Lysine β311 of Protein Geranylgeranyltransferase Type I Partially Replaces Magnesium*

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Protein geranylgeranyltransferase type I (GGTase I) catalyzes the attachment of a geranylgeranyl lipid group near the carboxyl terminus of protein substrates. Unlike protein farnesyltransferase (FTase) and protein geranylgeranyltransferase type II, which require both Zn(II) and Mg(II) for maximal turnover, GGTase I turnover is dependent only on Mg(II). In FTase, the magnesium ion is coordinated by aspartate β352 and the diphosphate of farnesyl diphosphate to stabilize the developing charge in the transition state (Pickett, J. S., Bowers, K. E., and Fierke, C. A. (2003) J. Biol. Chem. 278, 51243–51250). In GGTase I, lysine β311 is substituted for this aspartate and is proposed to replace the catalytic function of Mg(II) (Taylor, J. S., Reid, T. S., Terry, K. L., Casey, P. J., and Beece, L. S. (2003) EMBO J. 22, 5963–5974). Here we demonstrate that the prenylation rate constant catalyzed by wild type GGTase I (k_prenyly = 0.18 ± 0.02 s⁻¹) is not dependent on Mg(II), is ~20-fold slower than the maximal rate constant catalyzed by FTase, and has a single pK_a of 6.4 ± 0.1, likely reflecting deprotonation of the peptide thiol. Mutation of lysine β311 in GGTase I to alanine (Kβ311A) or aspartate (Kβ311D) decreases the k_prenyly in the absence of magnesium 9–41-fold without significantly affecting the binding affinity of either substrate. Furthermore, the geranylgeranylation rate constant is enhanced by the addition of Mg(II) for Kβ311A and Kβ311D GGTase I 2–5-fold compared with wild type GGTase I with k_Mg of 140 ± 10 mM and 6.4 ± 0.8 mM, respectively. These results demonstrate that lysine β311 of GGTase I partially replaces the catalytic function of Mg(II) observed in FTase.

Prenylation is a type of post-translational modification where a lipid group from either farnesyl diphosphate (FPP) or geranylgeranyl diphosphate (GGPP) is covalently attached via a thioether linkage to a conserved cysteine residue near the carboxyl terminus of a protein (1–6; for review, see Refs. 7 and 8). The attached lipid mediates membrane association and specific protein-protein interactions, which are obligatory for proper functioning of the modified protein (9, 10). Protein farnesyltransferase (FTase), protein geranylgeranyltransferase type I (GGTase I), and protein geranylgeranyltransferase type II (GGTase II) comprise the class of zinc-catalyzed sulfur alkyl transferases known as the protein prenyltransferases. All three protein prenyltransferases are α/β heterodimers; FTase and GGTase I share common α-subunits and possess β-subunits with 25% sequence identity (8, 11, 12). FTase and GGTase I prenylate proteins or peptides containing carboxyl-terminal CaaX (C refers to a conserved cysteine residue, a refers to an aliphatic amino acid, and X generally refers to leucine or phenylalanine for GGTase I, and methionine, serine, alanine, or glutamine for FTase) sequences (13–18). The proteins Ras, RhoB, CENP-E, and CENP-F are known substrates of FTase; GGTase I modifies the γ-subunits of most heterotrimeric G proteins as well as many Ras-related G proteins, including members of the Rac, Rap, and Rho families (9, 19–23). In a fashion distinct from FTase and GGTase I, GGTase II catalyzes the attachment of two geranylgeranyl groups to members of the Rab family of GTPases that contain carboxyl-terminal sequences of XXXC, CCXX, CXXX, and CCXX (C refers to a conserved cysteine residue, and X refers to any amino acid) (24).

Recently, much of the work on prenyltransferases has focused on FTase and the protein substrate, Ras, a small GTPase in the receptor tyrosine kinase signaling pathway that is a key regulator of cell division (25). 30% of all human cancers can be linked to mutations in the ras gene, which lead to constitutively activated Ras signaling (25). The post-translational attachment of a farnesyl group by FTase is required for the transforming activity of Ras oncoproteins (25). These observations prompted a search for FTase inhibitors as novel anticancer agents (25). Subsequent research demonstrated that the form of Ras most often mutated in human cancers is K-Ras4B, a protein substrate that can be farnesylated by FTase as well as geranylgeranylated by GGTase I (25). Moreover, geranylgeranylated G proteins in addition to Ras have been shown to play important roles in smooth muscle proliferation and apoptosis (25). The latter observations have initiated a search for GGTase I inhibitors for use in cancer therapy as well as for the treatment of cardiovascular disease (25).

The protein prenyltransferases catalyze thioether bond formation between the cysteine sulfur of the protein substrate and the carbon-1 of the isoprenoid substrate, a reaction requiring a

