Mutagenesis Studies of Protein Farnesyltransferase Implicate Aspartate β352 as a Magnesium Ligand*

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Protein farnesyltransferase (FTase) catalyzes the addition of a farnesyl chain onto the sulfur of a C-terminal cysteine of a protein substrate. Magnesium ions enhance farnesylation catalyzed by FTase by several hundred-fold, with a K_M value of 4 mM. The magnesium ion is proposed to coordinate the diphosphate leaving group of farnesylphosphate (FPP) to stabilize the developing charge in the farnesylation transition state. Here we further investigate the magnesium binding site using mutagenesis and biochemical studies. Free FPP binds to FTase, the K_M value of which is reduced by several orders of magnitude upon magnesium binding, whereas FPP-Mn complexes are much more stable. Furthermore, mutation of aspartate β352 to alanine (Dβ352A) or lysine (Dβ352K) in FTase drastically alters the Mg_2+ dependence of FTase activity. In Dβ352A FTase, the K_M increases 10-fold to 110 ± 30 mM, and the farnesylation rate constant at saturating Mg_2+ decreases 27-fold to 0.30 ± 0.05 s^{-1}. Substitution of a lysine for Asp-β352 removes the magnesium activation of farnesylation catalyzed by FTase but does not significantly enhance the rate constant for farnesylation in the absence of Mg_2+. In wild type FTase, Mg_2+ can be replaced by Mn_2+ with a 2-fold lower K_M (2 mM). These results suggest both that Mg_2+ coordinates the side chain carboxylate of Asp-β352 and that the role of magnesium in the reaction includes positioning the FPP prior to catalysis.

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The abbreviations used are: FTase, protein farnesyltransferase; FPP, farnesyl diphosphate; FMP, farnesyl monophosphate; GGTase I and II, protein geranylgeranyl transferase type I and type II; GGTase I also recognizes protein substrates with a C-terminal CaaX motif, but GGTase I substrates most frequently contain a leucine or phenylalanine at the X position (13, 14). FTase and GGTase I are structurally homologous heterodimers with identical α subunits and differing β subunits (15). Prenyltransferases contain an active site zinc ion that is required for activity; FTase and GGTase I also use a magnesium ion to facilitate catalysis (16–19). Surprisingly, GGTase I apparently catalyzes the same prenyl transfer reaction with no dependence on magnesium ions (20).

In FTase, the magnesium ion has been proposed to coordinate to the diphosphate of FPP, but the exact location of the magnesium binding site has not yet been identified (21). The bound zinc ion of FTase coordinates to the cysteine sulfur of the protein substrate, lowering the pK_a to form a zinc-thiolate at neutral pH (22, 23). In the proposed farnesylation transition state of FTase (Scheme 1), positive charge accumulates on the C-1 of FPP, and the diphosphate leaving group develops additional negative charge (24, 25). Bound magnesium is proposed to stabilize the negative charge buildup on the diphosphate moiety and activate the leaving group (21). Although magnesium is not absolutely required for FTase catalysis, millimolar concentrations of magnesium ions enhance the rate constant for product formation by 700-fold (24).

Several pieces of experimental evidence indicate that the diphosphate of FPP forms part of the magnesium binding site. X-ray crystallography of the FTase ternary complex in the presence of manganese showed a manganese ion bound near the diphosphate-like moiety of a FPP analog (26). Also, the
FTase-catalyzed farnesylation using farnesylmonophosphate (FMP) as a substrate is not activated by magnesium ions, indicating that the diphosphate moiety is required for magnesium-induced rate acceleration (21). In addition, the apparent magnesium affinity increases with increasing pH with a pK_m of 7.4, which is consistent with deprotonation of farnesylidiphosphate to enhance magnesium coordination (21). However, other aspects of FTase catalysis indicate that the magnesium binding site may consist of more than the diphosphate of FPP. The magnesium dependence of the reaction has a K_Mg of 2 mM, which is a weaker affinity than one would predict for a magnesium-diphosphate interaction (21). Also, if the magnesium interacts only with the diphosphate, one would predict that the GGTase I-catalyzed reaction should also be magnesium-dependent, since geranylgeranyldiphosphate would presumably have an affinity for magnesium that is similar to FPP.

