Ile–Met, corresponding to the K-RasB CaaX box, was 10-fold greater than that for peptide–Cys–Val–Leu–Ser, corresponding to the H-Ras CaaX box (Table V). The specificity constant for peptide–Cys–Val–Ile–Ser was 10-fold greater than that for peptide–Cys–Val–Leu–Ser, showing that the penultimate leucine has a substantial effect on substrate selectivity (Table V).

Zhang *et al.* (17) found that K-RasA and K-RasB proteins and peptides are good substrates for GGTase-I. They also demonstrated that the N-Ras protein but not peptide is a good substrate for GGTase. In contrast, our N-Ras peptide with the Cys–Ile–Ile–Met CaaX box had the highest k_{cat}/K_m value for GGTase-I of the Ras peptides examined (Table VI). This disparity is most likely due to the different upstream sequences of the peptides used. We found that the ratio of the specificity constants for peptide–Cys–Val–Ile–Leu (the best substrate for GGTase in our study) and peptide–Cys–Val–Ile–Met was 35 (Table II), but the ratio of the specificity constants of peptide–Cys–Val–Ile–Leu (Table II) and peptide–Cys–Val–Leu–Ser (Table VI) was 18,000. Thus, the ability of GGTase to modify peptide substrates containing the K-Ras CaaX box greatly exceeded its ability to modify substrates containing the H-Ras CaaX box. Our results and those of Zhang *et al.* (17) are consistent with the notion that FTase inhibitors may block H-Ras farnesylation, but K-RasA, K-RasB, or N-Ras may be modified by reactions catalyzed by GGTase-I and function as a surrogate *in vivo*.

It was initially surmised that FTase and GGTase-I recognized only the CaaX sequence and that extensions of the amino terminus were unimportant (14, 21, 36). However, the sequence upstream of the CaaX box (24) and the overall protein structure influences steady-state kinetic properties (17, 37, 38). For example, K-RasB is prenylated in reactions catalyzed by both GGTase-I and FTase (15, 17, 18). The low K_m of K-RasB protein for both FTase and GGTase-I is due to both the Cys–Val–Ile–Met CaaX box and an upstream polybasic sequence (15, 17).

CaaX box substrates ending in phenylalanine are considered to be substrates for GGTase. G25 protein, which ends with Cys–Cys–Ile–Phe, is geranylgeranylated *in vivo* (33), and a small peptide corresponding to this sequence is preferentially geranylgeranylated *in vitro* by GGTase compared with farnesylation catalyzed by FTase (39). However, TC21–Cys–Val–Ile–Phe, a Ras-related protein with oncogenic potential, is farnesylated in a reaction catalyzed by FTase and is geranylgeranylated in a reaction catalyzed by GGTase *in vitro* (40). Yokoyama *et al.* (35) showed that TC21 is farnesylated by FTase at 10% of the rate as a small peptide ending with Cys–Ala–Ile–Ser (FTase), and TC21 is geranylgeranylated by GGTase at 10% of the rate as a small peptide ending with Cys–Ala–Ile–Leu. In our study, the ratio of the specificity constants of FTase for peptide–Cys–Val–Ile–Met (200,000) and peptide–Cys–Val–Ile–Phe (12,000) was 17 (Table I), and the ratio of the specificity constants of GGTase for peptide–Cys–Val–Ile–Leu (22,000), the best GGTase-I substrate of this peptide family, and peptide–Cys–Val–Ile–Phe (600) was 36 (Table II). In the cell, TC21 will compete with the substrates for GGTase and the substrates for FTase (35). The nature of the TC21 product *in vivo* (farnesyl versus geranylgeranyl) has not yet been determined, but our results and those of previous workers (35, 40) suggest that TC21 and other CaaX box substrates with a carboxyterminal phenylalanine can be modified by GGTase-I or FTase.

Physiological substrates of FTase possess carboxyterminal methionine, cysteine, serine, alanine, or glutamine (1, 3), and these residues, except for serine, effectively promoted the binding of inhibitory tetrapeptides to FTase. Alanine and methionine are nonpolar residues, and serine, cysteine, and glutamine are polar residues. How FTase manifests specificity toward serine, cysteine, and glutamine but excludes asparagine is unclear. The affinity of the glutamine analog for FTase was 100 times that of the asparagine analog. The difference of 2.9 kcal/mol for the insertion of a $–CH_2–$ group into asparagine to produce glutamine is about the maximal value for the transfer of a methyl group substituent from water to enzyme (28, 41).

