Mammalian Protein Geranylgeranyltransferase-I: Substrate Specificity, Kinetic Mechanism, Metal Requirements, and Affinity Labeling†

Kohei Yokoyama, Paul McGeady, and Michael H. Gelb*

Departments of Chemistry and Biochemistry, University of Washington, Seattle, Washington 98195

Received May 23, 1994; Revised Manuscript Received October 5, 1994⊗

ABSTRACT: Protein geranylgeranyltransferase-I (PGGT-I) catalyzes the transfer of the 20-carbon prenyl group from geranylgeranyl pyrophosphate to the cysteine residue near the C-termini of a variety of eukaryotic proteins. Kinetic analysis of homogenous PGGT-I from bovine brain reveals that the reaction follows a sequential pathway in which either prenyl donor or acceptor can bind first to the enzyme and that the reaction operates at steady-state rather than at rapid equilibrium. Substrate inhibition by prenyl acceptor but not by prenyl donor suggests that geranylgeranyl pyrophosphate binding first to free enzyme is the kinetically preferred pathway. This is supported by isotope trapping experiments which show that the ternary complex goes on to products faster than the release of geranylgeranyl pyrophosphate from the complex. The K_M for the interaction of geranylgeranyl pyrophosphate with PGGT-I is markedly affected by the structure of the prenyl acceptor bound to the enzyme. A detailed analysis of the substrate specificity of PGGT-I reveals that peptides which contain a C-terminal leucine are preferred ($k_{cat}/K_M = 1-5 \times 10^5$ M^{-1} s^{-1}) to those that end in serine ($k_{cat}/K_M = 2-4 \times 10^3$ M^{-1} s^{-1}) or phenylalanine ($k_{cat}/K_M = 0.5 \times 10^3$ M^{-1} s^{-1}). PGGT-I also catalyzes the farnesylation of peptides that have a C-terminal leucine; k_{cat} for farnesylation and K_M for farnesyl pyrophosphate are similar to those for geranylgeranylation, but the K_M for the peptide is 30-fold higher. Geranyl pyrophosphate is utilized by PGGT-I but is a poor substrate. Optimal activity of PGGT-I is obtained in the presence of micromolar amounts of Zn^{2+} and mM amounts of Mg^{2+}. Mn^{2+} or Cd^{2+} but not Co^{2+} can substitute for Zn^{2+} and for Mg^{2+}. Metals are not required for tight-binding of geranylgeranyl pyrophosphate to PGGT-I, and the measured dissociation equilibrium constant for this binary complex is 16 nM. Photoaffinity analogues of geranylgeranyl pyrophosphate and farnesyl pyrophosphate were prepared and shown to exclusively label the β-subunit. The implication of the results for the substrate specificity of protein prenylation in cells is briefly discussed.

Protein prenyltransferases catalyze the transfer of prenyl groups from prenyl pyrophosphates to cysteine residues at the C-termini of a specific set of proteins in eukaryotic cells (Clarke, 1992; Reiss et al., 1992b; Yokoyama et al., 1992). Protein farnesyltransferase (PFT)[1] attaches a farnesyl group to the cysteine residue of proteins that contain the C-terminal motif Cys-Ali-Ali-Xaa where Ali is usually, but not always, an aliphatic amino acid and Xaa is glutamine, methionine, serine, cysteine, or possibly other residues (Gomez et al., 1992; Manne et al., 1990; Moores et al., 1991; Omer et al., 1993; Ray & Lopez, 1992; Reiss et al., 1990; Reiss et al., 1991b; Schafer et al., 1990). PGGT-I modifies proteins that end in Cys-Ali-Ali-Leu/Phe (Yokoyama et al., 1991; Casey et al., 1991; Finegold et al., 1991; Yoshida et al., 1991; Joly et al., 1991; Yokoyama & Gelb, 1993; Moomaw & Casey, 1992), whereas a distinct enzyme termed protein geranylgeranyltransferase-II or rab geranylgeranyltransferase prenylates rab proteins that have C-terminal sequences Cys-Xaa-Cys, Cys-Cys, or possibly Cys-Cys-Xaa-Xaa (Horiuchi et al., 1991; Seabra et al., 1992a; Seabra et al., 1992b).

PFT and PGGT-I are heterodimeric enzymes that share a common α-subunit but have distinct β-subunits (Chen et al., 1991a; Chen et al., 1991b; Kohl et al., 1991; Seabra et al., 1991; Zhang et al., 1994). Rab geranylgeranyltransferase contains two components, A and B, which dissociate in the presence of high salt (Seabra et al., 1992a; Seabra et al., 1992b). Component A is a single 95-kDa polypeptide and component B contains 38- and 60-kDa polypeptides (Andres et al., 1993; Armstrong et al., 1993). PFT and PGGT-1 require Zn^{2+} for catalytic activity, whereas rab geranylgeranyltransferase is inhibited by Zn^{2+}; all three enzymes require Mg^{2+} for catalytic activity (Chen et al., 1993; Moomaw & Casey, 1992; Reiss et al., 1992a; Seabra et al., 1992b).

PFT and PGGT-1 are able to form tight binary complexes with their prenyl pyrophosphate substrates that can be

† This work was supported by Grant CA52874 and Research Career Development Award GM562 from the National Institutes of Health to M.H.G. P.M. is the recipient of an NIH Postdoctoral Fellowship (GM16158).

* To whom correspondence should be addressed.

⊗ Abstract published in *Advance ACS Abstracts,* December 15, 1994.

[1] Abbreviations: biotin-CDC42(F), biotin-CONH-$(CH_2)_5$-CO-ALEPPETEPKRKCCIF; biotin-γ_6(L), biotin-CONH-$(CH_2)_5$-CO-NPFREKKFFCAIL; biotin-lamin B(S), biotin-CONH-$(CH_2)_5$-CO-GTPRASNRSCAIS; biotin-ras1(L), biotin-CONH-$(CH_2)_5$-CO-SSCAIL; [^{3}H]-DATFP-FPP, [12-^{3}H]-(*E,E,E*)-12-[(2-diazo-3,3,3-trifluoropropionyl)oxy]-3,7,11-trimethyl-2,6,10-dodecatriene; [^{3}H]-DATFP-GPP, [8-^{3}H]-(*E,E*)-8-[(2-diazo-3,3,3-trifluoropropionyl)oxy]-3,7-dimethyl-2,6-octadiene; [^{3}H]-FPP, [1-^{3}H]-*all-trans*-farnesyl pyrophosphate; [^{3}H]-GPP, [1-^{3}H]-*trans*-geranyl pyrophosphate; [^{3}H]-GGPP, [1-^{3}H]-*all-trans*-geranylgeranyl pyrophosphate; GST-CDC42(F), recombinant glutathione S-transferase fusion protein of CDC42Hs (with a C-terminal CCIF sequence); PD-83176, *N*-carbobenzoxy-L-histidyl-L-(*O*-benzyl)tyrosyl-L-(*O*-benzyl)seryl-L-tryptophyl-D-alaninamide; PFT, protein farnesyltransferase; PGGT-I, protein geranylgeranyltransferase type-I; 1-P-GGOH, racemic 1-phosphono-*all-trans*-geranylgeraniol; RAS1-CAIL, *S. cerevisiae* RAS1 with leucine at position 68 and containing the C-terminal sequence KSSCAIL after residue 185.

0006-2960/95/0434-1344$09.00/0

isolated by size exclusion chromatography (Reiss et al., 1991a; Yokoyama & Gelb, 1993). Peptide or protein acceptors become prenylated when added to these complexes, and this occurs without dissociation of the prenyl pyrophosphates from the enzymes. These facts together with the observation that PFT and PGGT-I bind to Cys-Ali-Ali-Xaa-containing peptides in the absence of prenyl pyrophosphates (Moomaw & Casey, 1992; Reiss et al., 1990; Yokoyama & Gelb, 1993) suggest that binding of substrates to the enzyme occurs in a random order. Steady-state kinetic studies of PFT have established a random sequential mechanism (Pompliano et al., 1992).

The discovery of inhibitors of PFT is an active area of research since such compounds may display antitumor properties stemming from their ability to inhibit the farnesylation of oncogenic ras proteins (Brown et al., 1992; Gibbs et al., 1993; Goldstein et al., 1991; Hara et al., 1993; Nigam et al., 1993; Omura et al., 1993; Reiss et al., 1990; Shiomi et al., 1993; Van der Pyl et al., 1992). In this context, a good understanding of the substrate specificity of the various protein prenyltransferases found in mammalian cells is crucial as it relates to the potential cell toxicity of PFT inhibitors.

In this paper, a detailed analysis of the substrate specificity of pure bovine brain PGGT-I is described, and the affinity of the enzyme for one of its substrates, geranylgeranyl pyrophosphate, has been measured. Kinetic analysis of the enzyme have been carried out in the presence and absence of competitive inhibitors to establish the steady-state mechanism. Finally, the ability of various metal ions to support the catalytic activity of PGGT-I has been explored.

EXPERIMENTAL PROCEDURES

Materials. All peptides were synthesized and analyzed by mass spectrometry as described previously (Jang et al., 1993; Yokoyama & Gelb, 1993; Yokoyama et al., 1991). The radiolabeled prenyl pyrophosphates [^{3}H]-FPP, [^{3}H]-GPP, [^{3}H]-GGPP, and [^{3}H]-dimethylallyl pyrophosphate (all 15 Ci/mmol) and nonlabeled FPP and GGPP were purchased from American Radiolabeled Chemicals. The purity of these compounds was routinely checked by thin layer chromatography (Yokoyama et al., 1991), and the compounds were not used if the purity was less than 80%. The pentapeptide PD-83176 and RAS1-CAIL were obtained as generous gifts from Dr. J. Sebolt-Leopold (Parke-Davis) and Dr. Charles Omer (Merck), respectively. High purity metals (99.999%) are from Aldrich ($ZnCl_2$ and $CoCl_2$) or Alfa ($MgCl_2$, $Cd(NO_3)_2$, and $MnCl_2$). The fusion protein GST-CDC42(F) was prepared as described (Aepfelbacher et al., 1994). The concentrations of RAS1-CAIL and GST-CDC42(F) were estimated from the OD280 using extinction coefficients calculated from their amino acid sequences (Perkins, 1986).