Analysis of binary FTase-FPP crystal structures and inactive ternary FTase-FPP-peptide crystal structures (27, 28), however, does not reveal residues that are located near the diphosphate group of FPP that could coordinate magnesium. In the crystal structure of an inactive FTase ternary complex with bound manganese, the only manganese ligands observed were the two oxygens of the diphosphate-like moiety of the FPP analog (26). In most enzymes that use a catalytic magnesium ion, there is at least one carboxylate ligand from the enzyme that coordinates the magnesium ion in the active site (29, 30). In the class I terpenoid synthase enzymes, which catalyze a second-order reaction, the two oxygens of the diphosphate-like moiety of the FPP analog (26) would predict for a magnesium-diphosphate interaction (21). Also, if the magnesium interacts only with the diphosphate, one would predict that the GGTase I-catalyzed reaction should also be magnesium-dependent, since geranylgeranyldiphosphate would presumably have an affinity for magnesium that is similar to FPP.

In the crystal structures of inactive ternary complexes of FTase, including the structure with bound manganese, the two reacting atoms, the C-1 of FPP and the peptide thiolate, are 7 Å apart, reflecting an inactive conformation (Fig. 1) (26, 35). An x-ray crystal structure of the FTase-product complex and mutagenesis studies of the diphosphate binding pocket suggest that an active structure may be achieved by a conformational change of FPP in which the first two prenyl groups rotate (37, 38). When this active conformation of FPP bound to FTase is modeled (38), the C-1 of FPP moves to within 2.4 Å of the peptide sulfur nucleophile. Furthermore, the diphosphate moiety moves slightly away from the positively charged diphosphate binding pocket and toward aspartate 352. This amino acid has not been previously studied by mutagenesis because it is not conserved in all prenyltransferases and is located −7 Å away from the FPP substrate in the ternary complex crystal structures (Fig. 1). In FTase and GGTase II, an aspartate is conserved across all organisms at position 352 (FTase) and 280 (GGTase II), whereas GGTase I has a conserved lysine at the corresponding position 311 (39). Long et al. (37) proposed that this lysine residue in GGTase I replaces magnesium in the geranylgeranylation reaction by appropriate placement of a positive charge. Therefore, the analogous aspartate residues are proposed to function as magnesium ligands in FTase and GGTase II.

In this study, we examine the role of Asp-β352 in magnesium binding through mutagenesis and biochemical experiments. These data demonstrate that the FTase-FPP-peptide ternary complex has a weaker affinity for magnesium than FPP alone in solution, indicating that the protein side chains modulate the magnesium affinity of the diphosphate moiety. Mutation of Asp-β352 to Ala has no effect on the rate constant for product formation in the absence of magnesium; however, the apparent magnesium affinity, K_Mg, decreases 28-fold, and the magnesium-activated farnesylation rate constant decreases 27-fold. The farnesylation rate constant catalyzed by the Dβ352K mutant is not enhanced by the addition of magnesium ions. Furthermore, the positive charge of the lysine side chain does not substitute for Mg2+ to enhance the farnesylation rate constant. Manganese, although more electrophilic, has only a modestly enhanced affinity for the FTase ternary complex than magnesium. In summary, these data provide evidence that at least one FTase side chain, Asp-β352, coordinates a catalytic Mg2+ in addition to coordination by the substrate diphosphate. Furthermore, the positively charged groups in the diphosphate binding pocket of FTase decrease the Mg2+ affinity of FPP more than 10-fold. Finally, the formation of the catalytic magnesium site is coupled to the formation of the active substrate conformation of FPP bound to FTase, where the diphosphate of FPP moves out of the diphosphate binding pocket toward Asp-β352, whereas C-1 of FPP moves within reacting distance of the zinc thiolate (37, 38). Therefore, Mg2+ may accelerate catalysis both by stabilizing developing negative charge in the transition state and by stabilizing the active site conformation prior to catalysis.

**EXPERIMENTAL PROCEDURES**

*Miscellaneous Methods*—All assays were performed at 25 °C. All curve fitting was performed with Kaleidagraph (Synergy Software).
FTase Magnesium Ligand Asp-352

[1H]FPP was purchased from Amersham Biosciences, vacuum-dried, washed with 100% methanol, and resuspended in 20 mM Hepes, pH 7.8, 0.5 mM 3–14 zwittergent, to a final concentration of 20 μM. [1H]FPP was obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO). The peptides GCVLS and danylated GCVLS were synthesized and purified by high pressure liquid chromatography by Bethyl Laboratories, Inc. (Montgomery, TX) and the University of Michigan Peptide Core (Ann Arbor, MI), respectively. The concentration of peptide was determined spectroscopically at 412 nm by reaction of the cysteine thiol with 5,5′-dithio-bis(2-nitrobenzoic acid), using an extinction coefficient of 14,150 M⁻¹ cm⁻¹ (40). FTase concentration was determined by absorbance at 280 nm using an extinction coefficient of 150,000 M⁻¹ cm⁻¹ (41). Farnesyl protein transferase inhibitor II (I2) was purchased from Calbiochem-Novabiochem. TLC plates were prerun in 100% acetone before use.