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The abbreviations used are: FPP, farnesyl diphosphate; Bes, 2-bis(2-hydroxyethyl)aminomethylsulfonic acid; Beie, N,N'-bis(2-hydroxyethyl)glycine; Caax, tetrapeptide sequence cysteine-aliphatic amino acid-aliphatic amino acid-X (serine glutamine, or methionine for FTase); leucine or phenylalanine for GGTase I; dansylated GCVLL, dansylated pentapeptide Gly-Cys-Val-Leu-Leu; FTase, protein farnesyltransferase; GGPP, geranylgeranyl diphosphate; GGTase I and II, protein geranylgeranyltransferase type I and II; Heppso, N-(2-hydroxyethyl)piperazine-N’-(2-hydroxypropanesulfonic acid); TCEP, tris(2-carboxyethyl)phosphine hydrochloride.
catalytically essential zinc ion (13–18). Zhang and Casey (8) demonstrated that Zn(I11) in GG/Tase I is required for peptide but not isoprenoid binding, suggesting that Zn(I11) plays a similar role in both FTase and GG/Tase I. The zinc ion in FTase coordinates the sulfur of the cysteine within the CxxC sequence of the protein substrate, stabilizing the nuclophilic thiolate (26). FTase and GG/Tase II also require Mg(I11) for maximal activity (27, 28). In FTase, Mg(I11) accelerates the chemical step 700-fold by coordinating the diphosphate group of the isoprenoid substrate, thereby stabilizing the negative charge buildup on the diphosphate leaving group (29–31). Mutagenesis studies provide evidence that Mg(I11) is also coordinated by an asparagine residue in FTase (32). Interestingly, the Mg(I11) dependence of GG/Tase I is not well understood. Initial studies indicated that, similar to FTase, GG/Tase I required Mg(I11) for maximal activity (33, 34). However, later studies demonstrated that metal-chelating reagents have little effect on the steady-state turnover rate of GG/Tase I, suggesting that Mg(I11) is not required for maximal activity (35). Sequence alignments and the crystal structures of FTase and GG/Tase I indicate that the aspartate in FTase that coordinates Mg(I11) is replaced by lysine 311 in the β-subunit of GG/Tase I, leading to the suggestion by Beece and colleagues (12, 36) that Lys-β311 in GG/Tase I represses the catalytic function of Mg(I11) in FTase.

Here we characterize the kinetics of wild type GG/Tase I and examine the role of residue Lys-β311 in catalysis using mutagenesis and biochemical experiments. These data demonstrate that the rate constant for geranylgeranylation catalyzed by GG/Tase I is slower than the farnesylation rate constant catalyzed by FTase and not enhanced by Mg(I11), suggesting differences in the catalytic mechanisms between FTase and GG/Tase I. When Lys-β311 in GG/Tase I is replaced by alanine or aspartate, geranylgeranylation is now enhanced by Mg(I11), indicating that Lys-β311 is important for blocking activation by Mg(I11) in wild type GG/Tase I. However, when the lysine side chain is removed in Kβ311A GG/Tase I, the rate constant for geranylgeranylation in the absence of Mg(I11) decreases 40-fold, indicating that Lys-β311 modestly enhances catalysis. Furthermore, the lysine side chain is not as effective at stabilizing the diphosphate leaving group as Mg(I11) in FTase, which enhances catalysis 700-fold; therefore, GG/Tase I is not as efficient as FTase in catalyzing prenylation (30).

EXPERIMENTAL PROCEDURES

Miscellaneous Methods—All assays were performed at 25 °C. All curve fitting was performed with Kaleidograph (Synergy Software, Reading, PA). Tritium-labeled farnesyl diphosphate ([1-H]FPP) and tritium-labeled geranylgeranylnyl diphosphate ([1-H]GGPP) were purchased from Amersham Biosciences. The peptides GCVLl and dantsylated GCVLL were synthesized and purified by high pressure liquid chromatography by Buthyl Laboratories (Dallas, TX). The concentration of peptide was determined spectroscopically at 412 nm by reaction of the cysteine thiol with 5,5′-dithiobis(2-nitrobenzoic acid), using an extinction coefficient of 14,150 M−1 cm−1 (37). GG/Tase I concentration was determined by absorbance at 280 nm using an extinction coefficient of 134,000 M−1 cm−1, which was calculated using the method of Edelhoch (38; data not shown). Thin layer chromatography (TLC) plates were pre-run in 100% acetone before use.

Subcloning of GG/Tase I—A bacterial expression vector for rat GG/Tase I was prepared in the following way. Mutagenesis of the high expression rat FTase pET23a-FPT plasmid (39) was performed using the QuickChange site-directed mutagenesis kit (Stratagene) to introduce an EcoRI restriction site into the stop/start region between the genes encoding the two subunits of FTase with the following codon changes: GAGGAGTTT to GAGGAATTC. The modified pET23a-FPT plasmid was cut using the restriction enzymes EcoRI and NdeI to remove a DNA fragment containing the β-subunit of FTase. The remaining plasmid fragment was purified from the DNA fragment encoding the FPT β-subunit using a 1.2% agarose gel. The gene encoding the β-subunit of GG/Tase I was amplified from the plasmid pET28a-GGT-I (33, 39, 40; a gift from Patrick Casey, Duke University) using PCR with primers that incorporated an NdeI restriction site at the 5′-end of the β-subunit and an EcoRI restriction site at the 3′-end of the β-subunit. This PCR fragment was purified using a 1.2% agarose gel, digested with the restriction enzymes EcoRI and NdeI, and repurified to obtain the DNA encoding the β-subunit of GG/Tase I. This DNA fragment was ligated into the NdeI/EcoRI-digested pET23a-FPT plasmid using a ligation premix (New England Biolabs, San Diego) to generate the plasmid pET23a-GGPT. The plasmid was transformed into XL-1 Blue cells, and the cells were grown in Luria-Bertani medium supplemented with 100 mg liter−1 ampicillin. The plated was purified using a Plasmid Midi Kit (Qiagen, Valencia, CA). The sequence of the entire gene was confirmed by DNA sequencing (University of Michigan DNA Sequencing Center, Ann Arbor).

