Substrate Binding Is Required for Release of Product from Mammalian Protein Farnesyltransferase*

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Protein farnesyltransferase (FTase) catalyzes the modification by a farnesyl lipid of Ras and several other key proteins involved in cellular regulation. Previous studies on this important enzyme have indicated that product dissociation is the rate-limiting step in catalysis. A detailed examination of this has now been performed, and the results provide surprising insights into the mechanism of the enzyme. Examination of the binding of a farnesylated peptide product to free enzyme revealed a binding affinity of ~1 μM. However, analysis of the product release step under single turnover conditions led to the surprising observation that the peptide product did not dissociate from the enzyme unless additional substrate was provided. Once additional substrate was provided, the enzyme released the farnesylated peptide product with rates comparable with that of overall catalysis by FTase. Additionally, stable FTase-farnesylated product complexes were formed using Ras proteins as substrates, and these complexes also require additional substrate for product release. These data have major implications in both our understanding of overall mechanism of this enzyme and in design of inhibitors against this therapeutic target.

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Protein farnesyltransferase (FTase)1 catalyzes the S-farnesylation of a number of key cellular regulatory proteins. Farnesylation is directed by a C-terminal CAAX motif, where C is cysteine, A is usually an aliphatic residue, and X is typically methionine, serine, glutamine, or alanine (1, 2). The farnesyl lipid is attached to the substrate protein via a thioether linkage to the cysteine residue using farnesyl diphosphate (FPP) as the prenyl donor. Among the substrates for FTase are the Ras family of proto-oncogenes, several γ subunits of heterotrimeric G proteins, and nuclear lamins (1, 3). Farnesylation of these proteins is required for their proper membrane localization and activity. In the case of oncogenic forms of Ras proteins, the finding that farnesylation is required for expression of their transforming activity has led to FTase becoming an important target for anticancer drug design (4). Both in cell culture (5, 6) and in animal models (7), specific inhibitors of FTase have been shown to reverse the oncogenic phenotype induced by mutationally activated Ras.

FTase has been purified to homogeneity from both rat and bovine brain by affinity purification on immobilized CAAX peptidase substrates (8, 9). The enzyme is a Zn2+-metalloenzyme that consists of α and β subunits that migrate on SDS-PAGE with apparent molecular masses of 48 and 46 kDa, respectively (8). Both subunits of the enzyme have been cloned (10–12), and their co-expression in either Sf9 (13) or E. coli (14) results in production of quantities of the enzyme required for detailed biochemical and structural analyses. Cross-linking experiments have provided strong evidence that the β subunit is involved in recognition of both the isoprenoid and protein substrates (15–17), although there is also evidence that the α subunit may participate (15, 18). In addition to its bound Zn2+, FTase also requires Mg2+ for activity. The Zn2+ is involved coordinating the thiol of the peptide substrate in the ternary complex of enzyme-isoprenoid-peptide substrate (19) and thus is presumed to play a direct role in catalysis. The role of Mg2+ is not yet known.

Steady-state kinetic studies indicate that mammalian FTase can bind either FPP or protein substrate independently, but product formation requires that the enzyme bind FPP first, giving FTase an ordered sequential mechanism (20–22). The overall kcat under steady-state conditions is a relatively sluggish 1–3 min−1 for the mammalian enzymes (8, 23), with product dissociation being rate determining in catalysis (22). The rate of the chemical step has been directly determined to be 17 s−1 through spectroscopic studies using enzyme containing a Co2+-for-Zn2+ substitution (19). This spectroscopic study also revealed that the sulfur atom of the product thioether remains coordinated to the metal atom, an observation that may in part explain the slow release of product in steady-state turnover.

To gain better insight into the product dissociation step in the mechanism of mammalian FTase, we have performed an examination of the binding and the release of both peptide- and protein-derived products. The results of the study have major implications in regard to the mechanism of mammalian FTase and design of inhibitors targeting this enzyme.

EXPERIMENTAL PROCEDURES

Materials—The isoprenoid substrates FPP and its 1H-labeled counterpart ([2H]FPP) were purchased from American Radiolabeled Company (St. Louis, MO). Peptides were synthesized by solid-state methods and purified by reverse-phase HPLC as described (24). Sephadex resins were obtained from Pharmacia Biotech Inc., and the immobilized nickel resin was from Qiagen. Recombinant rat FTase was produced in Sf9 cells and purified as described (13). The His-tagged K-Ras (H5-K-Ras) and H-Ras (H6-H-Ras) were produced by expression of the appropriate