Magnesium Binding Affinity for FPP—The affinity of the diphosphate of FPP for Mg²⁺ in the absence of enzyme was determined by competition assays in which the fluorescence of the Mg²⁺-5′-dihydroxyquinoline-5-sulfonic acid (HQS) complex decreases as the concentration of FPP increases, due to the formation of the nonfluorescent Mg²⁺-FPP complex. Increasing concentrations of FPP (0–2 mM) were titrated into a cuvette containing 30 mM MgCl₂ and 240 μM HQS in 50 mM Tris, pH 8, buffer. The fluorescence of the Mg²⁺-HQS was measured with an excitation wavelength of 390 nm and an emission wavelength of 500 nm (42, 43). The dissociation constant for magnesium binding to FPP was determined by fitting Equation 1 to the data, where ΔFL represents the observed fluorescence, IF is the initial fluorescence, EP is the fluorescence end point, Ko₅ is the dissociation constant for Mg²⁺ binding to HQS, and Kₘ₀ is the dissociation constant for Mg²⁺ binding to HQS. The Kₘ₀ was determined to be 240 μM by measuring the dependence of fluorescence on the magnesium concentration (not shown).

\[
\Delta FL = \frac{IF}{1 + \frac{K_{MI}}{[HQ5]}} \left(1 + \frac{[Mg2+]}{K_o}\right)^{-1} + EP \quad (\text{Eq. 1})
\]

Preparation of FTase—Mutagenesis of the FPT/pET23a plasmid (44) was performed using the QuikChange™ site-directed mutagenesis kit (Stratagene) with the following codon changes: Dj352A, GAC to GCC; and Dj352K, GAC to AAA. The changes were confirmed by DNA sequencing (University of Michigan DNA Sequencing Core). Recombinant rat FTase was overexpressed in Esherichia coli BL21(DE3) cells and purified as described previously (38, 44). After each column, the protein fractions were tested for FTase activity in a 15-well plate assay as described (38). The purified FTase 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 40 μM, and frozen at −80 °C.

Transient Kinetics—Single turnover assays were performed for FTase mutants Dj352A and Dj352K at varying magnesium or manganese concentrations in 50 mM Hepes, pH 7.8, as described previously (21). The MgCl₂ or MnCl₂ concentration was varied from 0.01 to 60 mM. The ionic strength in the reactions was kept constant at 0.2 M with the addition of NaCl. The reaction rates were determined using 0.8 μM FTase, 0.4 μM [1H]FPP, and 100 μM GCVLS (8-μl reaction volume). FTase was presaturated with [1H]FPP for 15 min, and then the reaction was initiated by the addition of GCVLS. The reactions were quenched at varying times (3 s to 3 h) by the addition of 8 μl of cold isopropanol alcohol and placed on ice. For reactions with product formation rates faster than 0.1 s⁻¹, a KinTek rapid quench apparatus was used (KinTek Corp., Austin, TX). The 30-μl reactions contained 0.2 μM FTase, 0.1 μM [1H]FPP, and 100 μM GCVLS. The reactions were quenched with 80% isopropanol alcohol, 20% acetic acid at varying times (0.005–600 s) then dried under vacuum and resuspended in 50% isopropanol alcohol.

The product was separated from substrate by thin layer chromatography on polyester-backed silica gel plates (Whatman PE SIL G) with an 8:1:1 (v/v/v) isopropanol alcohol/NH₄OH/H₂O mobile phase. The product migrates in this mobile phase, but the FPP substrate remains at the origin, so the plates were cut accordingly, and the radioactivity was quantified by scintillation counting. The radioactivity in the product was divided by the total radioactivity for each time point to calculate the fraction of product formed. The rate constant for product formation (kₘₐₓ) was determined by fitting Equation 2 to these data, where Pᵢ is the product formed at time t, and Pₖ is the calculated reaction end point, which varied from 65 to 90%.

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P_k = \frac{P_i}{1 - e^{-k_{max}t}}
\]

The magnesium or manganese dependence was determined by plotting the kₘₐₓ against the metal concentration and fitting Equation 3 to the data. Kₘₐₓ/Kₘₐₙ represents the apparent dissociation constant, kₘₐₓ/kₐₘₙ is the rate constant of the reaction at saturating metal concentration, and kₘₐₙ is the rate constant of the reaction in the absence of magnesium or manganese.

\[
k_{obs} = \frac{k_{max}}{1 + K_{MI}[Mg^{2+}]} + k_{n}
\]