The compound 1-P-GGOH was synthesized from geranylgeranal in a manner reported for the analogous farnesol analogue (Pompliano et al., 1992). The phosphonic acid dimethyl ester intermediate was purified by silica gel flash chromatography (Still et al., 1978) using ethyl acetate as solvent: ^{1}H-NMR ($CDCl_3$, 200 MHz) δ 1.50−1.75 (methyls, 15H), 1.85−2.20 (allylic methylenes, 12H), 3.78 (PO methyls, 6H), 4.68 (CHOH, 1H), 5.07 and 5.30 (vinyls, 4H). The phosphonic acid 1-P-GGOH was obtained by treating the dimethyl ester with trimethylsilyl bromide as described (Pompliano et al., 1992) except that a second treatment with trimethylsilyl bromide was needed to completely demethylate the starting material. Crude 1-P-GGOH was purified by silica gel column chromatography using 50% methanol in chloroform to give the desired product as an oil: ^{1}H-NMR ($CD_3SO_2CD_3$, 200 MHz) δ 1.50−1.75 (methyls, 15H), 1.85−2.15 (allylic methylenes, 12H), 4.26 (CHOH, 1H), 5.00 and 5.26 (vinyls, 4H).

DATFP-FPP was prepared essentially as described for DATFP-GPP (Allen & Baba, 1985; Baba et al., 1985). Briefly, *all-trans*-farnesol (1 g, Aldrich) was treated with chloroacetic anhydride to afford the ester, which was purified by suction filtration through a pad of silica gel with low boiling petroleum ether:ethyl acetate 100:1 and then 50:1 (yield 0.94 g). The ester (0.5 g) was oxygenated (Baba et al., 1985; Mann et al., 1977) to furnish the C12-oxo (0.15 g, silica TLC R_f 0.5, 10% ethyl acetate in petroleum ether) and C12-hydroxy (20 mg, TLC R_f 0.15, 10% ethyl acetate in petroleum ether) compounds after flash chromatography on silica gel with petroleum ether:ethyl acetate 20:1 and then 5:1. Aldehyde: NMR (200 MHz, $CDCl_3$) δ 1.58 (s, 3H), 1.70 (s, 3H), 1.73 (s, 3H), 1.95−2.25 (m, 6H), 2.42 (q, 2H), 4.03 (s, 2H), 4.63 (d, 2H), 5.08 (t, 1H), 5.30 (t, 1H), 6.43 (td, 1H), 9.34 (s, 1H). Alcohol: NMR (200 MHz, $CDCl_3$) δ 1.58 (s, 3H), 1.62 (s 3H), 1.65 (s, 3H), 1.90−2.20 (m, 8H), 3.95 (s, 2H), 4.03 (s, 2H), 4.63 (d, 2H), 5.05 (t, 1H), 5.32 (m, 2H). The alcohol (9.5 mg) was acylated with 2-diazo-3,3,3-trifluoropropionyl chloride, and the product (TLC R_f 0.85, 15% ethyl acetate in petroleum ether) was purified by flash chromatography on silica with petroleum ether:benzene 5:1 and then 2:1 to afford 7 mg of the ester. The chloroacetyl group was removed by treating with ice-cold 0.1 N NH_4OH in 90% methanol for 1 h on ice (prolonged treatment leads to loss of the 2-diazo-3-trifluoropropionyl group). The solvent was removed with a stream of argon to afford 4.5 mg of the alcohol (TLC R_f 0.3, 20% ethyl acetate in petroleum ether), which was purified by flash chromatography on silica gel with petroleum ether then 3% ethyl acetate in petroleum ether and then 10% ethyl acetate in petroleum ether: NMR (200 MHz, $CDCl_3$) δ 1.53 (s, 3H), 1.63 (s, 3H), 1.67 (s, 3H), 1.95−2.25 (m, 8H), 4.13 (t, 2H), 4.62 (s, 2H), 5.10 (t, 1H), 5.43 (m, 2H). The alcohol was converted to DATFP-FPP by Cramer phosphorylation (Popjak et al., 1962). The product was purified on a column of DE52 cellulose (37 g, Whatman, packed with 2.5 mM NH_4HCO_3 and then equilibrated with 5 mM NH_4HCO_3, pH 7.6). After loading the sample, which contained 5 μCi of [^{3}H]-GGPP as a tracer, the column was washed with 100 mL of 5 mM NH_4HCO_3, pH 7.6, and then eluted with a gradient (total volume 800 mL) of 5 mM NH_4HCO_3 (pH 7.6) to 120 mM NH_4HCO_3 (pH 8.5). Finally the column was washed with 100 mL of 120 mM NH_4HCO_3 (pH 8.5). Fractions containing radioactivity were pooled, and the solution was lyophilized: TLC, silica, R_f same as FPP, 1-propanol:concentrated NH_4OH:water (6:3:1). ^{31}P-NMR showed the expected pattern for a pyrophosphate monoester (2 peaks 2.5 ppm apart each split by 22 Hz).

[^{3}H]-DATFP-FPP and [^{3}H]-DATFP-GPP were prepared by treating 5.5 mg of chloroacetylated 12-oxofarnesol and 5.5 mg of chloroacetylated 8-oxogeraniol, respectively, in 5 mL of ice-cold absolute ethanol with 50 mCi of [^{3}H]$NaBH_4$ (dissolved in 0.5 mL of ice-cold absolute ethanol, 13.6 Ci/mmol, NEN). After 30 min with cooling on ice, 1 mL of 10% citric acid was added, and the reactions were incubated

on ice for an additional 20 min. The samples were concentrated to about 200 μL with a stream of argon, and 4 mL of ether was added. After addition of anhydrous $MgSO_4$, the samples were filtered, and the solvent was removed with a stream of argon. The samples were taken up in a small volume of ether and applied to TLC plates (silica gel 60, 20 × 20 cm, 250 μ, EM Science). Nonradiolabeled standards were applied at the edge of the plate. The plates were developed with ether:hexane (1:1), and the product-containing regions (viewed by iodine staining of the lanes containing nonlabeled standards, R_f values 0.29, 0.36) were scraped, and the products were eluted with ether. After acylation with 2-diazo-3,3,3-trifluoropropionyl chloride, the chloroacetyl group was removed with ammonia, and the products were purified on TLC plates with ethyl acetate:petroleum ether (1:4, R_f values 0.17, 0.25). The product bands were visualized by audoradiography. The products were eluted from the silica with ether and were submitted to Cramer phosphorylation. [^{3}H]-DATFP-FPP and [^{3}H]-DATFP-GPP were purified on columns (1 mL syringe barrels) of DE52 (1 mL of a 50% suspension in 5 mM NH_4HCO_3; suspension adjusted to pH 7.9). The samples were loaded in 5 mM NH_4HCO_3 (pH 7.8, not adjusted) and the columns were washed with 1.25 mL of 5 mM NH_4HCO_3. The columns were successively washed with 1 mL portions of 5, 10, 20, 30, and 40 mM NH_4HCO_3 and then successively with 2 × 1 mL portions of 50, 60, 70, 80, 90, and 100 mM NH_4HCO_3, and finally with 3 mL of 200 mM NH_4HCO_3. Fractions judged to have product by TLC (developed with 1-propanol: concentrated NH_4OH:water (6:3:1); [^{3}H]-FPP and [^{3}H]-GGPP as reference standards, visualized by fluorography using EN^{3}HANCE, NEN) were pooled, and the samples were lyophilized. Stock solutions of [^{3}H]-DATFP-FPP and [^{3}H]-DATFP-GPP were prepared in 5 mM NH_4HCO_3 and stored at −80 °C.

Bovine brain PGGT-I was purified as described previously (Yokoyama & Gelb, 1993) but with modifications. The purification was scaled up 2-fold by starting with 2 kg of bovine brain. After chromatography on Q-sepharose, the material was submitted to gel filtration on a column of AcA-34 (3 × 95 cm, Spectrum). The crude protein from the gel filtration column was loaded onto a Q-sepharose HP column (2.6 × 10 cm, Pharmacia), and PGGT-I was eluted as described previously for chromatography on Mono-Q, but scaled proportionally (Yokoyama & Gelb, 1993). At this stage, the PGGT-I was prepared for and submitted to chromatography on octyl-Sepharose CL-4B (2.6 × 23 cm, Pharmacia) as described previously. The PGGT-I fractions were pooled and dialyzed against 30 mM potassium phosphate, 1 mM DTT, 0.1% n-octyl β-glucoside, pH 7.7 (buffer B). The sample was concentrated to about 50 mL by ultrafiltration (PM-30 membrane, Amicon) and then applied to a column of 10 mL of SSCILL-sepharose CL-4B (Yokoyama & Gelb, 1993). The column was washed with buffer B (22 × 20 mL) by gravity flow and then washed with buffer B containing 0.1 M NaCl (18 × 20 mL). The PGGT-I was eluted using the buffer and protocol described previously (Yokoyama & Gelb, 1993). The PGGT-I-containing fractions were analyzed by SDS-PAGE (11% gel), and the fractions eluted at pH 6.5−6.0 were pooled as they contained apparently homogeneous enzyme and displayed the highest specific activity. The dilute enzyme was concentrated and submitted to one cycle of buffer exchange with 20 mM Tris-HCl, 1 mM DTT, 0.1% n-octyl β-glucoside, pH 7.7 by ultrafiltration (Centricon 30, Amicon). The PGGT-I (approximately 90 μg, estimated by SDS-PAGE analysis against standard amounts of bovine serum albumin) in 0.7 mL of buffer was stored in small aliquots at −80 °C. The specific activity is 72 microunits/μg where 1 microunit is the amount of enzyme that produces 1 pmol of product/min using biotin-γ_6(L) as substrate and the avidin-agarose assay (see below). The lower specific activity previously reported of 5.9 microunits/μg (Yokoyama & Gelb, 1993) was obtained in the presence of high peptide concentration such that significant substrate inhibition occurs (discussed below), and the assay was carried out for a period of time that extended beyond the region of linear progress.

