# PREFACE

Most presently used anticancer drugs were developed based on their antiproliferative rather than antioncogenic properties and consequently suffer from two major limitations. Many are cytotoxic and cause major thwarted effects owing to their ability to inhibit indiscriminately the growth of fast dividing cells. Drug resistance, the second major limitation of these drugs, arises primarily from the lack of activity against the more slowly growing solid tumors.

The recent explosion of knowledge gained from genes capable of causing cancer, and the pivotal role they play in growth factor signal transduction, have opened up new avenues for rationally designing novel anticancer drugs. One of the best studied signal transduction pathways, which contains a gold mine of anticancer drug discovery targets, is that of receptor tyrosine kinase signaling. A key molecular switch within this pathway is a small GTPase called Ras. Ras mediated the transfer of biological information from extracellular signals to the nucleus and is a major regulator of cell division. Oncogenic mutations in the ras gene are found in about 30% of all human cancers and result in a constitutively activated protein that sends uninterrupted signals to the nucleus. Over the last two decades several approaches have failed to reverse the constitutive activation of the Ras protein. Recently, however, the realization that farnesylation, a lipid posttranslational modification, of Ras is required for its cancer-causing activity, prompted an intense search for farnesyltransferase inhibitors as novel anticancer agents.

Farnesyltransferase Inhibitors in Cancer Therapy describes the efforts of several groups to design, synthesize, and evaluate the biological activities of farnesyltransferase inhibitors. Rational design of small organic molecules that mimic the carboxyl terminal tetrapeptide farnesylation site of Ras resulted in pharmacological agents capable of inhibiting Ras processing and selectively antagonizing oncogenic signaling and suppressing human tumor growth in mouse models without side effects. These agents are presently undergoing advanced preclinical studies. Several important issues, such as the mechanism of action of farnesyltransferase inhibitors and the potential mechanisms of resistance to inhibition of K-Ras farnesylation, are also discussed. Furthermore, the recent observation that K-Ras 4B, the most frequently mutated form of Ras in human tumors, can be geranylgeranylated and that, in addition to Ras, there are other geranylgeranylated small G-proteins that play an important role in smooth muscle proliferation and apoptosis, stimulated the search for inhibitors of a closely related enzyme, geranylgeranyltransferase I. Thus, the current volume also discusses geranylgeranyltransferase I inhibitors as modulators of cell cycle and apoptosis, and as potential therapeutic agents for cardiovascular disease.

Saïd M. Sebti
Andrew Hamilton

# 2 The Biochemistry of Farnesyltransferase and Geranylgeranyltransferase I

*Chih-Chin Huang, PHD, Carol A. Fierke, PHD, and Patrick J. Casey, PHD*

***CONTENTS***



## 1. INTRODUCTION TO PROTEIN PRENYLATION

Protein prenylation refers to a type of covalent posttranslational modification by lipids at cysteine residues near the C-terminus of a protein; either a 15-carbon farnesyl or a 20-carbon geranylgeranyl isoprenoid is attached to the protein via a thioether linkage *(1–3)* (Fig. 1). Protein prenylation is ubiquitous in the eukaryotic world, and most prenylated proteins are membrane-associated for at least part of their lifetime. The majority of prenylated proteins are involved in cellular signaling and/or regulatory events that occur at or near the cytoplasmic surfaces of cellular membranes *(4,5)*.

The first evidence for protein prenylation came from studies in Japan in the late 1970s from structural analysis of specific fungal mating factor peptides *(6,7)*. On some such mating factors, a 15-carbon farnesyl isoprenoid was found linked to the peptide through a thioether bond from a cysteine sulfhydryl to the C-1 carbon of the farnesyl. Biochemical data suggested that this modification was an important element of the mating factor peptide. The discovery of mammalian prenylated proteins arose from studies concerning the effects of inhibiting isoprenoid biosynthesis on cell growth. Inhibitors of HMG-CoA reductase, the rate-limiting enzyme in isoprenoid biosynthesis (Fig. 1), were found to block cell growth in a fashion that could not be reversed by adding exogenous sterols (the major end products of the isoprenoid pathway) to the media *(8,9)*. However, the effects could be reversed by addition of small amounts of mevalonate, suggesting that a nonsterol metabolite of mevalonate was involved in this cell growth control. When $^{3}$H-mevalonate