Steady State Kinetics—Steady state kinetic assays were performed with 24 mM wild type or mutant FTase, 1 μM [1H]FPP, and 10 μM GCVLS in 50 mM Hepes, pH 7.8, 1 mM TCEP, and 5 mM MgCl₂ as described previously (24). Under these conditions, both substrates are saturating, and a linear fit of the first 10% of the reaction reflects kₜₐₙ. Reaction with FMP—The reactions with [1H]FPP were measured at 6.1, the optimum pH of the reaction for wild type FTase (21). The assays were done as described above for [1H]FPP, with 50 mM Mes, pH 6.1, in place of 50 mM Hepes, pH 7.8, as the buffer. The TLC plates were run in a 7:2:1 (v/v/v) isopropanol alcohol/NH₄OH/H₂O mobile phase and treated as described above for the reaction with FPP. Equation 2 was fit to these data.

Peptide Binding Affinity—The affinity of the D352β mutants for the peptide substrate was determined as previously described by measuring the affinity of FTase-I2 for danyl-GCVLS (Dns-GCVLS) (22). The binding of Dns-GCVLS to FTase was observed by fluorescence resonance energy transfer, where the tryptophan residues of FTase are excited at 280 nm and the bound danyl group emits at 496 nm. The samples were prepared with 50 mM Hepes, pH 7.3, 2 mM TCEP, 1 mM MgCl₂, 40 mM NaCl, 20 mM Dj352A or Dj352K FTase, and 10 mM EDTA. The ionic strength of the samples was maintained at 0.1 M with NaCl. The sample was preincubated for 15 min and then aliquots of Dns-GCVLS (0–4 μM) were added with a 2-min stirred and a 1-min un-stirred incubation prior to each measurement. A weighted fit of Equation 4 to the data yielded the apparent dissociation constants, where ΔFL represents the observed fluorescence corrected for background, EP is the fluorescence end point, IF is the initial fluorescence, [Pep] is the concentration of Dns-GCVLS, and Kₚₑₚ is the dissociation constant for Dns-GCVLS.

\[
\Delta FL = \frac{EP}{1 + K_{Pep}^2 / [Pep]} + IF
\]

FPP Binding Affinity—The affinity of FPP for Dj352β mutant FTase was determined by equilibrium dialysis as previously described (24, 44). A 1-mL solution of 10–200 mM Dj352A or Dj352K FTase was preincubated with 20 mM [1H]FPP for 3 h at room temperature in buffer (50 mM Hepes, pH 7.5, 5 mM MgCl₂, and 1 mM TCEP). This solution was then dialyzed against 1 ml of the same buffer using a SpectraPor 25,000 molecular weight cut-off dialysis membrane. After 22 h, the radioactivity in several 100-μl samples from both sides of the membrane was determined by scintillation counting and averaged. The E-FPP concentration was determined by subtracting the counts on the buffer side from the counts on the enzyme side, dividing the difference by the total counts to get the fraction E-FPP, and then multiplying the fraction E-FPP by the initial FPP concentration (20 mM). The dissociation constant, Kₙ, was then determined by fitting Equation 5 to a plot of [FTase] versus [E-FPP]. The total FPP concentration was maintained at 20 mM.

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[E \cdot FPP] = \frac{(20 \text{ mM} + [\text{FTase}]) - \sqrt{(20 \text{ mM} + [\text{FTase}] + K_n^2 - (4 \times 20 \text{ mM} \times [\text{FTase}])}}}{2}
\]

RESULTS

Magnesium Affinity for FPP—Previous data have demonstrated that millimolar concentrations of magnesium enhance FTase catalysis several hundred-fold (K = 2 mM) (21). This enhancement has been proposed to be due to coordination of
FTase Magnesium Ligand Asp-β352

The dissociation constant of magnesium binding to FTase was determined by the addition of FPP to a fluorescent Mg$^{2+}$-HQ5 complex as described under "Experimental Procedures." A $K_D$ of 120 ± 10 μM was obtained by a fit of Equation 1 to the data.

Mg$^{2+}$ by the diphasophate of FPP, which stabilizes the developing charge in the transition state. Therefore, a first prediction would be that the $K_M$ for activation of FTase should be similar to the affinity of free FPP for Mg, if the protein does not significantly modulate this binding site. However, other small molecules containing diphasophate moieties coordinate magnesium with a significantly higher affinity than observed for FTase (such as ADP, $K_D^{ADP}=250 \mu M$, pH 8.0) (45). To determine how tightly magnesium binds to the FPP substrate in the absence of enzyme, the affinity of FPP for magnesium ($K_D^{Mg}$) was determined by competition using the fluorescent magnesium chelator HQS to be 120 ± 10 μM (Fig. 2). An identical $K_D^{Mg}$ value was determined for the compound dimethylallyldiphosphate (data not shown), suggesting that the high Mg$^{2+}$ affinity of FPP is not due to the formation of micelles. Therefore, the apparent affinity of the FTase ternary complex for Mg$^{2+}$ determined from kinetic studies is at least 10-fold weaker than the affinity of FPP for magnesium ions in the absence of enzyme, signifying that the enzyme modulates the magnesium binding site. The positively charged residues in the diphasophate binding pocket could certainly decrease the FPP-Mg$^{2+}$ interaction.