Methods. PGGT-I assays with biotinylated peptides were carried out using avidin-agarose as described previously (Yokoyama et al., 1991) except with the following modified washing procedure: The avidin-gel was washed twice with 50% ethanol, three times with 1% Triton X-100 in 50 mM potassium phosphate, pH 7.0, and once with 1 M NaCl in the same buffer. Assays with the protein substrate GST-CDC42(F) were carried out using the filter paper binding method (Pompliano et al., 1992). All reactions were carried out at 30 °C in polypropylene tubes with 20 μL of buffer A (30 mM potassium phosphate, 5 mM DTT, 0.5 mM $MgCl_2$, 20 μM $ZnCl_2$, pH 7.7) unless noted otherwise. Other conditions are given in the figure and table legends. The kinetic and equilibrium constants listed in Tables 1 and 2 and the dissociation equilibrium constant for the PGGT-I·3H-GGPP complex were obtained by fitting the data to the appropriate equation using nonlinear regression (fortran programs provided by Professor Paul Cook, Texas College of Osteopathic Medicine). In all cases, initial studies of the reaction time course were carried out to verify that the subsequent single time-point assays were obtained during the initial velocity period (linear progress curves, typically 5 min for peptides and 15 min for protein substrates).

All plasticware used in the preparation and handling of metal-free PGGT-I was soaked overnight in 10 mM EDTA, pH 7, rinsed with metal-free water (Milli-Q, Millipore), and oven dried. Metal-free PGGT-I was prepared by dialyzing octyl-Sepharose-purified enzyme for 2 days at 4 °C against 20 mM Tris-HCl, 1 mM DTT, 6 mM EDTA, 2 mM 1,10-phenanthroline (Aldrich), 0.01% n-octyl β-glucoside, pH 7.7 and then for 1 day at 4 °C against the same buffer except with 0.5 mM EDTA and 0.2 mM 1,10-phenanthroline. The apoenzyme was stored in small aliquots at −80 °C. Studies with metal-free PGGT-I were carried out in buffer C (30 mM HEPES-NaOH, 5 mM DTT, pH 7.7). Solutions of buffer and peptide substrates were rendered metal-free by extraction with dithizone (Lin et al., 1988). Stock solutions of metals were made by dissolving the solids in Milli-Q water. Stock solutions of [^{3}H]-GGPP and metal-free PGGT-I were not extracted with dithizone. Additional conditions are given in the figure and table legends.

The binary complex PGGT-I·[^{3}H]-GGPP was prepared and isolated by rapid gel filtration as described previously using octyl-Sepharose-purified enzyme (Yokoyama & Gelb, 1993). The metal-free binary complex was prepared as follows. Metal-free PGGT-I (octyl-Sepharose purified, 36 nanounits, 0.76 μg of protein) and 0.3 μCi (0.78 μM) [^{3}H]-GGPP were incubated in a final volume of 20 μL containing dithizone-treated buffer C containing 50 μM EDTA, 20 μM 1,10-

Table 1: Steady-State Kinetic Constants for the Utilization of Prenyl Pyrophosphates and Peptide Substrates by PGGT-I[a]

prenyl donor	prenyl acceptor	$^{PD}K_M$ (μM)[c]	$^{PA}K_M$ (μM)[c]	k_{cat} (s^{-1})[d]	$k_{cat}/^{PD}K_m$ (M^{-1} s^{-1})	$k_{cat}/^{PA}K_M$ (M^{-1} s^{-1})
GGPP	biotin-CDC42(F) (ALEPPETEPKRKCCIF)	0.03 ± 0.01 (0.05 ± 0.02)[b]	31 ± 2	0.075 ± 0.02	1.8×10^6	2.4×10^3
GGPP	GST-CDC42(F)	0.02 ± 0.006	16 ± 3	0.0057	2.9×10^5	0.36×10^3
GGPP	Ras1-CAIL	1.6 ± 0.3	2.0 ± 0.3	0.12 ± 0.03	7.5 ± 10^4	6.0×10^4
GGPP	biotin-ras1(L) (SSCAIL)	not determined	0.9 ± 0.2	0.09 ± 0.04	not determined	1.0×10^5
GGPP	biotin-γ6(L) (NPFREKKFFCAIL)	0.67 ± 0.15 (0.55 ± 0.13)	0.27 ± 0.07	0.12 ± 0.03	1.8×10^5	4.5×10^5
GGPP	biotin-γ6(S) (NPFREKKFFCAIS)	0.2 ± 0.02	3.2 ± 0.9	0.012 ± 0.0007	6.0×10^4	3.7×10^3
GGPP	biotin−lamin B(S) (GTPRASNRSCAIS)	1.4 ± 0.1 (1.1 ± 0.2)	3.0 ± 0.4	0.005 ± 0.0003	3.6×10^3	1.7×10^3
GGPP	biotin−lamin B(L) (GTPRASNRSCAIL)	0.23 ± 0.06	0.63 ± 0.2	0.31 ± 0.05	1.3×10^6	4.9×10^5
FPP	biotin-γ6(L)	0.66 ± 0.15 (0.89 ± 0.18)	7.8 ± 1.2 (4.8 ± 0.7)	0.10 ± 0.02	1.5×10^5	1.3×10^4
FPP	biotin−lamin B(S)	no detectable activity[e]				
GPP	biotin-γ6(L)	2.6 ± 0.8	25 ± 4	0.028 ± 0.003	1.1×10^4	1.2×10^3

[a] Assays were conducted with affinity column-purified PGGT-I. [b] Values in parentheses were obtained with octyl-sepharose-purified PGGT-I. [c] Obtained in the presence of saturating amounts of the other substrate. [d] Obtained in the presence of saturating amounts of both substrates. [e] Very low activity (<0.5% of the activity with 10 μM biotin-γ6(L) and 0.5 μM [^{3}H]-FPP) was measured with 10 μM peptide and 0.5 μM [^{3}H]-FPP, and thus the kinetic constants were not determined.

Table 2: Inhibition Patterns and K_I Values for Substrate Analogue Inhibitors of PGGT-I[a]

	with respect to GGPP		with respect to GST-CDC42	
inhibitor	inhibition type	K_I (μM)[b]	inhibition type	K_I (μM)[b]
1-P-GGOH	competitive	0.72 ± 0.15	noncompetitive	0.86 ± 0.12
PD-083176	noncompetitive	8.7 ± 1.3	competitive	8.2 ± 1.7

[a] Assays were conducted with octyl sepharose-purified PGGT-I and various concentrations of [^{3}H]-GGPP and GST-CDC42 as described in Figure 2. [b] Values of K_I were obtained by fitting the data to the appropriate inhibition equation using nonlinear regression.

phenanthroline, and 0.001% n-octyl β-glucoside. After incubation at 30 °C for 20 min, the solution was mixed with 30 μL of the dithizone-treated buffer C and subjected to rapid gel-filtration in a 0.5 mL spin-column of Sephadex G-50 as described previously (Yokoyama & Gelb, 1993).

The equilibrium dissociation constant for the PGGT-I·[^{3}H]-GGPP complex was measured by incubating enzyme with different concentrations of [^{3}H]-GGPP and subjecting the mixture to rapid gel filtration as described above. The amount of radioactivity in the void volume gives the amount of complex. [^{3}H]-GGPP does not dissociate from the enzyme during the gel filtration step (Yokoyama & Gelb, 1993), and thus the amount of complex eluting in the void volume is the same amount that is in equilibrium with free PGGT-I and [^{3}H]-GGPP in the mixture prior to gel filtration.

Isotopte trapping experiments (Rose, 1980) were carried out by adding PGGT-I·[^{3}H]-GGPP (600 cpm, prepared as described above) to buffer A with or without 10 μM unlabeled GGPP and containing various concentrations of RAS1-CAIL. After 15 s, the reactions were quenched and analyzed with the glass filter binding assay (Pompliano et al., 1992). The rate constant for the dissociation of [^{3}H]-GGPP from the PGGT-I·[^{3}H]GGPP binary complex ($^{GGPP}k_d$) is given by the equation $^{GGPP}k_d = (K_{1/2}/^{RAS}K_M)k_{cat}$, where $K_{1/2}$ is the concentration of RAS1-CAIL that leads to half maximal trapped [^{3}H]-GGPP (Rose, 1980); the latter was obtained from the fit of the data (cpm radiolabeled geranylgeranylated RAS1-CAIL versus [RAS1-CAIL]) to the equation of a hyperbola. $^{RAS}K_M$ is the apparent Michaelis constant for RAS1-CAIL obtained in the presence of saturating GGPP, and k_{cat} is the rate of the reaction per enzyme in the presence of saturating RAS1-CAIL and GGPP.

Photoaffinity labeling of PGGT-1 with [^{3}H]-DATFP-FPP and [^{3}H]-DATFP-GPP was carried out essentially as described for protein farnesyl transferase (Omer et al., 1993). A solution of PGGT-I (0.8 μg) in 20 μL of 50 mM HEPES, 1 mM DTT, 5 mM $MgCl_2$, 10 μM $ZnCl_2$, pH 7.5, with 10 μM [^{3}H]-DATFP-GPP or [^{3}H]-DATFP-FPP, was placed in a porcelain dish. The solution also contained GGPP (10 μM, 1 μL of a 200 μM stock solution in 14% ethanol, 20 mM NH_4HCO_3) or 1 μL of vehicle. The dish was placed on ice, and the sample was irradiated for 10 min with a germicidal lamp (8 W, General Electric G8T5) held at a distance of 2−3 cm. The sample was mixed with 5× Laemmli sample buffer and submitted to SDS-PAGE on an 11% gel. The gel was then submitted to fluorography. The gel was fixed in 50% methanol for 15 min and then in glacial acetic acid for 15 min. The gel was then soaked for 20 min in 15% 2,5-diphenyloxazole (Aldrich)/glacial acetic acid, rinsed with water, and then soaked for 5 min in a second portion of water to precipitate the fluor in the gel. The gel was dried for 95 min at 60 °C under vacuum, placed on X-ray film, and kept at −80 °C for 7 days.