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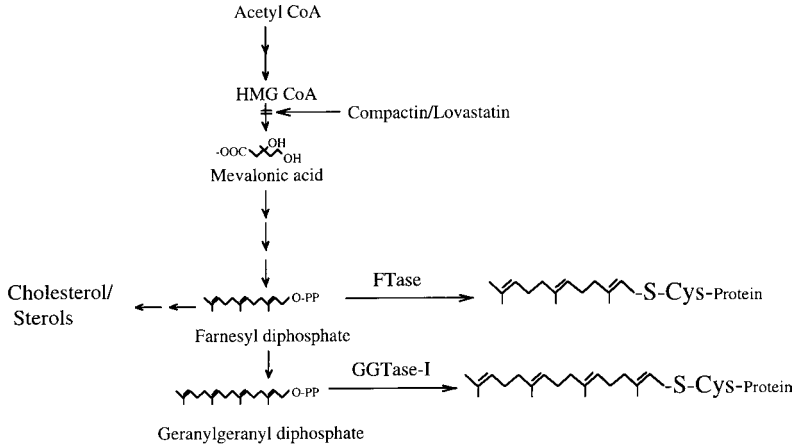


**Fig. 1.** Overview of the isoprenoid biosynthetic pathway and the structures of the prenyl groups attached to proteins by FTase and GGTase I.

was used in these types of experiments, the label was found to be incorporated into a number of cellular proteins, dubbed "prenylated proteins" *(10,11)*.

The first prenylated mammalian protein identified was the nuclear protein lamin B *(12,13)*. At about the same time, independent studies on the a-factor mating peptide of *Saccharomyces cerevisiae* revealed that this peptide was modified by a farnesyl isoprenoid *(14)*. The realization that both lamin B and the a-factor peptide contained a so-called "CAAX motif" at their carboxyl terminus (where "C" was the cysteine residue that served as the isoprenoid attachment site, "A" signified an aliphatic amino acid, and "X" denoted an undefined amino acid) prompted examination of other proteins containing the motif to determine if they too were prenylated. Foremost among the CAAX-containing proteins examined were the products of the Ras family of protooncogenes. The discoveries that Ras proteins were modified by farnesylation and that the modification was required for the oncogenic forms of these proteins to transform cells triggered an immediate and widespread interest in this form of lipid modification *(15–17)*. Subsequent studies have identified almost a hundred prenylated proteins in mammalian cells *(1,5,18)*, and revealed that, in addition to the 15-carbon farnesyl moiety, the 20-carbon geranylgeranyl isoprenoid could also be attached to proteins *(19,20)* (Fig. 1).

For CAAX-containing proteins, prenylation is but the first step in a series of three posttranslational modifications that occur at the C-terminus of most of these proteins. The three C-terminal amino acids (i.e., the -AAX) are subsequently removed by a membrane-bound protease, and finally a membrane-bound enzyme methylates the newly-formed carboxyl group to produce a methylester at the C-terminus *(21,22)*. Furthermore, in addition to the modifications at the CAAX motif, in some prenylated proteins other modifications such as palmitoylation and phosphorylation can occur in the C-terminal region just upstream of the CAAX motif *(5)*.

Monomeric guanine nucleotide (GTP)-binding proteins (G proteins) such as Ras, Rap, Rho, and Rab comprise the largest set of prenylated proteins *(5,23)*. Among these G proteins, Ras proteins have attracted particular attention because of the important role of Ras in carcinogenesis *(24,25)*. The normal functions of Ras proteins are in cellular signal

transduction pathways that are essential for cell growth and differentiation *(25–27)*. Moreover, specific mutations in Ras proteins render them oncogenic, and such mutations are found in about 30% of all human tumors, including over 90% of human pancreatic cancer and 50% of human colon cancer *(18,24)*. The dependence of the transformed phenotype on the constitutive activity of Ras prompted considerable speculation that blocking the Ras signaling pathway could provide a way to treat such cancers *(24)*. Hence, the finding that farnesylation is absolutely required for oncogenic Ras function identified a specific point in the process, i.e., the attachment of the isoprenoid, for which development of specific inhibitors might provide an approach to this type of cancer chemotherapy *(18,28–30)*.

## 2. FTASE AND GGTASE-I: THE CAAX PRENYLTRANSFERASES

### *2.1. General Features of the Enzymes*

The first identified protein prenyltransferase was protein farnesyltransferase (FTase), originally isolated from rat brain cytosol using an assay that followed the incorporation of radiolabel from $^{3}$H-FPP into a recombinant Ras protein *(31)*. The finding that CAAX proteins containing methionine or serine at their C-terminus were farnesylated, whereas those ending in leucine were modified by a geranylgeranyl moiety *(32–34)*, provided the initial evidence for the existence of a distinct enzyme that would catalyze the addition of geranylgeranyl to certain proteins in the CAAX class. Using an approach similar to that which led to the identification of FTase, an enzymatic activity capable of transferring the geranylgeranyl group from geranylgeranyl diphosphate to candidate proteins was identified *(35,36)*. This enzyme, protein geranylgeranyltransferase type I (GGTase I), exhibited properties similar to those of FTase (*see below*). The C-terminal leucine residue was shown to be responsible for the specific recognition of substrate proteins by GGTase I by producing a Ras protein with a leucine-for-serine switch at the COOH-terminal position, a switch that converted the Ras protein from a FTase to a GGTase I substrate *(35)*.