Mutagenesis of Asp-β352—Previous phylogenetic and structural data have suggested that the side chain of Asp-β352 could act as a magnesium ligand in FTase, similar to magnesium binding sites in other enzymes (37–39). To test this possibility, the aspartate at position 352 was changed to an alanine to remove the carboxylate functionality from the active site (D352A). The Asp-β352 in FTase was also mutated to lysine, the amino acid at the same position in GGTase I, to see if the addition of a positive charge in the active site at this position could accelerate the reaction in place of magnesium (D352K).

The mutant enzymes had expression levels in E. coli comparable with wild type (30 mg/liter of cell culture), and behaved similarly to the wild type enzyme during purification.

Ligand Binding Affinity—To begin characterization of the properties of these mutants, the binding affinity of the peptide substrate Dns-GCVLS was determined by fluorescence. The $K_D^{CVLS}$ values for mutant enzymes D352A and D352K were within 2-fold of the wild type value of 120 ± 10 nM (see Table I) (22). Equilibrium dialysis measurements determined that the affinity of the D352A mutant for FPP (21 ± 4 nM) was decreased 3-fold compared with the wild type value (6.5 ± 1.7 nM) (24), whereas the affinity of the D352K for FPP (60 ± 16 nM) was decreased 10-fold compared with wild type. These alterations in binding affinity could be caused by either alterations in the electrostatic environment in the active site or changes in the van der Waals volume of the side chains (decrease in D352A and increase in D352K) that slightly alter the structure of the active site (46). Nevertheless, these results show that changing the aspartate side chain to an alanine has a modest effect on ligand affinity, indicating that mutation of Asp-β352 does not dramatically alter the enzyme affinity for either substrate through disruption of a direct interaction of the substrates with the carboxylate side chain.

Effect of Mutagenesis on Product Formation—To determine the role of the Asp-β352 residue in the transition state of the FTase reaction, single turnover kinetic studies were used to measure the rate constant of product formation ($k_{cat}$), and steady state kinetic methods were used to determine the rate constant for multiple turnover reactions ($k_{cat}^M$). For mammalian FTase, $k_{cat}$ reflects the rate constant for product dissociation (47); therefore, it is necessary to measure the rate constant of the chemical step ($k_{ch}$) under single turnover conditions where the enzyme concentration is higher than the FPP concentration. Mutagenesis of Asp-β352 has little effect on either $k_{cat}$ or the magnesium-independent farnesylation rate constant. The rate constant of product formation in the absence of magnesium ($k_0$) of the FTase mutant D352A is equal to the $k_0$ value for wild type FTase, whereas the $k_0$ of D352K FTase is enhanced less than 2-fold compared with the wild type value (Table I). This indicates that mutation of Asp-β352 has little effect on the difference between the ground state ternary complex and the farnesylation transition state of the enzyme in the absence of magnesium. Furthermore, the lysine side chain at position 352 only modestly enhances catalysis and is not able to functionally replace Mg$^{2+}$ in this reaction. The D352A mutant also has a $k_{cat}$ value identical to that of wild type FTase (0.017 ± 0.002 s$^{-1}$), whereas D352K has a $k_{cat}$ value that is 6-fold slower (0.0027 ± 0.0003 s$^{-1}$). The slower $k_{cat}$ observed for D352K is consistent with the slightly higher affinity of this mutant for the peptide substrate; if the mutant also has a higher affinity for the farnesylated peptide, the product dissociation rate constant would be attenuated. The D352A mutation, however, has no effect on the product release rate constant, so the aspartate side chain at position 352 is not involved in catalyzing the reaction in the absence of magnesium or in facilitating product release.

Reactivity of FMP—Single turnover kinetic experiments using FMP as a substrate were performed with the D352A and D352K mutant enzymes to further investigate the effect of mutation of Asp-β352 on the transition state of the FTase-catalyzed farnesylation reaction. FTase catalysis of FMP is magnesium-independent in the wild type and the Asp-β352 mutants and has a pH maximum of 6.1 (21) (data not shown). The rate constant for product formation with the FMP substrate ($k_{cat}^{FMP}$) for D352A FTase is similar to the value for wild type FTase, whereas the $k_{cat}^{FMP}$ value for D352K FTase is decreased 3.5-fold (Fig. 3, Table I). The slight decrease in the rate constant in the D352K mutant suggests that the lysine at this position interferes with the catalytic reaction to some extent, perhaps by altering the position of the bound FMP. However, the absence of any effect on FMP catalysis by the D352A mutation further intimates that the aspartate side chain does not have a direct role in stabilizing the chemical step of farnesylation.