Preparation of Goat GG/Tase I—Recombinant GG/Tase I was overexpressed in Escherichia coli BL21(DE3) pET23a-GGPT cells and purified as described below. The cells were grown in rich induction medium (20 g liter−1 tryptone, 10 g liter−1 yeast extract, 5 g liter−1 NaCl, 50 mg liter−1 ampicillin, 1% glucose, 0.06 mM Na2SO4, 0.22 g liter−1 NaHPO4, 0.11 g liter−1 KH2PO4, 0.036 g liter−1 NH4Cl, pH 7.35) at 37 °C to an A 660 of 0.8, induced by the addition of 0.4 mM isopropyl β-d-thiogalactopyranoside and 0.5 mM ZnSO4, and incubated at 25 °C for 14 h (39). The harvested cells were lysed using a microfluidizer (Microfluidics, Newton, MA). The cell lysate from 2 liters of cells was loaded onto a DEAE-Sepharose (Amersham Biosciences, 30-ml bed volume) or DEAE-cellulose (Whatman) column (100-ml bed volume) column with HTZ buffer (50 mM Hepes, pH 7.8, 1 mM TCEP, 10 mM ZnCl2). The column was run by gravity with a linear gradient (400 ml) from 0 to 0.5 M NaCl in HTZ buffer. GG/Tase I eluted at 0.3 M NaCl. The fractions were tested for GG/Tase I activity using a 96-well plate format. Each well contained 50 μl Heppp, pH 7.5, 5 μM MgCl2, 1 mM TCEP, 0.2 μM GGPP (Sigma), 1 μM dantsylated GCVLL, and 10 μl of protein sample (100-μl total volume). The relative fluorescence of each fraction was measured after the addition of dantsylated GCVLL by the Polarstar Galaxy fluorescence plate reader (BMG Laboratory Technologies, Durham, NC; λex = 380 nm, λem = 490 nm). Fractions containing GG/Tase I were pooled and concentrated using Amicon Ultra centrifugal filter devices (Millipore, Bedford, MA). The pool was dialyzed against HTZ buffer overnight, then run over a Q-Sepharose HP column (Amersham Biosciences, 40-ml bed volume) using fast protein liquid chromatography (AKTA Prime, Amersham Biosciences) with a 0–0.5 M NaCl step gradient (400 ml). The active fractions were again pooled and concentrated using Amicon Ultra centrifugal filter devices (Millipore), and from this fraction 15 mg of purified GG/Tase I was obtained from 1 liter of cell culture.

Preparation of Mutant GG/Tase I—Mutagenesis of the pET23a-GGPT plasmid was performed using the QuikChange site-directed mutagenesis kit with the following codon changes: Kβ311A, AAA to GCA, and Kβ311D, AAA to GAC. The changes were confirmed by DNA sequencing (University of Michigan DNA Sequencing Core, Ann Arbor). Recombinant rat GG/Tase I mutants were overexpressed in E. coli and purified as described above except that the Q-Sepharose HP column was omitted. Instead, after the DEAE-cellulose column, the active fractions were loaded onto a Poros 20 HQ column (Applied Biosystems, Foster City, CA; 1.7-ml bed volume) and fractionated using a step gradient at 0.1, 0.5, and 2 mM NaCl (90 ml). The prepared GG/Tase I was determined by SDS-PAGE to be >90% pure. The protein was dialyzed against HT buffer (50 mM Hepes, pH 7.8, 1 mM TCEP), concentrated to 31 μM using Amicon Ultra centrifugal filter devices (Millipore), and from this fraction 15 mg of purified Mutant GG/Tase I was obtained from 1 liter of cell culture.

GGPP Binding Affinity—The affinity of wild type GG/Tase I for GGPP and FPP and Lys-β311 GG/Tase I mutants for GGPP was determined by equilibrium dialysis (30, 39). A 1-ml solution containing 33, 39, 40, or 42 nM enzyme was preformed with a constant concentration of [1-3H]GGPP or [1-3H]FPP (5, 10, or 20 nM) in the same buffer using a 25,000 molecular weight cutoff dialysis membrane (Dia-Cell, Spectra/Perm, 7, Spectrum Laboratories, Rancho Dominguez, CA). After 20 h, the radioactivity in 100-μl samples from both sides of the membrane was quantified in triplicate by scintillation counting and averaged. The fraction of [1-3H]GGPP bound to GG/Tase I (E:GGPP) was determined by subtracting the counts on the [1-3H]GGPP side from the counts on the enzyme side,
then dividing the difference by the total counts. The concentration of E:GGPP was calculated by multiplying the fraction of GGPP bound to E:GGPP by the initial [1-3H]GGPP concentration, [GGPP]GCVLL, (5–20 nM). The [E]free was calculated by subtracting [E:GGPP] from [E]total. The dissociation constant, K_d, was then determined by fitting Equation 1 to the dependence of E:GGPP/[GGPP]GCVLL on [E]free where EP refers to the fraction of GGPP bound to E:GGPP at saturation. The data were truncated at [E]free = 5K_D.

\[
\text{E:GGPP} = \frac{\text{EP}}{1 + \frac{\text{K}_D}{[\text{E}]_{\text{free}}}}
\]

(EP 1)

**Peptide Binding Affinity**—The affinity of GGTase I and Lys-β311 GGTase I mutants for datsylcyl-GGPP was determined by fluorescence anisotropy (26, 41), where the dansyl group of the peptide is excited at 340 nm (band pass = 16 nm), and its emission is monitored at 525 nm (band pass = 16 nm). The samples were prepared with 50 mM Hepes, pH 7.8, 2 mM TCEP, 2 mM datsylated GCVLL, and 10 mM EDTA. The samples were titrated with GGTase I (0–150 nM), and additional datsylated GCVLL was added to maintain the 2 nM concentration of peptide. For comparison, this titration was repeated in the absence of Mg(II) concentration; and EP refers to the fluorescence anisotropy at 525 nm corrected for background, EP is the fluorescence anisotropy end point, IF is the initial fluorescence anisotropy, [enzyme] is the concentration of GGTase I, and K_D,app is the dissociation constant for datsylcyl-GCVLL.

\[
\Delta A = \frac{\text{EP}}{1 + \frac{\text{K}_D,\text{app}}{[\text{enzyme}]} + \text{IF}}
\]