Mammalian FTase and GGTase I share many properties *(37)*. Both enzymes are heterodimers that contain a common subunit (designated the α-subunit) of 48 kDa and distinct β-subunits of 46 kDa (FTase) and 43 kDa (GGTase I) *(31,38,39)*. Both proteins are zinc metalloenzymes that operate through apparently quite similar kinetic and chemical mechanisms (*see below*). Both enzymes have been cloned, and sequence analysis has 1) confirmed that the α-subunits are the products of the same gene and 2) revealed that the β-subunits had ~35% sequence identity at the amino acid level *(40–42)*. The significance of the two enzymes sharing a common subunit is not yet clear, but the existence of an identical α-subunit and a highly homologous β-subunit for these two protein prenyltransferases provided the initial evidence that discrete segment(s) of the β-subunit would be responsible for the remarkable substrate specificities of the enzymes.

Structural information just recently has begun to emerge on the CAAX prenyltransferases. Data to date have come from analysis of mammalian FTase, whose X-ray crystal structure was determined at 2.2 Å resolution *(43)*. In this structure, which was of the free (i.e., unliganded) enzyme, the α-subunit was found to be folded into a crescent-shaped domain composed of seven successive pairs of coiled coils termed "helical hairpins," which contact a significant portion of the β-subunit. The existence of repeat motifs in this subunit was first predicted from sequence alignments of mammalian and fungal α-subunits *(44)*. The β-subunit was also found to consist largely of helical domains, with the

majority of the helices arranged into an α–α barrel structure. One end of the barrel was open to the solvent, while the other end was blocked by a short stretch of residues near the C-terminus of the β-subunit. This arrangement results in a structure containing a deep cleft in the center of the barrel that possesses all of the features expected for the active site of the enzyme, including the aforementioned bound zinc ion (*see* Subheading 2.4.1.). Quite recently, crystal structures of the complex of FTase with its isoprenoid substrate FPP have been reported that provide a quite detailed snapshot of the binding site for this substrate on the enzyme *(45,46)* (*see also* Chapter 3).

## *2.2. Recognition of Substrates*

### 2.2.1. Recognition of Isoprenoid Substrates

Binding of isoprenoid substrates by both CAAX prenyltransferases is of very high affinity with $K_D$ values being in the low n*M* range *(47–49)*; the initial realization of this property came from findings that the enzyme-isoprenoid complexes could be isolated by gel filtration *(50,51)*. The use of photoactivatable analogs of both FPP and GGPP revealed that the analogs could be specifically crosslinked to the β-subunits of FTase and GGTase I, respectively, upon activation *(52–54)*; these and related findings with peptide substrates (*see* Subheading 2.2.2.) provided the initial evidence that the active sites for the enzymes were, as expected, predominately associated with the β-subunits. The recent crystal structures determined for FTase-FPP complexes have provided the formal proof of this hypothesis *(45,46)*.

An early observation made with FTase was that the enzyme could bind both FPP and GGPP with relatively high affinity, although only FPP could serve as a substrate in the reaction *(50)*. A more detailed study of the FPP binding properties of FTase revealed that there is in fact a significant difference in the binding of the two isoprenoids, with GGPP binding being some 15-fold weaker than that of FPP *(49)*, although this still translates to an apparent affinity of ~100 n*M* for GGPP binding to FTase. A structural-based hypothesis why FTase exhibits such high affinity binding of GGPP to form a complex that is essentially catalytically inactive has been advanced *(46)*. Briefly, this hypothesis—discussed in detail in Chapter 3—is that the depth of a hydrophobic binding cavity in the β-subunit acts as a ruler that discriminates between the two isoprenoids based on their chain length. No structural information is yet available for GGTase I, although this enzyme exhibits much higher selectivity; binding of GGPP to the enzyme is several hundred-fold tighter than that of FPP *(48,49)*.

Analogs of FPP have been identified that bind to FTase with high affinity but cannot participate in catalysis *(55,56)*. These analogs have been quite useful in mechanistic studies of FTase, because they allow formation of an inactive FTase-isoprenoid binary complex (*see* Subheading 2.4.). Analogs of GGPP that should allow similar studies with GGTase I have also been described *(57,58)*.