Magnesium Dependence of Catalysis—The rate constant for product formation for wild type FTase at pH 7.8 increases 324-fold with increasing magnesium concentrations such that the concentration of magnesium at half-maximal reactivity ($K_M^{mag}$) is 4 mM (21). The value of $K_M^{mag}$ is weak and measured under single turnover conditions; hence, this measurement...
FTase Magnesium Ligand Asp-β352

Table I

| FTase       | k_{max} | k_{max} | k_0 | K_{Mg} | K_{Mn} | k_{cat} | K_{FMP}^{chem} | K_{FMP}^{tail} | K_{D}^{GCVLS} | k_{FMP}^{norm} |
|-------------|---------|---------|-----|--------|--------|---------|--------------|---------------|---------------|---------------|
| Wild type   | 8.1 ± 0.3 | 9.7 ± 0.3 | (2.5 ± 0.1) × 10^{-2} | 4.0 ± 0.3 | 2.2 ± 0.2 | (1.7 ± 0.2) × 10^{-2} | 6.5 ± 1.7 | 120 ± 10 | (7.0 ± 0.7) × 10^{-4} |
| D352A      | 0.30 ± 0.05 | 0.77 ± 0.07 | (2.4 ± 0.1) × 10^{-2} | 110 ± 30 | 37 ± 5 | (1.7 ± 0.2) × 10^{-2} | 21 ± 4 | 100 ± 20 | (6.0 ± 0.6) × 10^{-4} |
| D352K      | (3.2 ± 0.1 × 10^{-3}) | (3.7 ± 0.3) × 10^{-2} | (3.3 ± 0.1) × 10^{-3} | NA | NA | (2.7 ± 0.3) × 10^{-3} | 60 ± 16 | 62 ± 5 | (2.0 ± 0.2) × 10^{-4} |

* k_{max} and k_{max} are the rate constants for product formation at pH 7.8 at saturating concentrations of MgCl_2 and MnCl_2, respectively.
* k_0 is the rate constant for product formation in the absence of MgCl_2 or MnCl_2 at pH 7.8.
* Peptide dissociation constants were measured with Dns-GCVLS at pH 7.5.
* Data taken from Ref. 21.
* Data taken from Ref. 24.
* Data taken from Ref. 22.
* k_{obs} measured at 50 mM MgCl_2.
* k_{obs} measured at 50 mM MnCl_2.
* NA, not applicable due to the absence of metal activation of this mutant.

![Fig. 3. Single turnover kinetics of wild type and mutant FTase with FMP.](image)

probably reflects the dissociation constant for Mg^{2+} from the FTase-FPP-peptide ternary complex. To observe the effect of the mutations D352A and D352K on the magnesium dependence of farnesylation, the single turnover kinetic rate constant (k_{obs}) of the mutant enzymes was determined at varying concentrations of magnesium ions (Fig. 4). Deletion of the aspartate side chain has a dramatic effect on the concentration of magnesium ions required to accelerate farnesylation catalyzed by FTase, increasing the K_Mg of the D352A mutant ~25-fold to 110 ± 30 mM (Table I, Fig. 4). This decreased affinity for magnesium in the D352A mutant is unlikely to be caused by perturbation of the diadipate pK_a, because deletion of a negative charge should lower the diadipate pK_a, which would increase, not decrease, the magnesium affinity at pH 7.8. Additionally, the maximum rate constant of product formation (k_{max}) for the D352A mutant (0.30 ± 0.05 s^{-1}) is 27-fold slower than wild type. These data indicate that the side chain of Asp-β352 enhances both Mg^{2+} affinity and the farnesylation rate constant. Since the D352A mutation has no effect on farnesylation in the absence of Mg^{2+} or using FMP as the substrate, the simplest explanation for these data is that the carboxylate side chain directly coordinates the bound Mg^{2+}. A high concentration of Mg^{2+} can accelerate D352A FTase activity, but without the aspartate to properly position the magnesium ion, the activity can only be accelerated 12.5-fold by magnesium in the mutant, compared with the 324-fold acceleration observed in wild type FTase at pH 7.8. Upon mutation of aspartate to lysine in D352K, catalysis is not accelerated at all by the addition of up to 100 mM magnesium. Although a lysine at this position is proposed to accelerate catalysis in GGTagase I (37), the D352K substitution in the active site of FTase does not enhance the farnesylation rate constant.