(EP 2)

**Transient Kinetics**—Single turnover assays were performed for wild type and mutant GGTase I at multiple magnesium concentrations (0.0006–175 mM) in 50 mM Hepes, pH 7.8, 2 mM TCEP, with ionic strength kept constant at 0.2 M with NaCl up to 60 mM Mg(II) as described previously (31). Reactions with preincubations were initiated by the addition of 2 mM isodecanoyl treated datsylcyl-GCVLL (which should not bind to GGTase I). All samples were incubated for 5 min without stirring prior to each measurement at 25 °C. A weighted fit of Equation 2 to the data yields the apparent dissociation constants where ΔA represents the observed fluorescence anisotropy at 525 nm corrected for background, EP is the fluorescence anisotropy end point, IF is the initial fluorescence anisotropy, [E]free is the concentration of the limiting product/s where V refers to the velocity of the reaction in μM s⁻¹, K is the concentration of the limiting substrate, and F_max refers to the maximal fluorescence intensity at the end point (45). Under these conditions, both substrates are saturating so that V reflects V_max.

\[
\text{V} = \frac{R^tP}{F_{\max}}
\]

(EP 6)

**RESULTS**

**Mutagenesis of Lys-β311**—Previous mutagenesis studies of rat FTase suggest that the side chain of Asp-β352 coordinates a catalytic Mg(II) ion (32). An aspartate is conserved in the homologous position in GGTase II; however, sequence alignments and crystallographic analysis of GGTase I indicate that this aspartate is replaced by lysine (Lys-β311) in GGTase I (12, 32, 36). Therefore, the positive charge of the side chain of Lys-β311 has been proposed to replace the catalytic function of Mg(II) in GGTase I (12, 36). To test this possibility, we have examined the catalytic properties and Mg(II) dependence of wild type GGTase I and a mutant where the lysine at position β311 of GGTase I was changed to aspartate (Kp311D) for comparison with FTase. Lys-β311 of GGTase I was also altered to alanine (Kp311A) to observe the effect of removing the positive charge from the active site. Kp311A GGTase I had expression levels in E. coli similar to wild type (15 mg of purified protein/liter of cell culture) and behaved similarly to the wild type enzyme during purification. Kp311D GGTase I had expression levels in E. coli lower than wild type (3 mg of purified protein/liter of cell culture) but behaved similarly to the wild type enzyme during purification.

**Substrate Binding Affinity**—The binding affinity of the isoprenoid substrate, GGPP, was determined by equilibrium dialysis (Fig. 1). The affinities of the mutant enzymes for GGPP were within 2-fold of the wild type value of 340 nM (Table I). The affinity of wild type GGTase I for FPP (24 ± 5 nM, data not shown) was 30-fold weaker than the affinity of the wild type enzyme for GGPP but only 4-fold weaker than the affinity of wild type FTase for FPP (30, 6.5 ± 1.7 nM). The value of K_D for FPP is slightly tighter than the value of 3 nM reported previously by Yokoyama et al. (46), whereas K_D for GGPP is significantly tighter than the reported value of 1 μM. These values were determined using spin column gel filtration and competitive binding studies with buffer conditions different from the equilibrium dialysis experiments cited here, suggesting that the discrepancies may be related to the altered experimental conditions.
To examine peptide affinity and reactivity we are using the peptide sequence GCVLL, which corresponds to the CaaX sequence of the protein R-Ras, a known protein substrate of GGTase I (8). Wild type GGTase I and the GGTase I 3-aza-GGPP complex showed negligible affinity for the iodoacetamide-treated dansylated GCVLL (data not shown). The affinity of the GGTase I 3-aza-GGPP complex for dansylated GCVLL (9 ± 2 nM, data not shown) was slightly tighter than that for wild type GGTase I (15 ± 2 nM, Table I), as measured by fluorescence anisotropy (Fig. 2). The affinities of K311A and K311D GGTase I for dansylated GCVLL were decreased 2-fold (30 ± 3 nM and 20 ± 2 nM, respectively, Table I) compared with wild type GGTase I. These alterations in the binding affinity are modest and are likely explained by changes in the van der Waals volume of the altered side chain (32, 47).

Transient Kinetics of Wild Type GGTase I—The rate constant for geranylgeranylation catalyzed by GGTase I was determined under single turnover conditions, where the enzyme and peptide concentrations are in excess of the GGPP concentration. Previous kinetic data suggest that the kinetic scheme for GGTase I is similar to that of FTase (34, 35), including that the substrate binding is functionally ordered with GGPP binding before peptide and that the rate constant for geranylgeranylation is faster than product dissociation (Scheme 1). The kinetic and thermodynamic data that we have determined for GGTase I are consistent with this basic kinetic mechanism.

The rate constant measured by single turnover kinetics at saturating GGTase I and peptide concentrations is 0.18 ± 0.02 s⁻¹ (Fig. 3). This rate constant measures the formation of the GGTase I-product complex from the GGTase I-GGPP-peptide complex and could reflect either the geranylgeranylation reaction catalyzed by GGTase I or the isomerization of bound GGPP to form a reactive ternary complex, as observed in the crystal structure (12). We interpret this rate constant as reflecting chemistry because fluorinated GGPP substrates significantly decrease this step. Additionally, the value of this single turnover rate constant is 3-fold slower than a pre-steady-state rate constant measured by Yokoyama and colleagues (46) for geranylgeranylation of Biotin-CAIL catalyzed by rat GGTase I (0.56 s⁻¹). The difference between these values can most likely be attributed to differences in peptide sequence. Additionally, geranylgeranylation catalyzed by wild type GGTase I is not activated by Mg(II) (Fig. 3); the values of the geranylgeranylation rate constants are the same, within error, for data collected in the absence of Mg(II), in the presence of 50 mM EDTA and in the presence of 50 mM Mg(II) (0.18 ± 0.02, 0.18 ± 0.02, and 0.15 ± 0.02 s⁻¹, respectively). Therefore, GGTase I must stabilize the developing positive charge on the diphosphate leaving group in the transition state using a mechanism distinct from that of FTase (30, 31). Furthermore, prenylation catalyzed by GGTase I is 12–40-fold slower than the maximal Mg(II)-activated prenylation rate constant catalyzed by FTase (8.1 ± 0.3 s⁻¹), suggesting that GGTase I stabilizes the chemical transition state less efficiently than FTase in the presence of Mg(II) (31). On the other hand, in the absence of Mg(II) prenylation catalyzed by GGTase I is 20-fold faster than farnesylation catalyzed by FTase, indicating that active site residues in GGTase I partially compensate for the transition state stabilization provided by the Mg(II) ion in FTase.