### 2.2.2. Recognition of Protein Substrates

As noted in Subheading 1, mammalian cells contain a wide variety of proteins that are processed by CAAX prenyltransferases. Substrates of FTase include all four Ras proteins, nuclear lamins A and B, the γ-subunit of the retinal trimeric G protein transducin, and a variety of kinases and phosphatases *(18,59–63)*. Known targets of GGTase I include most identified γ-subunits of heterotrimeric G proteins and a multitude of Ras-related monomeric G proteins, including most members of the Rac, Rho, and Rap subfamilies

*(1,5)*. All these protein substrates contain a Cys residue precisely four amino acids from the C-terminus. Furthermore, as noted in Subheading 2.1., the identity of the C-terminal residue (i.e., the "X" of the CAAX motif) determines which of the two enzymes will act on the protein. FTase prefers proteins containing Ser, Met, Ala or Gln, whereas Leu at this position directs modification by GGTase I *(1,21)*. This property of the enzymes make it possible to predict with reasonable accuracy from its primary sequence which prenyl modification will be on a protein.

An important property of both FTase and GGTase I is that they can recognize short peptides containing appropriate CAAX motifs as substrates *(31,36,64)*. A quite detailed analysis of specificity in recognition of $Ca_1a_2X$ sequences by FTase indicates that the $a_1$ position has a relaxed amino acid specificity, while variability at $a_2$ and X are more restricted. Basic and aromatic side chains are tolerated at $a_1$ but much less so at $a_2$, whereas acidic residues are not well-tolerated at either position *(64,65)*. Moreover, substitution at the $a_2$ position by an aromatic residue in the context of a tetrapeptide creates a molecule that has been reported to be not a substrate for FTase but rather a competitive inhibitor *(66)*. One such peptide, CVFM, has served as the basis for design of peptidomimetic inhibitors of FTase *(67–69)*.

Binding of peptide substrates to FTase has been examined by nuclear magnetic resonance (NMR) using a resonance transfer approach. One such study reported that the CAAX sequence of a peptide substrate adopts a Type I β-turn conformation when bound to the enzyme *(70)*. However, a similar study of binding of a peptidomimetic inhibitor of FTase termed L-739,787 revealed a slightly different conformation most closely approximating a Type III β-turn *(71)*. A note of caution here is that, in both cases, the binding of the peptide/peptidomimetic was examined in the absence of bound isoprenoid on the enzyme. The recent realization that the kinetic mechanism is most likely an ordered one in which isoprenoid binding precedes that of the peptide/protein substrate *(47)*, and that the binding of the isoprenoid markedly increases the affinity for the peptide substrate *(72)* (*see* Subheading 2.3.) may have profound implications for this data, as the binding of the peptide/protein substrate to the enzyme-isoprenoid complex may be very different than its binding to free enzyme.

The zinc ion in both FTase and GGTase I is essential for the high affinity binding of the protein substrate (but not, however, for binding of the isoprenoid substrate) *(48,73)*, and recent studies indicate a direct coordination of the thiolate of the Cys residue of the protein substrate with the metal ion during catalysis *(72,74)*. Further evidence supporting a metal-substrate interaction in the enzymes comes from studies showing that the zinc ion can be replaced by cadmium, and the cadmium-substituted enzymes retain steady-state activity but have somewhat altered protein substrate specificities *(48,75)*. The location of the zinc ion was determined in the crystal structure of FTase to be in the β-subunit near the interface with the α-subunit *(43)*, consistent with the findings that both protein and peptide substrates can be crosslinked to the β-subunit of FTase *(50,76)*, and that short peptide substrates containing divalent affinity groups label both the α- and β-subunits upon photoactivation *(76)*.

### 2.2.3. Cross Prenylation by CAAX Prenyltransferases: Is it Important?

Although FTase and GGTase I seem to be quite selective for their substrates, cross-specificity (i.e., modification of a protein by either enzyme) has been observed *(36,65)*. However, whether this ability to modify alternate substrates is of physiological significance

is still somewhat unclear. The most compelling evidence in this regard comes from studies in fungal systems. Yeast lacking FTase are viable, although they exhibit growth defects *(77)*. Overexpression of GGTase I in these mutants can partially suppress the growth defects, suggesting that GGTase I can at some level prenylate substrates of FTase *(78)*. Although yeast lacking GGTase I are not viable, the phenotype can be rescued by overexpression of two essential G protein substrates of the enzyme *(78)*, suggesting that FTase can prenylate some substrates of GGTase I if the substrate proteins are overproduced.