**Effect of Manganese on Catalysis**—The importance of the properties of the metal ion in accelerating the FTase reaction can be explored by replacing magnesium with manganese. Manganese and magnesium have similar size and preferred coordination geometry, and manganese can activate many magnesium-dependent enzymes, including FTase (17, 48). To assess the enhancement of FTase catalysis by manganese, the k_{obs} values for farnesylation catalyzed by wild type, D352A, and D352K FTase were determined at varying manganese concentrations at pH 7.8 by single turnover kinetics. For wild type FTase, the maximal rate constant for product formation at saturating manganese (k_{max}^{Mn}) is only 1.2-fold faster than the value of k_{max} (Table I, Fig. 5A), indicating that changing the divalent metal ion has only a modest effect on the farnesylation rate constant. Furthermore, the concentration of manganese required to achieve half-maximal reactivity (K_{Mn}), is only 1.8-fold less than the value of K_{Mg} for wild type FTase, which is a smaller decrease than the value predicted from the enhanced affinity of the ADP diadipate moiety for manganese (6-fold) (45). These data imply that the Lewis acidity and avidity of the cation for the diadipate leaving group are not the dominating factors in facilitating catalysis in wild type FTase. In the D352A mutant, the effect of manganese is enhanced modestly; the value of K_{Mn} for D352A is 3-fold tighter than that of K_{Mg} and the value of k_{max} is 2.6-fold faster than that of k_{max} (Table I, Fig. 5B). However, the value of K_{Mn} is still 17-fold larger for
The Dβ352A mutant compared with wild type FTase, and the value of $k_{\text{obs}}$ was only $0.77 \pm 0.07$ s$^{-1}$, over 10-fold slower than the rate constant for wild type FTase with saturating magnesium or manganese. In the Dβ352K mutant, the addition of manganese (up to 50 mM) does not accelerate catalysis more than 10%, as observed with magnesium (Table 1). These data provide further evidence that mutation of Asp-β352 in FTase perturbs the Mg$^{2+}$ binding site.

**DISCUSSION**

**Magnesium Affinity**—In enzymes that use magnesium to facilitate catalysis, the magnesium ion can bind to the substrate and promote substrate binding and/or catalysis, bind to a site on the enzyme in the absence of substrate and play a catalytic role, or some combination of the two. If the magnesium is associated only with the substrate and the enzyme active site does not perturb this interaction, one would predict that the magnesium dependence of the reaction would be equal to the magnesium affinity for the substrate. Here we demonstrate that the affinity of the diphosphate of FPP for Mg$^{2+}$ is 10-fold tighter than the value of the $K_{Mg}$ for activation of FTase by Mg$^{2+}$. Since the $K_{Mg}$ probably reflects the Mg$^{2+}$ affinity of the FTase ternary complex, these data demonstrate that FTase does alter the FPP-Mg$^{2+}$ affinity. The crystal structures of FPP bound to FTase illustrate that the diphosphate moiety is surrounded by a pocket of positively charged amino acids that should lower the electrostatic interaction between FPP and Mg$^{2+}$.

**Role of Asp-β352 in FTase**—In the crystal structures of FPP bound to FTase, aspartate β352 is located ~7 Å away from the diphosphate-Mg$^{2+}$ complex. Mg$^{2+}$ bound to FTase rearranges into this active conformation. The crystal structure (38). These data demonstrate that the diphosphate group moves slightly out of the pocket of positively charged residues and closer to the side chain of aspartate β352, forming the high affinity metal binding site (38). The mutation Dβ352A does not have a deleterious effect on farnesylation by FTase in the absence of magnesium but requires much higher concentrations of magnesium to accelerate catalysis, reflecting the decrease in affinity of the enzyme...
for magnesium upon the removal of one or two metal ligands.

Prenyltransferases and Magnesium—Unexpectedly, the three enzymes in the prenyltransferase family that catalyze highly similar reactions do not have the same dependence on the presence of magnesium ions. FTase and GGTTase II both use magnesium ions to boost catalysis, whereas GGTTase I is apparently not activated by magnesium, although the main difference in the FTase and GGTTase I chemical reaction is the extra five carbons on the prenyl chain of GGPP (19, 20, 24). One possible explanation for this difference is found in the sequence of these enzymes: the magnesium- utilizing enzymes FTase and GGTTase II contain a conserved aspartate, which is replaced by lysine in GGTTase. Our data provide evidence that Asp-β352 does coordinate magnesium in FTase. However, when this aspartate is mutated to lysine in FTase, as found in GGTTase I, the activity of Djβ352K is equal to that of Djβ352A, providing no evidence that a lysine at this position could replace magnesium. The active sites of FTase and GGTTase I do have other differences, however, so it remains possible that lysine can replace magnesium in GGTTase I, due to proper placement of the positive charge that cannot be replicated in FTase.