The pH dependence of $k_{\text{max}}$ for geranylgeranylation catalyzed by wild type GGTase I was measured (Fig. 4), and a single $pK_a$ of 6.4 ± 0.1 was observed. This ionization most likely reflects deprotonation of the thiol of the bound peptide cysteine, as observed for FTase where the value of this $pK_a$ is also 6.4 ± 0.1 (26). This result suggests that the thiolate form of the peptide cysteine is present at physiological pH in the GGTase I-GGPP-peptide complex and that Zn(II) plays a similar role in both FTase and GGTase I, that of stabilizing and orienting the thiolate form of the cysteine of the peptide substrate (26).

Effect of Alterations at Lys-311 on Catalysis—To investigate whether Lys-311 in GGTase I is responsible for the different catalytic properties of FTase and GGTase I, we examined the catalytic properties of mutants at this position. Removal of the lysine side chain (K311A GGTase I) decreases the geranylgeranylation rate constant (0.0044 ± 0.0004 s⁻¹) 40-fold compared with wild type GGTase I (0.18 ± 0.02 s⁻¹) in the absence of Mg(II) (Fig. 5). These data demonstrate that the positively charged side chain of Lys-311 stabilizes the transition state for prenylation catalyzed by GGTase I and partially replaces the Mg(II) stabilization observed with FTase. In fact, this side chain more than accounts for the 20-fold enhancement of prenylation catalyzed by GGTase I compared with FTase in the absence of Mg(II). However, the geranylgeranylation rate constant in the absence of Mg(II) for the K311D mutant (0.022 ± 0.007 s⁻¹) is 5-fold faster than the K311A mutant, suggesting perhaps that van der Waals interactions and the positive charge of the side chain at position 311 each enhance prenylation by 5–10-fold. Moreover, K311A and K311D mutants of GGTase I are activated by Mg(II); K311A GGTase I is accelerated ~70-fold in the presence of saturating Mg(II) with a $K_{\text{Mg}} = 140 ± 10$ mM, whereas K311D GGTase I is accelerated ~40-fold in the presence of saturating Mg(II) with a $K_{\text{Mg}} = 6.4 ± 0.8$ mM. Interestingly, the rate constants reflecting geranylgeranylation catalyzed by K311A and K311D GGTase I mutants in the presence of saturating magnesium concentrations are both faster than wild type GGTase I (0.16 ± 0.02 s⁻¹) with $k_{\text{max}}$ values of 0.33 ± 0.01 and 0.84 ± 0.03 s⁻¹, respectively (Fig. 5). However, these rate constants are still slower than the maximal farnesylation rate constant catalyzed by FTase in the presence of magnesium. Furthermore, the
these data together suggest that the magnesium ion likely
observed for wild type FTase (data not shown; 31, 32). All of
GGPP. Similarly, farnesylation catalyzed by GGTase I is de-
shown) only 5-fold more slowly than geranylgeranylation using
The dissociation constants for dansylated GCVLL were
determined by fluorescence anisotropy (KD = 0.8 ± 0.2 nM), K
pitent, Table I), suggesting that the lysine side chain stabilizes

dication of Mg(II) Dependence of GGTase I Mutants

Therefore, Table I), suggesting that the lysine side chain stabilizes

| GGTase I | KdGPP | KdGGPP | kcat/GPP-Me<sub>max</sub> | K<sub>Mg</sub> | kcat/GPP<sub>max</sub> | kcat/FPP<sub>max</sub> |
|----------|-------|--------|--------------------------|------------|-----------------|-----------------|
| Wild type| 0.8 ± 0.2 | 15 ± 2<sup>a</sup> | 0.18 ± 0.02<sup>b</sup> | 0.16 ± 0.02<sup>d</sup> | >50<sup>c</sup> | (33 ± 8) × 10<sup>-3</sup> |
| Kβ311A | 1.6 ± 0.5 | 30 ± 3 | 0.0044 ± 0.0004 | 0.33 ± 0.01 | 140 ± 10 | (0.96 ± 0.08) × 10<sup>-3</sup> |
| Kβ311D | 1.1 ± 0.2 | 20 ± 2 | 0.022 ± 0.007 | 0.84 ± 0.03 | 6.4 ± 0.8 | (3.4 ± 0.6) × 10<sup>-3</sup> |

<sup>a</sup> Peptide dissociation constants were measured with dansylated GCVLL at pH 7.8.
<sup>b</sup> k<sub>c</sub> is the single turnover rate constant for product formation at pH 7.8 in the absence of MgCl<sub>2</sub>.
<sup>c</sup> k<sub>Mg</sub> is the single turnover rate constant for product formation at pH 7.8 at saturating concentrations of MgCl<sub>2</sub>.
<sup>d</sup> k<sub>cat</sub> at 50 mM MgCl<sub>2</sub>.
<sup>e</sup> k<sub>cat</sub> at 175 mM MgCl<sub>2</sub>.