In terms of mammalian CAAX proteins, two Ras isoforms—K-Ras4B and N-Ras—can serve as substrates for both FTase and GGTase I in vitro, although they are much better substrates for FTase *(79,80)*. Although under normal conditions these two Ras isoforms seem to be modified solely by the farnesyl group, geranylgeranylation of the proteins can be detected in cells if FTase is inhibited *(81,82)*. The primary determinant for this type of cross-prenylation appears to be the existence of a Met as the C-terminal residue of these proteins *(80)*. Although these studies do not provide evidence to support the notion that cross-prenylation has significance under normal physiological conditions, it is certainly a concern in terms of the biology associated with FTase inhibition. The discussion of these concerns can be found in Chapters 5,13, and 15.

There do appear to be some mammalian proteins that can be normally modified by either isoprenoid. One such example is the Ras-related small G protein TC21, where the presence of Phe as the C-terminal residue apparently allows modification by either enzyme *(83)*. Additionally, another Ras-related small G protein, RhoB, has been shown to be farnesylated as well as geranylgeranylated even though its C-terminal residue is Leu *(84,85)*; farnesylation of this protein is most likely due to an ability to be processed by FTase, rather than an alternate activity of GGTase I *(86)*. How RhoB gets recognized and farnesylated by FTase is not yet clear, although a Lys residue in the second position of the CAAX motif may be partly responsible. Whatever property is responsible for this "dual prenylation," the differently prenylated forms of RhoB apparently have unique functions, as suppressing the farnesylated population by treatment of cells with a FTase inhibitor suppresses RhoB-dependent cell growth *(86)*.

### *2.3. Kinetic Mechanism*

Mammalian CAAX prenyltransferases are quite slow enzymes, with $k_{cat}$ values in the range of 0.05 $s^{-1}$*(31,87)*. Steady-state kinetics of mammalian FTase were initially interpreted as indicating a random-order binding mechanism in which either substrate could bind first *(87)*. However, the failure to trap enzyme-bound protein or peptide substrate in transient kinetic experiments suggested that either substrate binding is actually ordered or that the dissociation rate constant of the protein/peptide substrate is so fast and the affinity is so weak that farnesyl diphosphate (FPP) binding first is the kinetically preferred pathway *(47,87)* (Fig. 2). Consistent with this functionally ordered mechanism, the affinity of FPP for FTase is in the low n*M* range; whereas the affinity of the peptide substrate in the absence of bound FPP is relatively weak but this affinity is increased several hundred-fold by the binding of FPP analogs *(72)*. The aforementioned pre-steady-state kinetic studies also revealed that the association of the peptide substrate with the FTase · FPP binary complex was effectively irreversible with a $k_{assoc}$ of $2 \times 10^5$ $M^{-1}s^{-1}$ *(47)*. While the rate constant for product formation could not be accurately determined in these studies, a lower limit of $> 12$ $s^{-1}$ was established using protein fluorescence *(47)*. A more precise determination of the rate constant for product formation has come from

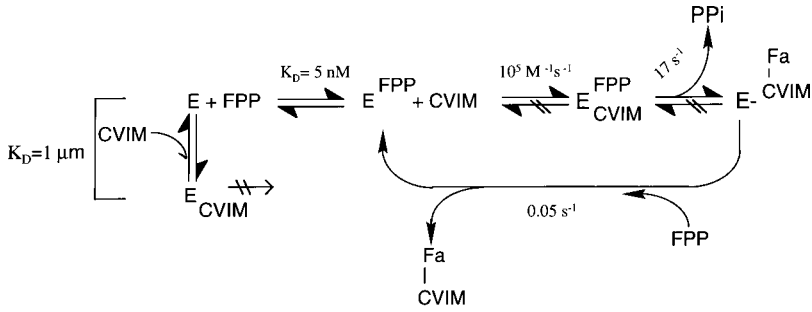


**Fig. 2.** Kinetic scheme for FTase. The overall kinetic scheme for the FTase reaction is shown; the available data indicates that the kinetic pathway for GGTase I will be quite similar. The abbreviations used are: E, FTase; CVIM, the C-terminal peptide of protein substrates (in the case shown this is K-Ras); FPP, farnesyl diphosphate; Fa, the 15-carbon farnesyl group attached to the Cys residue of the protein/peptide substrate. (*See text* for details.)

measurements of changes in the absorption spectrum of cobalt-substituted FTase during the catalytic process *(74)*, in which a value of 17 $s^{-1}$ was obtained. The finding that the rate constant for the product formation was much greater than the steady-state $k_{cat}$ revealed that the rate-limiting step under steady-state conditions was a step after formation of the thioether product, most likely product dissociation. In fact, in the absence of excess substrates, the product dissociation rate constant is so slow that the adduct of FTase with bound product can be isolated *(88)*. However, product dissociation can be enhanced by the addition of either substrate, with FPP being the most efficient in this regard (Fig. 2). Surprisingly, the affinity of FTase for the thioether product is weak (>1 m*M*), suggesting that product dissociation is kinetically controlled by an associated step that is triggered by substrate binding *(88)*.