RESULTS

Substrate Specificity Studies. Peptide and protein substrates were used for substrate specificity studies. Previous substrate specificity studies using partially purified PGGT-I led to the conclusion that the C-terminal residue of the prenyl acceptor is a major determinant of the type of prenyl group that is transferred (Casey et al., 1991; Seabra et al., 1991; Yokoyama et al., 1991). Thus, biotin-γ_6(L), which contains a C-terminal leucine, is preferentially geranylgeranylated *in vitro*, but the analogous peptide containing a C-terminal serine is farnesylated. Likewise, the farnesyl acceptor biotin-lamin B(S) is mainly geranylgeranylated when the C-terminal serine is changed to leucine. When high concentrations of

peptides (50 μM) are used in cell-free reactions, this specificity is lost; both biotin-γ_6(L) and biotin-lamin B(S) are acceptors for both farnesyl and geranylgeranyl groups (Yokoyama et al., 1991).

A detailed kinetic analysis of the substrate specificity of affinity-purified homogeneous PGGT-I was undertaken in part to understand the origin of the previously reported results. The steady-state kinetic parameters for a number of PGGT-I substrates were determined under the standard assay condition (Methods) and by fitting the initial velocity versus substrate concentration data to the Michaelis−Menten equation using nonlinear regression analysis. The measurement of the K_M for one substrate was carried out in the presence of saturating amounts of the other substrate. The results are summarized in Table 1. Because substrate inhibition occurs in the presence of high concentrations of prenyl acceptor (described below), the apparent K_M values of the prenyl acceptors ($^{PA}K_M$) were obtained by using the hyperbolic portion of the velocity versus substrate concentration curve. For all studies, affinity-purified PGGT-I was used; however, essentially identical results were obtained with enzyme that had been purified through the octyl-Sepharose step (Table 1). The latter was used for studies of the steady-state mechanism and metal requirements (described below).

The results show that values of the apparent K_M for the interaction of prenyl donor [^{3}H]-GGPP with PGGT-I·prenyl acceptor complexes ($^{PD}K_M$) vary by up to 50-fold depending on the structure of the prenyl acceptor bound to the enzyme. When the C-terminal leucine of biotin-γ_6(L) is replaced with serine, the $^{PD}K_M$ for [^{3}H]-GGPP changes only modestly. The same is seen when the C-terminal serine of biotin-lamin B(S) is changed to leucine. The biggest effect on the $^{PD}K_M$ of [^{3}H]-GGPP is seen with the enzyme bound to biotin-CDC42-(F) or GST-CDC42(F), which contain a C-terminal phenylalanine. Farnesylation of biotin-lamin(B) by PGGT-I is barely detectable but biotin-γ_6(L) can serve as a respectable farnesyl or geranyl acceptor. In the presence of saturating amounts of biotin-γ_6(L), values of $^{PD}K_M$ for [^{3}H]-GGPP and [^{3}H]-FPP are similar but the $^{PD}K_M$ for [^{3}H]-GPP is 4-fold higher. [^{3}H]-Dimethylallyl pyrophosphate (0.8 μM) was not detectably transferred to biotin-γ_6(L) by PGGT-I.

Values of $^{PA}K_M$ for the interaction of the prenyl acceptors with PGGT-I in the presence of saturating amounts of prenyl donor depend on the structure of the prenyl acceptor and of the prenyl donor. Values of $^{PA}K_M$ for prenyl acceptors ending in leucine are 20−70-fold smaller than for those ending in phenylalanine and are 5 to 10-fold smaller than those that end in serine. The $^{PA}K_M$ for biotin-γ_6(L) increases 30-fold and 100-fold in the presence of saturating amounts of [^{3}H]-FPP and [^{3}H]-GPP, respectively, versus [^{3}H]-GGPP.

Values of k_{cat} for geranylgeranylation, farnesylation, and geranylation of peptides were also determined by measuring the rate of reaction in the presence of saturating amounts of both prenyl acceptor and prenyl donor (Table 1). When the C-terminal leucine of biotin-γ_6(L) is changed to serine, k_{cat} for geranylgeranylation decreases 10-fold, and k_{cat} for biotin-lamin B(S) is 60-fold lower than that of biotin-lamin B(L). Values of k_{cat} for geranylgeranylation measured in the presence of biotin-CDC42(F) or GST-CDC42(F) are 8-fold and 20-fold, respectively, below that of biotin-γ_6(L). Interestingly, k_{cat} for the farnesylation of biotin-γ_6(L) is essentially the same as that for the geranylgeranylation of the same peptide, whereas k_{cat} is 4-fold smaller in the presence of ^{3}H-GPP. As will be discussed later, the substrate specificity of these prenylation reactions is governed by the ratio $k_{cat}/^{PA}K_M$ and not by the individual steady-state constants. Finally the kinetic parameters for biotin-γ_6(L), RAS1-CAIL, and biotin-RAS1(L) are very similar.

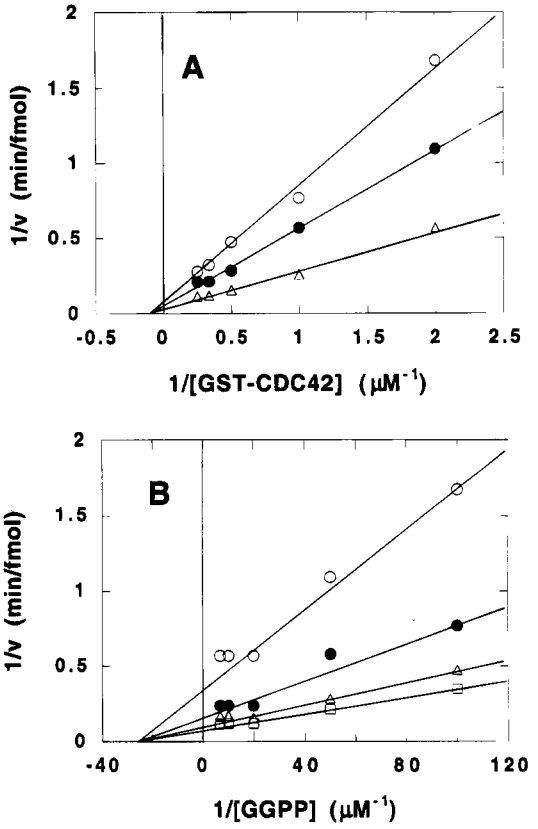

FIGURE 1: Double reciprocal plots of initial velocity versus [^{3}H]-GGPP or GST-CDC42(F) concentration at various fixed concentrations of the other substrate. (A) PGGT-I (20 nanounits, purified through the octyl-Sepharose step) was incubated with 10 nM (○), 20 nM (●), or 50 nM (△) [^{3}H]-GGPP and the indicated concentrations of GST-CDC42(F) under the standard conditions. (B) The assay was carried out with 0.5 μM (○), 1 μM (●), 2 μM (△) or 3 μM (□) GST-CDC42(F) and the indicated concentrations of [^{3}H]-GGPP. The kinetic constants are given in Table 1.

The PGGT-I-catalyzed radio-geranylgeranylation of 5 μM biotin-γ_6(L) in the presence of 1 μM [^{3}H]-GGPP was inhibited only by 25% when a 10-fold excess (10 μM) of nonlabeled FPP was added. In contrast, the radio-farnesylation of 20 μM biotin-γ_6(L) in the presence of 1 μM [^{3}H]-FPP was completely inhibited by addition of the same amount (1 μM) of nonlabeled GGPP.

Steady-State Mechanism. The glass filter binding assay developed for PFT (Pompliano et al., 1992) was found to be a reliable and rapid assay of PGGT-I and was used for studies to unravel the steady-state mechanism of PGGT-I using the protein substrate GST-CDC42(F). CDC42 is a member of the low MW GTP-binding proteins (Munemitsu et al., 1990) and is produced as a GST-fusion protein in *E. coli* and readily purified by affinity chromatography (Aepfelbacher et al., 1994). Double reciprocal plots of the initial reaction velocity versus the concentration of GST-CDC42 (F) in the presence of various fixed amounts of the other substrate [^{3}H]-GGPP for the reaction catalyzed by bovine brain PGGT-I are shown in Figure 1A. The intersecting line pattern establishes that the reaction is sequential with both

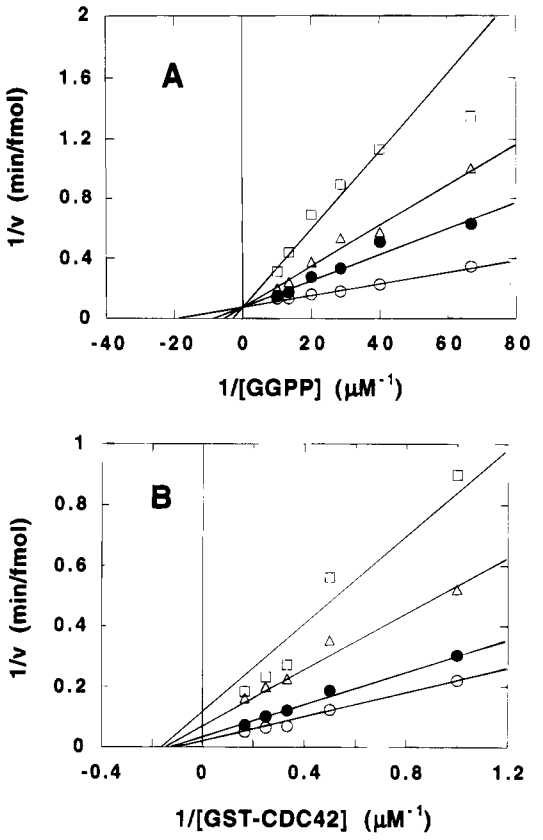

FIGURE 2: Inhibition of PGGT-I by 1-P-GGOH. (A) Double reciprocal plot of initial velocity versus [^{3}H]-GGPP concentration at 0 μM (○), 2 μM (●), 5 μM (△), or 10 μM (□) 1-P-GGOH. Each assay was performed with 20 nanounits PGGT-I (purified through the octyl-Sepharose step) and 3 μM GST-CDC42(F) under the standard conditions. (B) Double reciprocal plot of initial velocity versus GST-CDC42(F) concentration at 0 μM (○), 2 μM (●), 5 μM (△), or 10 μM (■) 1-P-GGOH. Each assay mixture contained 20 nanounits PGGT-I and 78 nM [^{3}H]-GGPP. The inhibition constants are given in Table 2.

substrates adding to the enzyme to form a ternary complex prior to prenyl transfer. An intersecting line pattern is also seen in the double reciprocal plots of the initial reaction velocity versus the concentration of [^{3}H]-GGPP in the presence of various fixed amounts of GST-CDC42(F) (Figure 1B). Such results rule out a nonsequential mechanism in which the geranylgeranyl moiety is first transferred to the enzyme followed by a second half-reaction in which the geranylgeranyl moiety is transferred to the protein acceptor.