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RESULTS AND DISCUSSION

As noted in the Introduction, kinetic analyses have shown that the rate-limiting step in FTase is the release of product from mammalian FTase, with a \( k_{\text{cat}} \) of \(-2\) min\(^{-1}\) at 30 °C. Although this result would seem to imply a high affinity binding of product to the enzyme, previous steady-state analysis of FTase indicated that a farnesylated peptide product was a very poor competitor of the reaction with a \( K_i \) of \(-5\) µM (20). Because steady-state kinetics are an indirect measure of affinity, we chose to directly determine the binding affinity of a farnesylated peptide to FTase. The method used was that of Hummel and Dreyer (28), which employs equilibrium gel filtration. A graphical depiction of this method is shown in Fig. 1. A Sephadex G-25 column is equilibrated with buffer containing radiolabeled ligand (shaded solution), and then the binding protein (i.e., FTase) is applied to the column in a small volume at a concentration that is much higher than that of the ligand in the buffer. As the enzyme moves through the column it binds ligand, with the result that a peak of radioactivity is observed in the void volume where the enzyme-ligand complex elutes (stippled black band); this peak is followed by a “trough” in the profile where the buffer that has been depleted of radiolabeled ligand (white solution) emerges. Equilibration of the binding protein and ligand within the column is indicated by the resolution of the peak and trough in the elution profile (28). From this type of profile it is possible to extract an equilibrium binding constant of the enzyme-ligand complex.

We performed this type of analysis on the binding of \([^{3}H]\)farnesylated-CVIM (\([^{3}H]\)CVIM) to FTase, a typical profile is shown in Fig. 1. Analysis of such profiles (see “Experimental Procedures”) yielded a \( K_d \) for the interaction of 0.78 µM (range of 0.5–1.0 µM). Furthermore, addition of excess FPP or peptide substrate to the running buffer of the column did not affect the binding constant (not shown).

We next sought to directly examine the dissociation of product formed on the enzyme during catalysis. To investigate this step in the catalytic process, the FTase-FPP complex was prepared and isolated, and then a stoichiometric amount of a tetrapeptide substrate was added. Previous studies have shown that the reaction occurs quite rapidly under these conditions (10 m M FTase, 50 m M FPP, 5 m M MgCl\(_2\), pH 8.0 at 30 °C) (22). Formation of the FTase-product complex under these single-turnover conditions allowed a direct examination of product dissociation, which was determined by rapid separation of the complex from free product on Sephadex G-25 spin chromatography columns. The results of this analysis, shown in Fig. 2A, revealed that there was no appreciable dissociation of product from the enzyme even after 10 min of incubation. This inability to detect product release was not simply due to its release and re-binding, be-
cause addition of a large excess of unlabeled product to the reaction mixture after product formation but prior to the separation procedure did not result in any exchange with the radiolabeled product formed on the enzyme. Surprisingly, however, addition of excess peptide or isoprenoid to the reaction did trigger product release (Fig. 2A). FPP was slightly more efficient than peptide substrate in this regard; $k_{\text{off}}$ values for product release were 0.13 and 0.08 min$^{-1}$, respectively, in the presence of the two substrates. Because these experiments were performed at 10 °C, we determined the $k_{\text{cat}}$ under the same conditions. The results of this analysis, shown in Fig. 2B, gave a turnover number of 0.11 min$^{-1}$, a value in close agreement with the product release under the same conditions (see above). From these data, we conclude that FTase must bind an additional substrate molecule before it can release its product; the implications of this finding are discussed below.

We then asked the question of whether the requirement of substrate binding for product release also applied to modification of authentic protein substrates by FTase. To assess this, we developed an affinity co-precipitation method to examine the formation and dissociation of the enzyme-product complex (see "Experimental Procedures"). The FTase-FPP was prepared as before (except that unlabeled FPP was used), and the complex was then incubated with a substoichiometric amount of His-tagged Ras substrates. Following catalysis, the reaction mixture was incubated with a resin of immobilized nickel to precipitate the Ras and the associated enzyme. Proteins bound to the nickel resin were eluted with imidazole and analyzed by SDS-PAGE gel followed by immunoblotting using antisera directed against both FTase and Ras. Formation of a stable complex between FTase and Ras is thus detected by the appearance of the enzyme in the affinity precipitate. The results of this type of product dissociation analysis using protein substrates are shown in Fig. 3. The analysis was initially performed with His-tagged K-Ras as the substrate and clearly indicated that FTase and H$_2$-K-Ras do in fact form a stable complex under conditions where the K-Ras is subject to farnesylation (Fig. 3A, lane 3). As seen with the experiments using the peptide substrate of FTase, addition of excess FPP resulted in product release as demonstrated by the absence of FTase in the affinity precipitate under these conditions (Fig. 3A, lane 4). Additionally, FTase did not stably bind the nickel
A 10-fold higher Km the X residue and the absence of the polybasic region results in different from K-Ras in that its CAA sequence is CVLS and it does not contain the polybasic region just upstream of the carrier protein. Immobilized nickel resin (10 m37 °C for 30 min, at which point 30 μg of cytochrome c was added as a carrier protein. Immobilized nickel resin (10 μl) was then added, and the incubation mixture was rocked for 30 min at 4 °C. The resin was pelleted and then washed as described under “Experimental Procedures,” and bound proteins eluted with imidazole buffer. The eluate was pelleted and then washed as described under “Experimental Procedures.” The eluate was subjected to SDS-PAGE and immunoblot analysis using antisera directed against both FTase (upper strip) and Ras (lower strip). B, analysis with H-Ras substrate. The experiment was performed as described for A except that H6-H-Ras was used as the substrate protein instead of H2-K-Ras. For both panels, the standard (Std) lanes contain the amount of FTase that could be theoretically precipitated assuming stoichiometric conversion to product and the amount of Ras protein present in the reactions, respectively.