The kinetics of yeast FTase differ from mammalian FTase in several aspects. For the yeast enzyme, steady-state kinetics clearly show that the mechanism is ordered with FPP binding first, because yeast FTase is inhibited by the peptide substrate *(89)*. In addition, the dissociation rate constant of the peptide substrate (33 $s^{-1}$) is faster than the chemical step (10.5 $s^{-1}$) *(90)*. Most importantly, the $k_{cat}$ (4.5 $s^{-1}$) is much faster than that of the mammalian enzyme, such that product dissociation is not the sole rate-limiting step for steady-state turnover at saturating substrate concentrations *(89)*.

GGTase I has been less well studied than FTase, because only recently has there been much interest in this enzyme as a drug target (*see* Chapters 15 and 16). The catalytic activity of mammalian GGTase I is very similar to that of mammalian FTase, with the exception that this enzyme does not require added magnesium for optimal turnover *(48)*. The steady-state kinetic parameters of GGTase I are also comparable to those of FTase *(53,57)*. Yeast GGTase I, similar to yeast FTase, follows an ordered binding mechanism; however, steady-state turnover at saturating substrate ($k_{cat}$ = 0.34 $s^{-1}$) is 10-fold slower than that of yeast FTase *(91)*.

## *2.4. Chemical Mechanism*

### 2.4.1. Role of Zinc in Catalysis

In zinc proteins, the major role of the zinc ion can be either catalytic or structural. A catalytic zinc is involved in the chemical reaction directly, whereas a structural zinc is only required for the structural stability of the protein. A catalytic zinc ion is located at

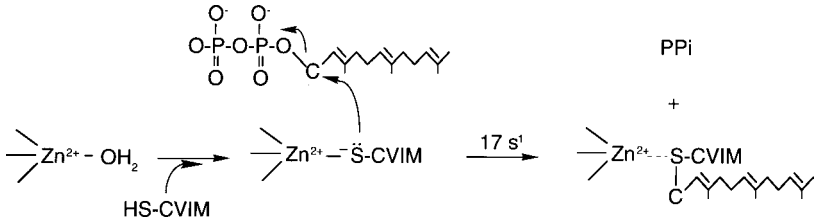


**Fig. 3.** The basic mechanism of FTase. Shown is the active site zinc ion with an open coordination sphere (i.e., with a water molecule providing one of the metal ligands). The peptide/protein substrate is designated HS-CVIM, although the thiolate anion form is the zinc-bound species that participates in the catalytic step. The net result is the formation of the enzyme-bound thioether product. (*See text* for details.)

the active site of an enzyme, where it participates directly in the catalytic mechanism. A unique feature of a catalytic zinc site is the existence of an open coordination sphere; that is, the zinc binding polyhedron contains at least one water molecule in addition to three or four protein ligands *(92)*; such an open coordination sphere for the bound zinc in FTase was revealed in the initial crystal structure of the enzyme *(43)*. The accessibility of the open coordination sphere to solvent and substrates implies that the zinc site can function in a catalytic manner. Unlike other first-row transition metals, the zinc ion ($Zn^{2+}$) contains a filled d orbital ($d^{10}$) and therefore does not participate in redox reactions, but rather functions as a Lewis acid to accept a pair of electrons *(93)*. The zinc-bound water is a critical component for a catalytic zinc site, because it can be either ionized to zinc-bound hydroxide (e.g., in carbonic anhydrase), polarized by a general base (e.g., in carboxypeptidase A) to generate a nucleophile for catalysis, or displaced by the substrate (e.g., in alkaline phosphatase) *(94)*.

Zinc is a metal of borderline "softness;" therefore, it can coordinate ligands comprised of either oxygen, nitrogen, or sulfur atoms *(95)*. In most catalytic zinc sites, the zinc ion is coordinated by different combinations of protein ligands, including the nitrogen of histidine, the oxygen of aspartate or glutamate, and the sulfur of cysteine; among these, histidine is most commonly observed *(96)*; in FTase, the three protein-derived zinc ligands are Asp297, Cys299, and His362 *(43,97)*. This spacing between the protein ligands is also characteristic of many catalytic zinc sites, which show a regular pattern that is not observed for structural zinc sites. This pattern consists of a short spacer (1–3 amino acids) between the first and the second ligands, and a long spacer (20–120 amino acids) between the second and the third ligands. The short spacer, with a rigid arrangement, may constitute a nucleus for the zinc binding site; whereas the third ligand, distant from the other two ligands in the linear sequence, may be responsible for the spatial formation of the active site and increase the stability of zinc coordination. The long spacer may also imply that flexibility is essential for the change of geometry and number of ligands that occurs in the zinc polyhedron during catalysis *(96)*.