**Fig. 2. Affinity of wild type and mutant GGTase I for dansylated GCVLL.** The dissociation constants for dansylated GCVLL were determined by fluorescence anisotropy (λ<sub>ex</sub> = 340 nm, λ<sub>em</sub> = 525 nm), as described under “Experimental Procedures.” Assays contained 2 nM dansylated GCVLL in 50 mM Hepes, pH 7.8, 2 mM TCEP, and 10 mM EDTA. GGTase I (wild type (A), Kβ311A (B), or Kβ311D (C)) and additional dansylated GCVLL were titrated into the peptide solution. The values of K<sub>d</sub> shown in Table I were calculated from a weighted fit of Equation 2 to these data.

**Scheme 1.** Basic one-step peptide binding mechanism for GGTase I.

![Image](https://example.com/image1)

**Fig. 3. Single turnover kinetics of wild type GGTase I.** Single turnover rate constants were measured as described under “Experimental Procedures.” Assays contained 0.8 μM GGTase I, 0.4 μM [1-<sup>3</sup>H]-GGPP, 100 μM GCVLL, 50 mM Hepes, pH 7.8, and 2 mM TCEP, with ionic strength kept constant at 0.2 M with NaCl. Assays were performed in the absence of Mg(II) (A), in the presence of 50 mM EDTA (B), and in the presence of 50 mM Mg(II) (C). The data were normalized for clarity using the formula (fraction product < fraction product<sub>max</sub>)/(fraction product<sub>max</sub> - fraction product<sub>min</sub>). The geranylgeranylation rate constant k<sub>cat</sub> was calculated by a fit of Equation 3 to the data for these three conditions to be 0.18 ± 0.02, 0.18 ± 0.02, and 0.15 ± 0.02 s<sup>-1</sup>, respectively.

**Fig. 4. pH dependence of k<sub>cat</sub> for geranylgeranylation of wild type GGTase I.** The single turnover rate constant at saturating concentrations of GGTase I and GCVLL was measured as described in the legend of Fig. 3 at pH 6–9 using the buffers described under “Experimental Procedures.” The pH<sub>K</sub> of the single ionization was calculated to be 6.4 ± 0.1 using Equation 5.

Mg(II) dependence of GGTase I Mutants

apparent magnesium affinity of these mutants is only slightly weaker than FTase with the comparable side chain at this position, K<sub>Mg</sub> = 4.0 ± 0.3 mM and K<sub>Mg</sub> = 110 ± 30 mM for wild type and Dβ352A FTase, respectively (31, 32). For Kβ311D GGTase I, like wild type FTase (31, 32), the apparent magnesium affinity, K<sub>Mg</sub>, and the chemical rate constant, k<sub>cat</sub> vary little as the pH varies from 7.8 to 8.3 (data not shown). However, the value of K<sub>Mg</sub> increases as pH decreases, as is also observed for wild type FTase (data not shown; 31, 32). All of these data together suggest that the magnesium ion likely binds to the Kβ311D in a position similar to that observed for FTase (Fig. 6B), interacting with the diphosphate of GGPP and the side chain of Kβ311D.

To determine whether the Mg(II) dependence of prenylation varies with the length of the prenyl chain, we also measured the reactivity of GGTase I with FPP. Wild type GGTase I catalyzes farnesylation using FPP (0.033 ± 0.008 s<sup>-1</sup>, data not shown) only 5-fold more slowly than geranylgeranylation using GGPP. Similarly, farnesylation catalyzed by GGTase I is decreased to an extent comparable with geranylgeranylation by the Kβ311A and Kβ311D mutants (34-fold and 10-fold, respectively, Table I), suggesting that the lysine side chain stabilizes the farnesylation and geranylgeranylation transition states similarly. In these mutants, the addition of Mg(II) also enhances farnesylation to rate constants that are comparable with or faster than those catalyzed by wild type GGTase I. Addition of 175 mM Mg(II) increases farnesylation catalyzed by Kβ311A GGTase I by ~30-fold to 0.032 ± 0.006 s<sup>-1</sup> and Kβ311D GGTase I by ~60-fold to 0.20 ± 0.04 s<sup>-1</sup>. The rate constants for farnesylation catalyzed by wild type, Kβ311A, and Kβ311D GGTase I are generally 5–10-fold slower than their respective geranylgeranylation rate constants, indicating that mutations at position β311 do not affect the selectivity of GGTase I for the isoprenoid substrate.

Effect of Mutagenesis on Steady-state Turnover—The steady-state parameter, k<sub>cat</sub>, was measured using a continuous fluorescence assay with a dansylated peptide substrate (43, 44, 48).
Faster product release rate constant observed for K parameter reflects a step that occurs after product is formed, such as product dissociation (46). The K parameter is consistent with the slightly lower affinity of this mutant for the GGPP and peptide substrates. If this mutant also has a lower affinity for the geranylgeranylated product, the product dissociation rate constant, and kcat, should also increase. Because the Kβ311A mutation has no effect on kcat, the lysine side chain neither facilitates product release nor stabilizes the affinity of the prenylated peptide product. This is a reasonable result because we propose that the lysine side chain interacts with the diphosphate leaving group.