The studies summarized in Figure 1 cannot be used to distinguish whether the substrates add to the enzyme in a random versus an ordered manner. Studies employing dead-end reversible inhibitors of the PGGT-I can be used to make this distinction. A GGPP analogue, 1-P-GGOH, competes with [^{3}H]-GGPP for the binding to PGGT-I since double reciprocal plots of the reaction velocity versus the concentration of [^{3}H]-GGPP in the presence of various fixed concentrations of 1-P-GGOH and a fixed subsaturating concentration of GST-CDC42(F) intersect on the *Y*-axis (Figure 2A). Surprisingly, 1-P-GGOH is a poor inhibitor of PGGT-I (K_I = 1 μM) compared to the analogous FPP analogue as a PFT inhibitor (K_I = 50 nM) (Pompliano et al., 1992). A noncompetitive inhibition pattern (intersecting lines near the *X*-axis) is seen in double reciprocal plots of the reaction velocity versus the concentration of GST-CDC42(F) in the presence of various fixed concentrations of 1-P-GGOH and a fixed subsaturating concentration of [^{3}H]-GGPP (Figure 2B).

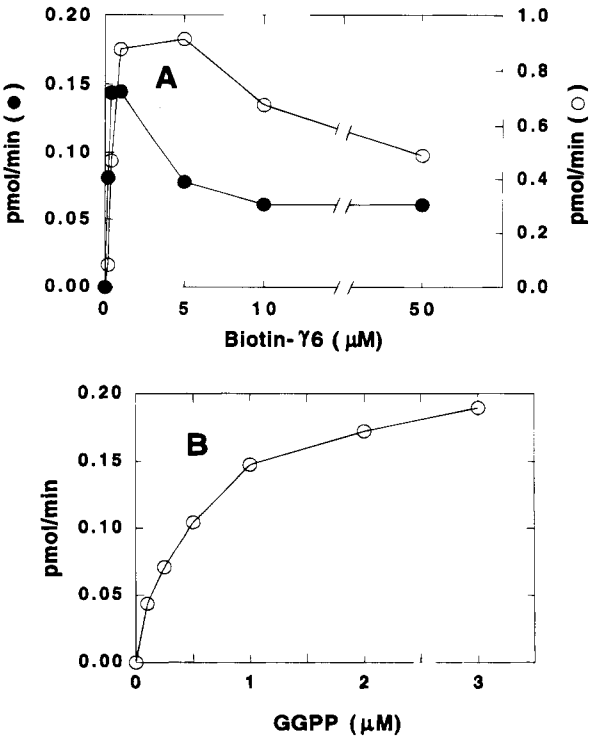

FIGURE 3: Substrate inhibition. (A) PGGT-I (120 nanounits, purified through the octyl-Sepharose step) was assayed in the presence of 0.05 μM (●) or 1.5 μM (○) [^{3}H]-GGPP and the indicated concentrations of biotin-γ_6(L). (B) Assays were carried out in the presence of 0.3 μM of biotin-γ_6(L) and the indicated concentrations of [^{3}H]-GGPP.

The pentapeptide PD-83176 is a potent inhibitor of protein farnesyltransferase (Leonard et al., 1994; Sebolt et al., 1994). PD-83176 was found to be a modest PGGT-1 inhibitor (Table 1). From a set of double reciprocal plots analogous to those shown in Figure 2A,B (not shown) the peptide PD-83176 was found to be a competitive inhibitor with respect to GST-CDC42(F) and a noncompetitive inhibitor with respect to [^{3}H]-GGPP. The inhibition patterns and dissociation constants are listed in Table 2. This data is consistent only with a random sequential mechanism (Segel, 1975); either substrate can bind first to the enzyme followed by the other substrate to form the ternary complex. Pyrophosphate is a weak inhibitor of PGGT-I and is competitive with respect to [^{3}H]-GGPP (50% inhibition at approximately 0.5 mM in the presence of 1 μM biotin-γ_6(L) and 1 μM [^{3}H]-GGPP). Under the same conditions, the other product, geranylgeranylated biotin-γ_6(L), produces 50% inhibition at a concentration of 3 μM and is competitive with respect to bitoin-γ_6(L).

Substrate Inhibition. As shown in Figure 3A, substrate inhibition of PGGT-I is seen at high concentrations of biotin-γ_6(L), and the onset of this inhibition occurs at lower peptide concentration when the [^{3}H]-GGPP concentration is lowered. As the concentration of biotin-γ_6(L) is increased, the velocity does not fall to zero but rather reaches a limiting value. Substrate inhibition is not seen with high concentrations of [^{3}H]-GGPP in the presence of a fixed subsaturating concentration of biotin-γ_6(L) (Figure 3B).

Interaction of PGGT-1 with GGPP. The equilibrium dissociation constant for the PGGT-I·[^{3}H]-GGPP complex of 10 ± 0.8 nM was obtained as described in Methods.

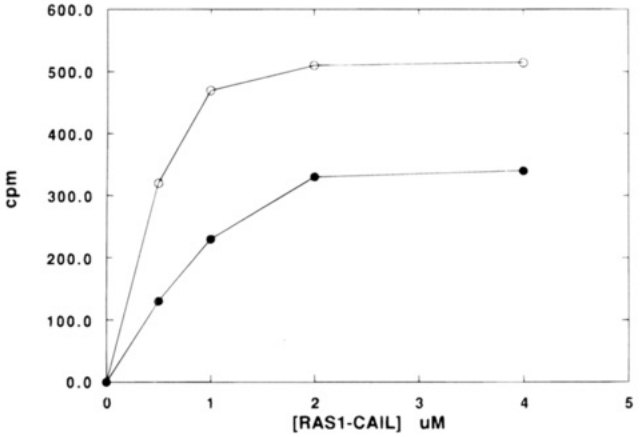

FIGURE 4: [^{3}H]-GGPP trapping. The binary complex PGGT-I•[^{3}H]-GGPP was mixed with the indicated concentrations of RAS1-CAIL either in the absence (○) or presence (●) of 10 μM unlabeled GGPP. The cpm of radioprenylated protein after a 15 s incubation is given along the abscissa.

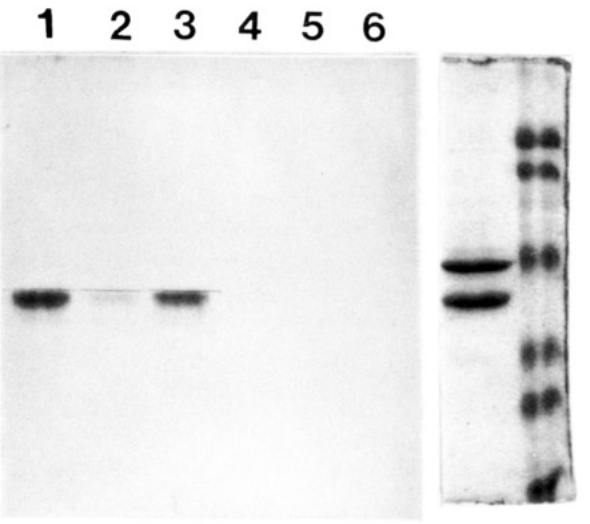

FIGURE 5: Photoaffinity labeling of PGGT-I β-subunit by [^{3}H]-DATFP-FPP and [^{3}H]-DATFP-GPP. Lanes 1−6 are a fluorograph of the SDS-PAGE gel. Lane 1, PPGT-I + 10 μM [^{3}H]-DATFP-FPP; lane 2, PGGT-I + 10 μM [^{3}H]-DATFP-FPP + 10 μM GGPP; lane 3, PGGT-I + 10 μM [^{3}H]-DATFP-GPP; lane 4, PGGT-I + 10 μM [^{3}H]-DATFP-GPP + 10 μM GGPP; lane 5, PGGT-I + 10 μM [^{3}H]-DATFP-FPP not irridiated; lane 6, PGGT-I + 10 μM [^{3}H]-DATFP-GPP not irradiated. After fluorography, the gel was rehydrated, and the proteins were visualized by Coomassie staining. Shown on right is the Coomassie stain of lane 6 (upper band is PGGT-I α-subunit, lower band is PGGT-I β-subunit) and the M_r markers (BioRad prestained, 106, 80, 49.5, 32.5, 27.5, and 18.5 kDa).

Isotope trapping experiments were carried out on the PGGT-I•[^{3}H]-GGPP complex to probe whether the random sequential mechanism is operating at rapid equilibrium or at steady-state. As shown in Figure 4, a maximum of 77% of the [^{3}H]-GGPP bound in the binary complex PGGT-I•[^{3}H]-GGPP is transfered to RAS1-CAIL without dissociating from the enzyme. A $K_{1/2}$ of 0.31 μM is obtained from the data, and a value for the rate constant for dissociation of ^{3}H-GGPP from PGGT-I•[^{3}H]-GGPP, $^{GGPP}k_d$, of 0.015 s^{-1}, is obtained using the equation given in Methods.