Because all of the studies to this point were performed either with K-Ras or peptides encompassing the C terminus of K-Ras, we also examined a distinct substrate of FTase to determine whether this inability to release product was a general property of FTase or whether it was something unique to the properties of K-Ras as a substrate. For these studies, we selected His-tagged H-Ras as the protein substrate; this protein is quite different from K-Ras in that its CAAX sequence is CVLS and it does not contain the polybasic region just upstream of the CAAX box that is found in K-Ras. This combination of Ser as the X residue and the absence of the polybasic region results in a 10-fold higher Km for H-Ras as a substrate for FTase as compared with K-Ras (25). Addition of the H6-H-Ras to the FTase-FPP complex did indeed result in formation of a FTase-product complex that could be precipitated with the nickel resin (Fig. 3B, lane 3). Again, addition of FPP prior to the affinity precipitation resulted in release of the product by the enzyme, as demonstrated by the inability to detect FTase in the precipitate under these conditions (Fig. 3B, lane 4). The control experiments again confirmed that no stable complex was formed under conditions where FTase-product complex could not be formed (Fig. 3B, lanes 1 and 2).

What is the significance of the finding that FTase does not release its product until there is additional substrate present for it to bind? From a mechanistic viewpoint, these data suggest the presence of two distinct binding conformations for product on the enzyme. One of these conformations, to which product binds relatively weakly with a $K_d$ of around 1 μM,

![Fig. 3. Affinity precipitation of FTase-Ras product complexes.](image)

A, analysis with K-Ras substrate. FTase (250 nM), either as the free enzyme (lanes 1 and 2) or as the enzyme-FPP complex (lanes 3 and 4) were incubated with (lanes 2, 3, and 4) or without (lane 1) 50 nM H2-K-Ras. In the experiment in lane 4, additional FPP (275 μM) was added during the initial incubation. The incubation was carried out at 37 °C for 30 min, at which point 30 μg of cytochrome c was added as a carrier protein. Immobilized nickel resin (10 μl) was then added, and the incubation mixture was rocked for 30 min at 4 °C. The resin was pelleted and then washed as described under “Experimental Procedures,” and bound proteins eluted with imidazole buffer. The eluate was pelleted and then washed as described under “Experimental Procedures.” The eluate was subjected to SDS-PAGE and immunoblot analysis using antisera directed against both FTase (upper strip) and Ras (lower strip). B, analysis with H-Ras substrate. The experiment was performed as described for A except that H6-H-Ras was used as the substrate protein instead of H2-K-Ras. For both panels, the standard (Std) lanes contain the amount of FTase that could be theoretically precipitated assuming stoichiometric conversion to product and the amount of Ras protein present in the reactions, respectively.

![Fig. 4. Kinetic scheme for FTase.](image)

A simplified version of the overall kinetic scheme for FTase is shown. For simplicity, FPP binding to the free enzyme is shown as a single step, although it is best described as a two-step process (22). In this scheme, however, FPP binding to free enzyme is considered of minimal importance in vivo because the enzyme cycles directly to the E-FPP complex after catalyzing the farnesylolation of a protein substrate (a Ras protein in the case depicted here). This direct conversion of the FTase-product complex to the FTase-FPP complex is the hypothesized consequence of FPP-trigged release of the farnesylated Ras protein (Ras-S[C15]). See text for further details.

Figure 4: Kinetic scheme for FTase. A simplified version of the overall kinetic scheme for FTase is shown. For simplicity, FPP binding to the free enzyme is shown as a single step, although it is best described as a two-step process (22). In this scheme, however, FPP binding to free enzyme is considered of minimal importance in vivo because the enzyme cycles directly to the E-FPP complex after catalyzing the farnesylolation of a protein substrate (a Ras protein in the case depicted here). This direct conversion of the FTase-product complex to the FTase-FPP complex is the hypothesized consequence of FPP-trigged release of the farnesylated Ras protein (Ras-S[C15]). See text for further details.
Product Release from Mammalian FTase

critical processing step. One can certainly envision the design of compounds capable of interacting with the product binding site in a very tight fashion. Elucidation of the structural basis of this interaction could lead to the design of highly effective product-based inhibitors. Another significant implication of the findings that FPP binding by FTase results in formation of a relatively stable FTase-FPP complex and that the enzyme does not release product until an additional substrate molecule is encountered is that the enzyme in vivo probably never exists for any appreciable period of time as a free (i.e. unliganded) species (see Fig. 4). Thus, it seems likely that the many types of FTase inhibitors that are under development as therapeutic agents are actually targeting the E-FPP complex rather than the free enzyme; such a realization could provide for strategies to optimize design of even more effective compounds. Additionally, if FTase is involved in the delivery of its product to another cellular protein or membrane (see above), defining this process could identify new targets for design of agents that can block subcellular trafficking and thus perturb the activities of specific products of the enzyme, e.g. oncogenic Ras proteins.

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