**DISCUSSION**

**Function of Lys-β311 in Rat GGTase I**—The lysine side chain at position β311 activates prenylation catalyzed by GGTase I (Table I). In the absence of magnesium, the Lys-β311 side chain in GGTase I stabilizes the free energy change of the single turnover transition state by 9.2 kJ mol⁻¹ compared with that of Kβ311A GGTase I where the side chain is deleted.

\[
\Delta \Delta G^\ddagger = \Delta G^\ddagger_{\text{WT}} - \Delta G^\ddagger_{\text{MU}} = -RT \ln(k_{\text{cat,WT}}/k_{\text{cat,MU}}) \quad (\text{Eq. 7})
\]

Likewise, ΔΔG‡ for the prenylation step catalyzed by GGTase I is stabilized by 4.9 kJ mol⁻¹ compared with FTase in the absence of Mg(II). These values for ΔΔG‡ suggest that the lysine side chain at position β311 enhances stabilization of the prenylation transition state by GGTase I. In FTase, the developing negative charge on the diphosphate in the farnesylation transition state is stabilized by both positively charged side chains and a magnesium ion that coordinates with the diphosphate group (29, 31, 32). The side chain of Lys-β311 in GGTase I could similarly stabilize the developing negative charge on the diphosphate leaving group. In the crystal structure of the GGTase I ternary complex (GGTase I 3-azaGGPP-peptide), the charged amino group of Lys-β311 is located 5.3 Å from one of the nonbridging oxygens of the β-phosphate of 3-azaGGPP (Fig. 6A). This is suboptimal positioning of the lysine side chain for stabilization of the developing charge on the diphosphate.

In fact, the prenylation rate constant catalyzed by wild type GGTase I is slower than wild type FTase as well as the Kβ311D GGTase I mutant in the presence of saturating Mg(II), suggesting that the lysine side chain at position β311 of GGTase I does not stabilize the developing charge as effectively as the diphosphate-coordinated Mg(II) ion in FTase and Kβ311D GGTase I (32). In the presence of saturating Mg(II), ΔΔG‡ for GGTase I is destabilized by −9.7 kJ mol⁻¹ compared with FTase, indicating that lysine does not stabilize the transition state as effectively as Mg(II). This diminished stabilization of the developing charge on the diphosphate leaving group is likely related to the decreased positive charge of the lysine side chain compared with Mg(II).

**Mg(II) Binding Site**—Rat FTase and GGTase I are highly related enzymes; they share a common α-subunit, and the β-subunits are highly homologous with 25% identical and 32% similar amino acids (12). Nonetheless, these enzymes have different preferences for both the isoprenoid and the peptide substrates (27). All three enzymes in the protein prenyltransferase family require a divalent zinc ion for catalysis, and the amino acids that coordinate zinc are strictly conserved. This conservation, along with a variety of biochemical and structural data, suggests that these enzymes share a common catalytic mechanism where the zinc ion coordinates the sulfur of the peptide substrate (12, 26, 31, 35, 50, 51). This interaction enhances catalysis by lowering the pKcat of the peptide thiol, as well as positioning and, perhaps, activating the thiolate for nucleophilic attack on the isoprenoid diphosphate (26, 31, 51).

In contrast, the protein prenyltransferases have different requirements for magnesium; FTase and GGTase II are activated by magnesium, whereas the activity of GGTase I is unaffected by the addition of magnesium (35) (Fig. 5). Biochem-

**FIG. 5. Comparison of the single turnover kinetics of wild type and mutant GGTase I at various Mg(II) concentrations.** The single turnover rate constant at saturating concentrations of GGTase I (wild type (A), Kβ311A ( ), or Kβ311D ( )) and GCVLL was measured as described in the legend of Fig. 3 at concentrations of Mg(II) varying from 0.0006 to 175 mM in 50 mM Hepes, pH 7.8, 2 mM TCEP. The ionic strength was maintained at 0.2 M with NaCl up to 60 mM Mg(II). The values for kmax and Kcat were determined by a weighted fit of Equation 4 to the data and are listed in Table I.

**FIG. 6. Models of active site residues of FTase and GGTase I.** A, crystallographic model of GGTase I active site residues with bound 3-aza-GGPP and peptide KKKSTKCVIL (Protein Data Bank code 1N4Q) (12). B, model of proposed active ternary complex of FTase (32) with bound magnesium created by overlaying the two crystal structures FTase-FPP-L-759,750 (Protein Data Bank code 1JQ) (52) and FTase-FPT inhibitor II-KRas peptide (Protein Data Bank code 1D8D) (50) and then adjusting the position of the diphosphate moiety.
ical and mutagenesis studies have demonstrated that in the FTase-FPP-peptide ternary complex magnesium is coordinated by the diphosphate of FPP and the carboxylate side chain of Asp-β352 and postulated to interact with the side chain of Asp-β297 and a water molecule (32, Fig. 6B). Replacement of Asp-β352 by lysine completely abrogates activation by magnesium. Here we have demonstrated the reciprocal relationship in GGTase I. In wild type GGTase I, a lysine is substituted at the position homologous to Asp-β352 (Lys-β311), and no activation by magnesium is observed (Fig. 5). However, when Lys-β311 is altered to aspartate, the activity of this GGTase I mutant is enhanced by magnesium, indicating that a magnesium binding site has now been restored. These data demonstrate that the aspartate to lysine substitution at this position is the main determinant of the differential magnesium dependence in these two enzymes. However, this is likely not the sole structural difference, as the GGTase I Kβ311D mutant still has a 1.5-fold weaker affinity for magnesium and a 10-fold lower prenylation rate constant than wild type FTase.