An attempt was made to detect the binary complex PGGT-I•biotin-γ_6(L) by incubating the enzyme with 20 μM peptide and subjecting the mixture to rapid gel filtration. Very little, if any, product (<10% of the theoretical maximum) was detected when the void volume solution was incubated with 0.8 μM [^{3}H]-GGPP for 20 min; 8000 cpm of product would have been formed if 1 equiv of peptide came through the column with enzyme.

Since [^{3}H]-GGPP interacts tightly with PGGT-I, it should be possible to specifically label the PGGT-I with a photoaffinity analogue of this substrate. Allen and co-workers have described a photoaffinity analogue of FPP that contains a 2-diazo-3,3,3-trifluoropropionyloxy group at the end opposite the pyrophosphate (DATFP-GPP) (Allen & Baba, 1985; Baba et al., 1985). The analogous [^{3}H]-GGPP analogoue, [^{3}H]-DATFP-FPP, was prepared as described in Materials. As shown in Figure 5, irradiating a mixture of PGGT-I with [^{3}H]-DATFP or [^{3}H]-DATFP-FPP results in exclusive labeling of the β-subunit. The labeling is completely suppressed if excess GGPP is present as a protectant. [^{3}H]-DATFP-GPP has recently been shown to label the β-subunit of protein farnesyltransferases (Omer et al., 1993).

Metal Ion Requirements of PGGT-I. Casey and co-workers have reported that removal of bound metals from PGGT-I results in loss of catalytic activity. Activity is restored when both Zn^{2+} (10−20 μM) and Mg^{2+} (1 mM) are added to the apoenzyme (Moomaw & Casey, 1992). Similar results have been reported for PFT (Chen et al., 1993; Reiss et al., 1992a). In the present study other metals were tested for their ability to restore the activity of metal-free PGGT-I, and the role of metals in supporting the binding of [^{3}H]-GGPP to the enzyme was investigated. Metals bound to PGGT-I were removed by extensive dialysis against metal chelators as described in Methods. In handling the apoenzyme, care was taken to avoid contamination by metals. Thus, all buffers, reagent solutions, and plasticware were rendered metal-free by extraction with dithiazone as described in Methods. As shown in Figure 6A no PGGT-I activity was detected in the absence of metal. About 20% of maximal activity was detected in the presence of 0.5 mM Mg^{2+} and in the absence of Zn^{2+}. Addition of 2 μM Zn^{2+} or Cd^{2+} but not Co^{2+} fully restored the enzyme activity.

As shown in Figure 6B, in the presence of 10 μM Zn^{2+} alone, no activity was detected; full activity was restored when either 0.1 mM Mg^{2+}, Mn^{2+}, or Cd^{2+} was additionally present, and no activity was detected in the copresence of 0.1−0.5 mM Co^{2+}. Studies with only one metal present are summarized in Figure 6C. In the presence of either 0.1 mM Cd^{2+} or 0.2 mM Mn^{2+} alone, 60−80% activity was restored, 20% activity was seen with 0.5 mM Mg^{2+} alone, and no activity was detected in the presence of up to 0.5 mM Co^{2+} alone.

The metal requirements for the farnesylation of biotin-γ_6-(L) by the PGGT-I were also investigated. In the presence of 0.2 mM $MgCl_2$, full activity is seen in the presence of 10 μM Zn^{2+} (data not shown). Thus, the concentration of Zn^{2+} required for maximal farnesylation of biotin-γ_6(L) by PGGT-I is about 5-fold higher than that required for geranylgeranylation of biotin-γ_6(L) by the same enzyme.

The role of metal ions in promoting the tight association of [^{3}H]-GGPP with PGGT-I was investigated. The binary complex PGGT-I•[^{3}H]-GGPP can be isolated by rapid gel-filtration, and addition of biotin-γ_6(L) to this complex leads to near complete transfer of the geranylgeranyl group to the peptide (Yokoyama & Gelb, 1993). Metal-free PGGT-I was incubated with [^{3}H]-GGPP in the absence of metals, and the binary complex was isolated by rapid gel-filtration. The

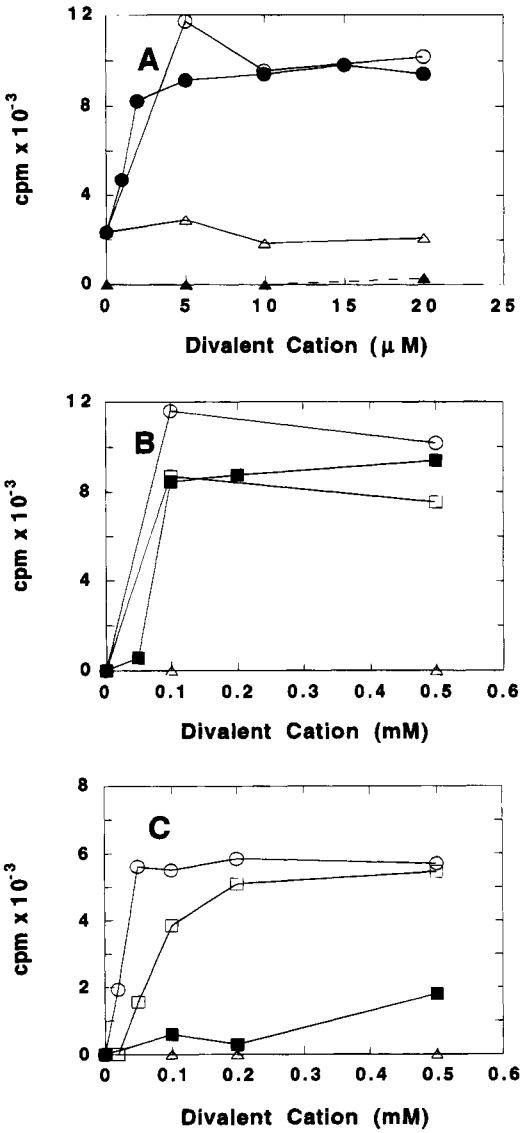

FIGURE 6: Metal ion requirements of PGGT-I. Metal-free PGGT-I (Methods) (40 nanounits, purified through the octyl-Sepharose step) was incubated at 30 °C for 15 min with 0.78 μM [^{3}H]-GGPP and 20 μM dithizone-treated biotin-γ_6(L) peptide in 20 μL of dithizone-treated buffer containing 30 mM Hepes−NaOH, 5 mM DTT, 50 μM EDTA, 20 μM 1,10-phenanthroline, and 0.001% n-octyl β-glucoside, pH 7.7, containing the following metals: (A) 0.5 mM $MgCl_2$ plus either of $ZnCl_2$ (●), $Cd(NO_3)_2$ (○), and $CoCl_2$ (△), or $ZnCl_2$ in the absence of $MgCl_2$ (▲) at the indicated concentrations. (B) 10 μM $ZnCl_2$ plus either of $MgCl_2$ (■), $Cd(NO_3)_2$ (○), $MnCl_2$ (□), or $CoCl_2$ (△) at the indicated concentrations. (C) $Cd(NO_3)_2$ (○), $MnCl_2$ (□), $MgCl_2$ (■), or $CoCl_2$ (△) at the indicated concentrations.

amount of radioactivity appearing in the void volume is an indication of the amount of binary complex formed. As shown in Table 3, the formation of binary complex in the absence of metals was the same as that formed in the presence of either Zn^{2+}, Mg^{2+}, Mn^{2+}, Cd^{2+}, Co^{2+}, or Zn^{2+} and Mg^{2+} together. In the absence of metals, addition of 20 μM biotin-γ_6(L) to the metal-free PGGT-I·[^{3}H]-GGPP complex did not result in the prenylation of the peptide, but the reaction did occur when both Zn^{2+} and Mg^{2+} were added (data not shown). The equilibrium dissociation constant for the PGGT-I·[^{3}H]-GGPP complex was measured as described in Methods, and a value of 16 nM was obtained.

Table 3: Effect of Metal Ions on PGGT-I·[^{3}H]-GGPP Complex Formation[a]

addition	formation of PGGT-I·[^{3}H]-GGPP complex (cpm)
none	7888
2 mM EDTA	6148
10 μM $ZnCl_2$	7614
0.1 mM $ZnCl_2$	7506
0.2 mM $MgCl_2$	7704
0.2 mM $Cd(NO_3)_2$	7772
0.2 mM $CoCl_2$	8178
10 μM $ZnCl_2$, 0.2 mM $MgCl_2$	7220
10 μM $ZnCl_2$, 0.2 mM $MgCl_2$ (no enzyme)	580

[a] Metal-free PGGT-I was mixed with [^{3}H]-GGPP in buffer containing the indicated metal ions. The amount of PGGT-I·[^{3}H]-GGPP complex formed is given in the table as the cpm that elute in the void volume of a gel-filtration spin-column (Methods).

DISCUSSION

The results of this study establish that the reaction catalyzed by PGGT-I, like PFT (Pompliano et al., 1992), proceeds via random sequential mechanisms and that both enzymes operate under steady-state, rather than rapid-equilibrium, conditions. The latter is apparent from the following arguments. For a rapid-equilibrium mechanism in which free enzyme and all of its complexes with substrates are in equilibrium, intersection of lines in double reciprocal plots of the type shown in Figures 1A,B on the *X*-axis implies that the equilibrium constants for the interaction of one substrate with the free enzyme and with the complex of the enzyme bound to the other substrate are the same. Under the rapid-equilibrium assumption, the K_M values in Table 1 would actually be equilibrium constants, and it is clear from the data that the affinity of the enzyme for one substrate is influenced by the presence of the other substrate. This seemingly paradoxical situation can be accommodated by a random-sequential steady-state mechanism but not by a rapid-equilibrium mechanism as pointed out by Pompliano and co-workers (Pompliano et al., 1993). In addition, the isotope trapping experiments demonstrate that the rate of the chemical transformation from the ternary complex PGGT-I·[^{3}H]-GGPP·biotin-γ_6(L) is significantly faster than the release of [^{3}H]-GGPP from this complex, and thus the rapid-equilibrium condition does not apply. No trapping of enzyme-bound peptide was observed, which demonstrates that the prenyl acceptor rapidly dissociates from the enzyme. Thus the reaction PGGT-I·[^{3}H]-GGPP + prenyl acceptor ⇌ PGGT-I·[^{3}H]-GGPP·prenyl acceptor may be operating at or close to rapid equilibrium.