The CAAX prenyltransferases are members of a new class of zinc metalloproteins that possess a previously-unappreciated catalytic function of the zinc ion: to enhance the nucleophilicity of a thiol group at neutral pH *(98)* (Fig. 3). The "founding member" of this family is a DNA repair protein termed "Ada." The function of Ada is to remove irreversibly the methyl group from the $S_p$ diastereomer of DNA methylphosphotriesters *(99)*. Recently, the bound zinc ion found at the N-terminal domain of Ada has been proposed

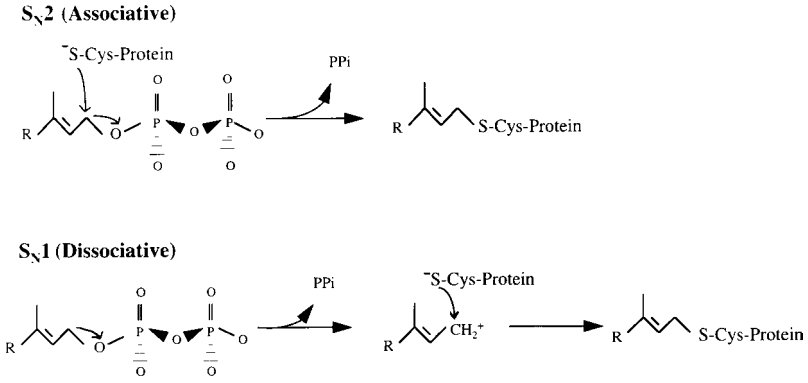


**Fig. 4.** Possible chemical mechanisms of CAAX prenyltransferases. Shown are the two extremes for potential mechanisms of catalysis, either a purely nucleophilic attack of the thiolate anion at C-1 of the isoprenoid substrate (the $S_N2$ mechanism) or an electrophilic reaction that involves preliminary formation of a carbocation at C-1 of the isoprenoid that then "captures" the appropriately positioned thiolate anion (the $S_N1$ mechanism). (*See text* for details.)

to catalyze the methyl transfer reaction in addition to stabilizing the structure of Ada *(99)*. The zinc ion is coordinated by the sulfur atoms of four cysteines, and one of the zinc ligands, $Cys_{69}$, is the residue that is methylated to form a thioether bond during the reaction with the methylphosphotriesters. The primary function proposed for this zinc ion is to coordinate the cysteine thiolate to lower the $pK_a$ and perhaps enhance the reactivity of this group *(99)*. A similar mechanism has been recently proposed for several additional zinc-containing enzymes that catalyze S-alkylation reactions; these include, in addition to the CAAX prenyltransferases, the enzymes cobalamin-dependent methionine synthase, cobalamin-independent methionine synthase, and methanol:coenzyme M *(98,100–103)*.

### 2.4.2. Mechanism of Catalysis by FTase

Both electrophilic and nucleophilic mechanisms have been proposed for CAAX prenyltransferases *(37,104)* (Fig. 4). The electrophilic mechanism was first suggested by analogy to the somewhat similar enzymes such as prenyl diphosphate synthases and cyclases that use prenyl diphosphate as a substrate *(105–107)*; reactions catalyzed by these enzymes proceed predominately through formation of a carbocation at the site of bond formation. However, a conserved aspartate-rich region in the active sites of these enzymes that is important for catalysis was not observed in FTase, suggesting that the mechanism of the protein prenyltransferases may be somewhat different *(43,107)*. Furthermore, predominately nucleophilic mechanisms have been proposed for the other zinc-containing enzymes and proteins involved in S-alkylation reactions (i.e., Ada, the methionine synthases, and methanol:coenzyme M methyltransferase) noted in Subheading 2.4.1. *(98)*.