**Structure of Active Ternary Complex**—The crystal structures of FTase and GGTase I indicate that the substrates bound in their respective ternary and product complexes have a similar overall conformation (12, 50, 52). The first three isoprene units of 3-aza-GGPP are arranged along a straight line in the central cavity of the β-subunit of GGTase I, with the first isoprene unit shifted 1 Å relative to the crystal structure of an FPP analog bound to FTase (12). Unlike the extended isoprenoid conforma-

tion observed for FTase, the fourth isoprene unit of 3-aza-

GGPP is observed as orthogonal to the axis formed by the first three in the crystal structure of GGTase I (12). In both FTase and GGTase I, the structures of the ternary complexes depict inactive substrate conformations in which the carbon-1 of the prenyl diphosphate is located 7–8 Å away from the peptide 

thiolate. Structures of the product complexes indicate that the first two isoprene units of the isoprenoid substrate rotate to place the carbon-1 of the prenyl diphosphate within reacting distance of the cysteine sulfur of the peptide substrate (12, 36, 53). The observed, inactive substrate conformation likely reflects an intermediate along the reaction pathway in both of these enzymes. Potentially, the conformational change required to form the active substrate conformation could be slow and limit the prenylation rate constant. However, this is not likely to limit prenylation catalyzed by either wild type FTase or GGTase I because fluorinated substrates (monofluoromethylfarnesyl diphosphate or 2-fluorogeranylgeranyl diphosphate) decrease the prenylation rate constant significantly (60-fold)² (30).

Mutagenesis studies indicate that the position of the diphosphate moiety of FPP also changes in the active substrate complex, accompanying the rotation of the isoprene units of farnesyl diphosphate (53). In particular, these data suggest that the hydroxyl group of Tyr-β300 forms a hydrogen bond with an oxygen attached to the α-phosphate, rather than the β-phosphate, of FPP, whereas Lys-α164 interacts with a β-phosphate oxygen. Pickett and colleagues (32) also suggested that the formation of the active substrate conformation in FTase is coupled to the formation of the catalytic Mg(II) binding site because in the crystal structure of the substrate ternary complex the groups that are proposed to form the magnesium binding site, including the side chain carboxyl group of Asp-β352 and the diphosphate mimic of FPP, are located ~7 Å apart (32). To account for all of these data, a model of the structure of the substrates in an active conformation bound to FTase was developed (32, Fig. 6B). In this model, the groups that coordinate Mg(II) in FTase consist of one oxygen of the carboxyl side chain of Asp-β297, two oxygens of the carboxyl side chain of Asp-β352, two diphosphate oxygens of FPP, and a water molecule (32). Residue Asp-β352 in FTase corresponds to residue Lys-β311 in GGTase I, as discussed previously. The similar response of FTase and GGTase I to mutations (Lys, Asp, and Ala) at this site suggests that the prenyl diphosphate may be bound in a similar position in the active ternary complex in both enzymes. When the diphosphate of GGPP is moved to a similar position, the side chain of Lys-β311 moves to within hydrogen bonding distance of one of the β-phosphoryl oxygen atoms of GGPP where it is positioned to stabilize the developing negative charge on the diphosphate leaving group.

However, the prenylation rate constant and the magnesium affinity of the Kβ311D mutation in GGTase I are not identical to that of wild type FTase. This could be caused by alterations in the structure of the active ternary complex or by additional amino acid changes between the two enzymes. Most of the active site residues are conserved between GGTase I and FTase (Fig. 6). However, a number of the residues near the proposed Mg(II) binding site for FTase are not conserved in GGTase I (32). Sequence alignments and crystal structure data indicate that residues Asp-β297, Asp-β352, Lys-β353, and Lys-β356 of FTase correspond to the respective residues Asp-β269, Lys-

β311, Trp-β312, and Ser-β315 in GGTase I. Amino acid substitution at any of these sites could affect the position and affinity of the prenyl diphosphate or magnesium ion bound to the Kβ311D GGTase I mutant. In particular, Lys-β353 is completely conserved in FTase and proposed to form a hydrogen bond with a magnesium-bound water to enhance the magnesium affinity (32, Fig. 6B). Therefore, substitution of Trp-β312 with lysine in GGTase I could serve to increase the Mg(II) affinity of the enzyme by orienting the proposed Mg(II)-bound water molecule in the correct location and, perhaps further increase the catalytic efficiency of GGTase I.

**Implications for Peptide Specificity**—As discussed, substitution of the magnesium cofactor in FTase with a lysine residue in GGTase I decreases the prenylation rate constant for GGTase I compared with FTase. This decreased prenylation rate constant may have important implications for the in vivo specificity of farnesylation compared with geranylgeranylation in light of the dual substrate specificity for a number of CaaX proteins (25). In general, the ratio of farnesylation to geranylgeranylation should depend on the ratio of V_max/K_m for FTase and GGTase I for a given peptide substrate. The decreased prenylation rate constant of GGTase I also leads to a decrease in V_max/K_m in many cases, allowing FTase to compete effectively for farnesylation of these substrates. This situation would also allow for GGTase I to compensate for FTase if FTase-catalyzed prenylation becomes slow or inhibited, such as has been observed with the substrate K-Ras4B (25). Moreover, GGTase I can utilize FPP as a substrate, suggesting that even if FTase activity were repressed, farnesylation could still occur, albeit slowly.

**Mg(II) Regulation**—We speculate that the substitution of Lys-β311 activation in GGTase I for Mg(II) activation in FTase could allow for regulation of these enzymes by changes in the concentration of Mg(II). For example, a dual specificity substrate could be farnesylated at high concentrations of magnesium and geranylgeranylated at low magnesium, which could affect the fate of the cell. The regulation of cellular Mg(II) is not well understood, nor is it yet known whether changes in magnesium concentrations influence cellular signal transduction (54). However, intracellular concentrations of Mg(II) have been shown to be affected by such factors as transport, sequestration in organelles, and binding, particularly to ATP (55). ATP is hypothesized to play an important role in storing and releasing Mg(II); perhaps the ambient concentration of Mg(II) changes in
response to signaling events that affect metabolic activity and alter the concentration of ATP (55).

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Lysine β311 of Protein Geranylgeranyltransferase Type I Partially Replaces Magnesium
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