Our quantitative data substantiate the notion that FTase exhibits much broader specificity than GGTase-I. FTase recognized compounds with a variety of nonpolar and polar residues in the X position, and GGTase-I recognized nonpolar residues. Although GGTase-I preferred hydrophobic and branched residues and FTase preferred linear residues, there is cross-specificity *in vitro* that can be manifested when high concentrations of synthetic peptides are used, as in this study. Charged residues in the X position had deleterious effects on peptide binding for both enzymes.

Our data indicate that methionine and its sulfur heteroatom in the X residue play a defining role in prenyltransferase enzymology. The methylene group ($–CH_2–$) and sulfur group (–S–) are considered isofunc-

tional, except in highly unusual circumstances, and aminohexanoate usually mimics methionine in its properties (31). However, the behavior of the aminohexanoate-containing compounds and the methionine-containing compounds toward FTase and GGTase-I differed. Although the methionine-containing inhibitor bound 3-fold more tightly to FTase than the aminohexanoate-containing inhibitor, the methionine-containing substrate had a 30-fold greater k_{cat}/K_m than the aminohexanoate-containing substrate. In contrast, the methionine-containing inhibitor bound 10-fold less tightly to GGTase-I than the aminohexanoate-containing inhibitor, and the k_{cat}/K_m of the methionine-containing substrate was one-third that of the aminohexanoate-containing substrate. The sulfur in the methionine-containing substrate seems to play a special role in catalysis.

The rates of the reactions catalyzed by FTase and GGTase-I are unusually slow. With Ras proteins as substrates, k_{cat} values are on the order of 0.02—0.1 s^{-1} (23, 26, 42). With peptides as substrates, the rates of the enzyme-catalyzed reactions are also slow (27, 30). In the most comprehensive study of the kinetics of FTase using a biotinylated peptide with a Cys–Val–Val–Met CaaX box, Furfine *et al.* (30) reported that the chemistry for the farnesylation of their peptide was 0.44 s^{-1} but the k_{cat} of 0.06 s^{-1} at 298 K was limited by the rate of release of product from the enzyme. Several of our peptides had k_{cat} values on the order of 1.5 s^{-1} (Table I). Our higher k_{cat} values may be related to a higher temperature (310 versus 298 K) or to the different peptides used. Our peptides with the high k_{cat} values had high K_m values, and consequently they had low k_{cat}/K_m values. With the variation in k_{cat} of the various peptides over a range of two orders of magnitude, it is possible that the rate-limiting step may differ among the peptides.

The IC_{50} value for a competitive inhibitor is given by the following expression: $IC_{50} = K_i(1 + S/K_m)$ (43). The IC_{50} value of a competitive inhibitor varies with the substrate concentration and underestimates the affinity of inhibitor for the enzyme (43). This analysis explains, at least in part, the higher IC_{50} value that Zhang and co-workers (17) observed for their inhibitor of FTase using equal concentrations of K-RasB protein or peptide compared with K-RasA, N-Ras, and H-Ras proteins and peptides at equal concentrations because the K_m for the K-RasB protein or peptide is eightfold lower than that of the alternative substrates.

Lerner *et al.* (20) reported that inhibition of GGTase-I in NIH 3T3 cells blocked transformation caused by K-*ras*. Inhibition of both FTase and GGTase-I might be necessary to overcome K-*ras* transformation (18, 20). The idea that K-RasB may be modified by geranylgeranylation mediated by GGTase-I was not considered when the first inhibitors of Ras prenylation were developed; many of these inhibitors contain a methionine residue to provide selectivity for FTase. With both the penicillamine and the phenylalanine groups of inhibitors, aminohexanoate, phenylalanine, and valine residues were the least selective in distinguishing between FTase and GGTase-I (Tables III and IV). Thus, these residues might yield compounds that are effective against both FTase and GGTase-I.

ACKNOWLEDGMENTS

We are grateful to Drs. Patrick W. Casey and John Moomaw for the generous gift of recombinant rat FTase and GGTase.

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