Substrate inhibition for the PGGT-I-catalyzed geranylgeranylation of biotin-γ_6(L) is seen at high peptide concentration and is more pronounced at a lower [^{3}H]-GGPP concentration (Figure 3A). Similar behavior has been reported for PFT (Pompliano et al., 1993). Although it cannot be ruled out that the inhibition is due to binding of the peptide to a site on the enzyme distinct from the catalytic site, a more likely explanation is that the reaction is operating at steady-state, rather than at rapid-equilibrium, and that the pathway involving binding of [^{3}H]-GGPP to the free enzyme followed by peptide binding is the kinetically-preferred route (Pompliano et al., 1993). Thus, with high concentrations of biotin-γ_6(L), the reaction will be forced to operate via the slower pathway with peptide binding before [^{3}H]-GGPP. Such a

situation is not possible if the enzyme is operating at rapid-equilibrium. This model also predicts that the substrate inhibition will not be 100%, but rather it will approach a limiting value (Figure 3A) when every catalytic reaction proceeds through the slower pathway. The model is also consistent with the observations that substrate inhibition is more pronounced when the concentration of [^{3}H]-GGPP is lower.

The data in Table 1 were collected under conditions where the kinetics are hyperbolic (i.e. not at the very high peptide concentrations where deviations from hyperbolic kinetics are observed, Figure 3A). These data can be used to evaluate the substrate preferences of PGGT-I. Substrate specificity of enzymatic reactions is quantitatively described in terms of the ratio of steady-state constants $k_{cat}/^{PA}K_M$ according to eq 1.

$$v_{PA1}/v_{PA2} = ({}^1k_{cat}/{}^{PA1}K_M)/({}^2k_{cat}/{}^{PA2}K_M) \times [PA1]/[PA2] \quad (1)$$

Thus, if two prenyl acceptors, PA1 and PA2, are competing for geranylgeranylation by PGGT-I and if both substrates are present at equal concentration, the relative reaction velocity for the two reactions (v_{PA1}/v_{PA2}) is dictated by the relative values of $k_{cat}/^{PA}K_M$. Here, $^{PA1}K_M$ and $^{PA2}K_M$ are the Michaelis constants for prenyl acceptors 1 and 2, respectively in the presence of saturating amounts of prenyl pyrophosphate, and $^1k_{cat}$ and $^2k_{cat}$ are the turnover numbers in the presence of saturating prenyl donor and saturating prenyl acceptors 1 and 2, respectively. It is important to note that the peptide specificity of PGGT-I is independent of the concentration of [^{3}H]-GGPP and the $^{PD}K_M$ for this substrate.

On the basis of the $k_{cat}/^{PA}K_M$ data is Table 1, biotin-γ_6(L) with a C-terminal leucine is 120-fold preferred over the corresponding peptide with a C-terminal serine, and biotin-lamin B(L) is 290-fold preferred over biotin-lamin B(S). These differences are due to a combination of higher values of $^{PA}K_M$ (10-fold) and lower values of k_{cat} (10 to 60-fold) for peptides that have a C-terminal serine instead of leucine. These large differences in kinetic parameters are presumably the reason why γ_6 and lamin B proteins are predominantly, if not exclusively, geranylgeranylated or farnesylated, respectively, in the cell by PGGT-I and PFT, respectively (Yamane et al., 1990, Farnsworth et al., 1989).

The $k_{cat}/^{PA}K_M$ for biotin-CDC42(F), which has a C-terminal phenylalanine, is 930-fold smaller than that for biotin-γ_6-(L). This is due to the relatively small value of k_{cat} for the geranylgeranylation of this peptide. Thus, biotin-CDC42-(F) is a poor substrate for PGGT-I; according to eq 1 this is true even though the K_M for [^{3}H]-GGPP is 22-fold smaller when the enzyme is saturated with biotin-CDC42(F) versus biotin-γ_6(L). The cDNA-inferred amino acid sequence of CDC42 from human placenta contains a C-terminal leucine (Shinjo et al., 1990), whereas the form from human brain ends in phenylalanine (Munemitsu et al., 1990). Although it has been shown that bovine brain CDC42 is geranylgeranylated (Yamane et al., 1991), the C-terminal amino acid sequence of this protein is not known. In light of the present results, further studies will be needed to determine whether proteins that contain a Cys-Ali-Ali-Phe sequence are prenylated. In vitro studies have shown that biotin-CDC42(F) is not a substrate of PFT (Yokoyama et al., 1991).

The results in Table 1 also show that the kinetic parameters of the two proteins GST-CDC42(F) and RAS1-CAIL are not significantly different than those of the corresponding peptides biotin-CDC42(F) and biotin-RAS1(L) which suggests that PGGT-I like PFT interacts mainly with the Cys-Ali-Ali-Xaa portions of their substrates. In marked contrast, protein geranylgeranyltransferase-II, also known as rab geranylgeranyltransferase, forms much more extensive interactions with its rab prenyl acceptors that extend to other regions besides their C-termini (Beranger et al., 1994).

One could write an equation analogous to eq 1 that describes the specificity of PGGT-I for the prenyl donor. In this case the prenyl donor specificity would be given by the ratio of $^{GGPP}k_{cat}/^{GGPP}K_M$ to $^{FPP}k_{cat}/^{FPP}K_M$. The data in Table 1 show that, for peptides or proteins that have a C-terminal leucine, $^{GGPP}K_M$ is similar in value to $^{FPP}K_M$ and likewise for $^{GGPP}k_{cat}$ and $^{FPP}k_{cat}$. Thus, this argument would suggest that PGGT-I lacks specificity for the prenyl donor. However, this is not the case. The specificity of PGGT-I for GGPP over FPP lies in the fact that the reaction preferentially proceeds via the pathway involving prenyl donor binding to free enzyme before prenyl acceptor and the fact that GGPP binds to the enzyme much tighter than FPP (Yokoyama & Gelb, 1993). In the presence of very high concentrations of prenyl acceptor the prenyl donor selectively is lost since the K_M's for GGPP and FPP interacting with the binary complex PGGT-I•prenyl acceptor are similar in magnitude, as are the k_{cat}'s for the prenyl transfers. This presumably explains the earlier observation that the prenyl donor specificity is lost in the presence of high concentrations of peptide prenyl acceptors (Yokoyama et al., 1991).

Numerous recent studies have been reported in which genes carrying a mutation that changes the C-terminal residue (Xaa of Cys-Ali-Ali-Xaa) of prenylated proteins have been expressed in cells, and the functional consequences of the change of the prenyl group (farnesyl versus geranylgeranyl) have been examined. In most cases the protein of interest has been overexpressed. Given the fact that PGGT-I catalyzes the farnesylation of proteins especially in the presence of high protein concentrations (Table 1), such structure/function studies in cells should be interpreted with caution. Even if analytical methods were used to confirm the identity of the protein prenyl group, it is difficult to rule out that the undesired prenyl group may be present in small amounts.

Although the addition of Zn^{2+} and Mg^{2+} is sufficient to restore the enzymatic activity of metal-free PGGT-I, full enzymatic activity is seen in the presence of other metals present either in combination or individually. The activation of other prenyltransferases by a variety of divalent metals is not without precedence. Farnesyl-, octaperenyl-, solanesyl-, and decaprenyl pyrophosphate synthetases, which catalyze the transfer of prenyl groups from allylic pyrophosphates to isopentenyl pyrophosphate, require Mg^{2+} for activity, but other divalent metals including Mn^{2+}, Zn^{2+}, Co^{2+}, and Ni^{2+} support 10−100% of the enzymatic activities measured in the presence of Mg^{2+} (Ishii et al., 1985; Ohnuma et al., 1993; Sagami et al., 1984). Metals are not needed for complex formation between PGGT-I and [^{3}H]-GGPP, but addition of prenyl acceptor to this binary complex does not result in prenyl transfer unless both Mg^{2+} and Zn^{2+} are added. PFT displays similar behavior in its interaction with [^{3}H]-FPP (Reiss et al., 1992a). The farnesyl acceptor H-ras can be

cross-linked to the β-subunit of PFT, and this requires the presence of Zn^{2+} suggesting that this metal plays a structural role in anchoring the prenyl acceptor to the enzyme (Reiss et al., 1992a), but definitive evidence for this must await detailed structural analysis of enzyme−substrate complexes.

ACKNOWLEDGMENT

We are grateful to Professor Paul Cook for helpful discussion.

REFERENCES

Aepfelbacher, M., Vauti, F., Weber, P. C., & Glomset, J. A. (1994) *Proc. Natl. Acad. Sci. U.S.A.* in press.

Allen, C. M., & Baba, T. (1985) *Methods Enzymol. 110*, 117−124.

Andres, D. A., Seabra, M. C., Brown, M. S., Armstrong, S. A., Smeland, T. E., Cremers, F. P., & Goldstein, J. L. (1993) *Cell 73*, 1091−9.

Armstrong, S. A., Seabra, M. C., Sudhof, T. C., Goldstein, J. L., & Brown, M. S. (1993) *J. Biol. Chem. 268*, 12221−9.

Baba, T., Muth, J., & Allen, C. M. (1985) *J. Biol. Chem. 260*, 10467−10473.

Beranger, F., Cadwallader, K., Porfiri, E., Powers, S., Evans, T., de Gunzburg, J., & Hancock, J. F. (1994) *J. Biol. Chem. 269*, 13637−13643.