Role of Magnesium in the FTase Transition State—In FTase, magnesium has previously been proposed to stabilize the buildup of negative charge on the diphosphate moiety in the chemical transition state. The decreased reactivity of fluorene-substituted FPP analogs (24, 25) indicates that positive charge builds up on C-1 of FPP in the transition state. Therefore, negative charge should also build up on the diphosphate leaving group. Magnesium ions have been proposed to activate catalysis in FTase by coordinating the diphosphate leaving group from studies demonstrating that substrates lacking the diphosphate moiety (i.e. FMP) are not activated by Mg^2+ and that the Mg^2+-dependent pK_a is consistent with the deprotonation of diphosphate (21). Additionally, this is a common mode of activation by magnesium for enzyme-catalyzed reactions of diphosphate substrates (30). On the other hand, the manganese affinity for the FTase ternary complex is only 1.8-fold tighter than magnesium, whereas manganese binds to diphosphate of ADP with a 6-fold tighter affinity than magnesium (45). Also, substitution of magnesium with manganese only increases the maximum farnesylation rate constant for FTase 1.2-fold. Therefore, changing the electronic properties of the divalent metal ion in the FTase reaction does not translate to an equivalent effect on the metal affinity and catalysis of farnesylation. One explanation for these results is that the magnesium binding site consists of more than the diphosphate moiety. These data also suggest that the role of magnesium ion in activating catalysis may be more than just charge stabilization. The decreased reactivity of the Djβ352A mutant indicates that the magnesium ion may also be important for promoting formation of the active conformation of FPP prior to farnesylation by providing an impetus for the diphosphate to move out of the positively charged binding pocket, allowing the prenyl groups to rotate so that the C-1 carbon can approach the peptide thiolate. In the Djβ352A mutant, the 12-fold catalytic enhancement by Mg^2+ (Table 1) may reflect mainly electrostatic stabilization of the transition state. Consistent with this, the substitution of the more electrophilic manganese for magnesium has a greater effect on catalysis in this mutant; the maximum rate of catalysis increases 2.6-fold, and the manganese affinity for the ternary complex is 3-fold tighter. Another possibility is that manganese, as a softer transition metal ion, can adapt to alterations in binding geometry more readily than magnesium and therefore demonstrates a less severe decrease in affinity for the enzyme upon mutation of a ligand. The 27-fold decreased catalytic activity of the Djβ352A mutant at saturating Mg^2+ compared with wild type may reflect mainly the role of Mg^2+ in repositioning the FPP molecule in the ternary complex for optimal reaction geometry.

Proposed Magnesium Binding Site—We speculate that the magnesium binding site of FTase probably resembles the metal binding sites found in GDP-mannose mannosyl hydrolase and 2C-methyl-d-erythritol 2,4-cyclodiphosphate synthase, where a divalent metal ion coordinates two nonbridging oxygens of the diphosphate ligand and one carboxylate side chain of the enzyme (49, 50). In 2C-methyl-d-erythritol 2,4-cyclodiphosphate synthase, the bound manganese ion is only 5.7 Å away from the active site zinc ion. In adenylyl cyclase, a similar dimetal binding site is observed with zinc and manganese, and the two metals share an oxygen ligand (51). In FTase, the zinc ligand aspartate β297 is also potentially in a position to coordinate the magnesium ion (28). Furthermore, a lysine residue in this pocket of FTase, Lys-β353 (and in rat FTase, an additional lysine β356), could form hydrogen bonds with additional water molecules to complete the octahedral magnesium binding sphere. Therefore, we propose a magnesium binding site for FTase that is similar to the one modeled in Fig. 6. This binding site would probably only be formed in the presence of both FTase substrates, since the diphosphate of FPP is proposed to coordinate Mg^2+, and the presence of the peptide substrate may trigger the formation of the “active” conformation of bound FPP. The position of this binding site indicates the importance of magnesium for stabilizing the proper conformation of the diphosphate of FPP prior to chemistry.

Our proposed binding site is probably only observable in an active ternary complex of FTase. Therefore, examination of the magnesium environment by spectroscopic and crystallographic methods will be very challenging. Spectroscopic studies could be performed using freeze-quetch techniques to capture the enzyme in an active ternary complex, or with next generation substrate mimics that can more closely approximate the “active substrate conformation” of FTase.

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