Brown, M. S., Goldstein, J. L., Paris, K. J., Burnier, J. P., & Marsters, J. C. J. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 8313−6.

Casey, P. J., Thissen, J. A., & Moomaw, J. F. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 8631−8635.

Chen, W.-J., Andres, D. A., Goldstein, J. L., Russell, D. W., & Brown, M. S. (1991a) *Cell 66*, 327−334.

Chen, W. J., Andres, D. A., Goldstein, J. L., & Brown, M. S. (1991b). *Proc. Natl. Acad. Sci. U.S.A. 88*, 11368−72.

Chen, W. J., Moomaw, J. F., Overton, L., Kost, T. A., & Casey, P. J. (1993) *J. Biol. Chem. 268*, 9675−80.

Clarke, S. (1992) *Annu. Rev. Biochem. 61*, 355−386.

Farnsworth, C. C., Wolda, S. L., Gelb, M. H., & Glomset, J. A. (1989) *J. Biol. Chem. 264*, 20422−20429.

Finegold, A. A., Johnson, D. I., Farnsworth, C. C., Gelb, M. H., Judd, S. R., Glomset, J. A., & Tamanoi, F. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 4448−4452.

Gibbs, J. B., Pompliano, D. L., Mosser, S. D., Rands, E., Lingham, R. B., Singh, S. B., Scolnick, E. M., Kohl, N. E., & Oliff, A. (1993) *J. Biol. Chem. 268*, 7617−20.

Goldstein, J. L., Brown, M. S., Stradley, S. J., Reiss, Y., & Gierasch, L. M. (1991) *J. Biol. Chem. 266*, 15575−15578.

Gomez, R., Goodman, L. E., Tripathy, S. K., O'Rourke, E., Manne, V., & Tamanoi, F. (1993) *Biochem. J. 289*, 25−31.

Hara, M., Akasaka, K., Akinaga, S., Okabe, M., Nakano, H., Gomez, R., Wood, D., Uh, M., & Tamanoi, F. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 2281−5.

Horiuchi, H., Kawata, M., Katayama, M., Yoshida, Y., Musha, T., Ando, S., & Takai, Y. (1991) *J. Biol. Chem. 266*, 16981−16984.

Ishii, K., Sagami, H., & Ogura, K. (1985) *Biochim. Biophys. Acta 835*, 291−297.

Jang, G.-F., Yokoyama, K., & Gelb, M. H. (1993) *Biochemistry 32*, 9500−9507.

Joly, A., Popjak, G., & Edwards, P. A. (1991) *J. Biol. Chem. 266*, 13495−13498.

Kohl, N. E., Diehl, R. E., Schaber, M. D., Rands, E., Soderman, D. D., He, B., Moores, S. L., Pompliano, D. L., Ferro-Novick, S., Powers, S., Thomas, K. A., & Gibbs, J. B. (1991) *J. Biol. Chem. 266*, 18884−18888.

Leonard, D. M., Eaton, S. R., Sawyer, T. K., Bolton, G., Hodges, J., Gowan, R., & Sebolt-Leopold, J. S. (1994) 207th American Chemical Society National Meeting March 13−17, 1994, San Diego, American Chemical Society: Washington, DC; Abstract MEDI281.

Lin, Y. K., Myhrman, R., Schrag, M. L., & Gelb, M. H. (1988) *J. Biol. Chem. 263*, 1622−7.

Mann, K. R., Lewis, N. S., Miskowski, V. M., Erwin, D. K., Hammond, G. S., & Gray, H. B. (1977) *J. Am. Chem. Soc. 99*, 5526−5528.

Manne, V., Roberts, D., Tobin, A., O'Rourke, E., De, V. M., Meyers, C., Ahmed, N., Kurz, B., Resh, M., Kung, H. F., et al. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 7541−5.

Moomaw, J. F., & Casey, P. J. (1992) *J. Biol. Chem. 267*, 17438−43.

Moores, S. L., Schaber, M. D., Mosser, S. D., Rands, E., O'Hara, M. B., Garsky, V. M., Marshall, M. S., Pompliano, D. L., & Gibbs, J. B. (1991) *J. Biol. Chem. 266*, 14603−14610.

Munemitsu, S., Innis, M. A., Clark, R., McCormick, F., Ullrich, A., & Polakis, P. (1990) *Mol. Cell. Biol. 10*, 5977−5982.

Nigam, M., Seong, C. M., Qian, Y., Hamilton, A. D., & Sebti, S. M. (1993) *J. Biol. Chem. 268*, 20695−8.

Ohnuma, S., Koyama, T., & Ogura, K. (1993) *Biochem. Biophys. Res. Commun. 192*, 407−412.

Omer, C. A., Kral, A. M., Diehl, R. E., Prendergast, G. C., Powers, S., Allen, C. M., Gibbs, J. B., & Kohl, N. E. (1993) *Biochemistry 32*, 5167−76.

Omura, S., Van der Pyl, D., Inokoshi, J., Takahashi, Y., & Takeshima, H. (1993) *J. Antibiot. (Tokyo) 46*, 222−8.

Perkins, S. J. (1986) *Eur. J. Biochem. 85*, 169−180.

Pompliano, D. L., Rands, E., Schaber, M. D., Mosser, S. D., Anthony, N. J., & Gibbs, J. B. (1992) *Biochemistry 31*, 3800−7.

Pompliano, D. L., Schaber, M. D., Mosser, S. D., Omer, C. A., Shafer, J. A., & Gibbs, J. B. (1993) *Biochemistry 32*, 8341−8347.

Popjak, G., Cornforth, J. W., Cornforth, R. H., Ryhage, R. H., & Goodman, D. S. (1962) *J. Biol. Chem. 237*, 56.

Ray, K. P., & Lopez, B. J. (1992) *Biochem. Soc. Trans. 20*, 494−7.

Reiss, Y., Brown, M. S., & Goldstein, J. L. (1992a) *J. Biol. Chem. 267*, 6403−6408.

Reiss, Y., Goldstein, J. L., Seabra, M. C., Casey, P. J., & Brown, M. S. (1990) *Cell 62*, 81−88.

Reiss, Y., Seabra, M. C., Armstrong, S. A., Slaughter, C. A., Goldstein, J. L., & Brown, M. S. (1991a) *J. Biol. Chem. 266*, 10672−7.

Reiss, Y., Seabra, M. C., Brown, M. S., & Goldstein, J. L. (1992b) *Biochem. Soc. Trans. 20*, 487−8.

Reiss, Y., Stradley, S. J., Gierasch, L. M., Brown, M. S., & Goldstein, J. L. (1991b) *Proc. Natl. Acad. Sci. U.S.A. 88*, 732−736.

Rose, I. A. (1980) *Methods Enzymol. 64*, 47−59.

Sagami, H., Ogura, K., Weiner, A., & Poulter, C. D. (1984) *Biochem. Int. 8*, 661−667.

Schafer, W. R., Trueblood, C. E., Yang, C.-C., Mayer, M. P., Rosenberg, S., Poulter, C. D., Kim, S.-H., & Rine, J. (1990) *Science 249*, 1133−1139.

Seabra, M. C., Brown, M. S., Slaughter, C. A., Sudhof, T. C., & Goldstein, J. L. (1992a) *Cell 70*, 1049−57.

Seabra, M. C., Goldstein, J. L., Sudhof, T. C., & Brown, M. S. (1992b) *J. Biol. Chem. 267*, 14497−503.

Seabra, M. C., Reiss, Y., Casey, P. J., Brown, M. S., & Goldstein, J. L. (1991) *Cell 65*, 429−34.

Sebolt, J. S., Gowan, R., Su, T.-Z., Leonard, D., Sawyer, T., Bolton, G., Hodges, J., & Hupe, D. (1994) *Proc. Am. Assoc. Cancer Res. 35*, Abstr. 3535.

Segel, I. H. (1975) *Enzyme Kinetics* Wiley-Interscience, New York.

Shinjo, K., Koland, J. G., Hart, M. J., Narasimhan, V., Johnson, D. I., Evans, T., & Cerione, R. A. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 9853−9857.

Shiomi, K., Yang, H., Inokoshi, J., Van der Pyl, D., Nakagawa, A., Takeshima, H., & Omura, S. (1993) *J. Antibiot. (Tokyo) 46*, 229−34.

Still, W. C., Kahn, M., & Mitra, A. (1978) *J. Org. Chem. 43*, 2923.

Van der Pyl, D., Inokoshi, J., Shiomi, K., Yang, H., Takeshima, H., & Omura, S. (1992) *J. Antibiot. (Tokyo) 45*, 1802−5.

Yamane, H. K., Farnsworth, C. C., Xie, H., Evans, T., Gelb, M. H., Glomset, J. A., Clarke, S., & Fung, B. K.-K. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 286−290.

Yamane, H. K., Farnsworth, C. C., Xie, H., Howald, W., Fung, B. K.-K., Clarke, S., Gelb, M. H., & Glomset, J. A. (1990) *Proc. Nat. Acad. Sci. U.S.A. 87*, 5868−5872.

Yokoyama, K., & Gelb, M. H. (1993a) *J. Biol. Chem. 268*, 4055−4060.

Yokoyama, K., Goodwin, G. W., Ghomashchi, F., Glomset, J., & Gelb, M. H. (1992) *Biochem. Soc. Trans. 20*, 489−94.

Yokoyama, K., Goodwin, G. W., Ghomashchi, F., Glomset, J. A., & Gelb, M. H. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 5302−5306.

Yoshida, Y., Kawata, M., Katayama, M., Horiuchi, H., Kita, Y., & Takai, Y. (1991) *Biochem. Biophys. Res. Commun. 175*, 720−728.

Zhang, F. L., Diehl, R. E., Kohl, N. E., Gibbs, J. B., Giros, B., Casey, P. J., & Omer, C. A. (1994) *J. Biol. Chem. 269*, 3175−3180.

BI941120K