A nucleophilic mechanism for FTase was first suggested when it was found that the bound zinc ion in the enzyme was required both for catalysis as well as for the high affinity of peptide and protein substrates *(39,73)*. The initial direct evidence supporting a mechanism with substantial nucleophilic character came from the finding that the metal ion coordinated the thiol(ate) of the peptide substrate in the FTase · isoprenoid · CAAX ternary complex of cobalt-substituted FTase *(74)*; this finding was consistent with a model

where the substrate thiol is activated by binding to the metal. Additional evidence in this regard comes from an examination of the pH dependence of peptide substrate binding to FTase. These studies revealed that the peptide thiol coordinates the metal as the thiolate anion, and that the $pK_a$ of the -SH group is shifted from ~8 in the free peptide to ≤6.5 in the enzyme-bound peptide *(72)*. Stereochemical studies using FPP containing chiral deuterium-for-hydrogen substitutions at its C-1 carbon have demonstrated that FTasecarries out the reaction with inversion of configuration at the C-1 farnesyl center *(108,109)*. Although this finding is consistent with a $S_N2$-type nucleophilic displacement, it could also result from a $S_N1$-type mechanism in which the configuration of carbocation formed was subject to steric hindrance.

In a recent study, a transient kinetic analysis of FTase in which the zinc ion was substituted with cadmium ion was used to investigate further whether the metal-coordinated substrate thiolate played a nucleophilic role in catalysis by mammalian FTase *(109a)*. Cadmium is a softer metal than zinc, hence it has higher affinity for sulfur atom *(95)*. In model studies using small thiol compounds, $Cd^{2+}$ was found to bind the thiolate 1–2 orders of magnitude more tightly than $Zn^{2+}$ *(110)*. In fact, the affinity of the FTase · isoprenoid binary complex for peptide substrates is increased >10-fold for Cd-FTase compared to Zn-FTase *(109a)*, a finding consistent with the notion that $Cd^{2+}$ enhances the binding of peptide substrate through stronger coordination of the substrate thiolate. Furthermore, in single-turnover experiments, the rate of product formation catalyzed by Cd-FTase was decreased ~sixfold compared to that of Zn-FTase, suggesting that the metal-thiolate nucleophile is important in the transition state *(109a)*.

Evidence has also been obtained for an electrophilic component in the mechanism of FTase. This evidence, initially using the yeast enzyme, came from the use of FPP substrate analogs that contained fluorine substitutions at the $C_3$ methyl position; fluorine, an electron-withdrawing group, would be expected to destabilize the carbocation in the transition state *(104)*. A decreased activity of yeast FTase with the $C_3$ fluoromethyl-FPP analogs was in fact observed; furthermore, the decrease in activity paralleled the number of fluorines in the substrate *(104)*. Similar results have recently been obtained for mammalian FTase using the $C_3$ fluoromethyl FPP analogs (C.C. Huang and C.A. Fierke, unpublished results). In both the yeast and mammalian enzymes, the rate constant for the chemical step of the FTase reaction was significantly decreased with these compounds and the decrease paralleled the number of fluorines in the substrate, strongly suggesting that the transition state has carbocation character. Because the fluoro-substituted FPPs affect a step after the metal-thiolate coordination, the carbocation apparently does not form until the peptide substrate binds. Importantly, the decreases in reactivity caused by fluorine substitution in FPP analogs are significantly smaller than the effects of fluorine substitution on either the solvolysis of dimethylallyl *p*-methoxybenzensulfonates or the reactivity of farnesyl pyrophosphate synthase, both of which proceed via an $S_N1$ reaction *(104,111)*. In fact, the effects of the fluorine substitution on FTase reactivity more closely parallel the effects on solvolysis reactions in the presence of a potent nucleophile, such as azide, which proceed with significant $S_N2$ character through what has been termed an open "exploded" transition state *(112)*.

Taken together, the data summarized above suggest that the mechanism of FTase is a carbocation-nucleophile combination reaction (Fig. 5). It seems that neither the nucleophilic nature of the metal-thiolate nor the carbocation character of the $C_1$ of FPP can be ignored. For such a carbocation-nucleophile combination reaction, whether the reaction

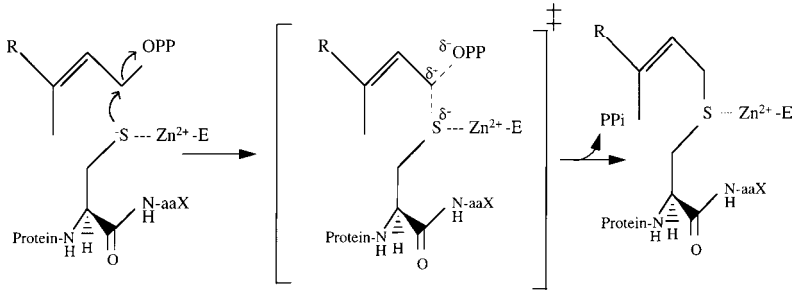


**Fig. 5.** Proposed mechanism for FTase. Shown is the carbocation-nucleophile combination mechanism proposed for FTase. In this mechanism, the role of the enzyme-bound zinc ion is to both facilitate formation of the substrate protein thiolate anion and position it for attack at the developing carbocation at C-1 of the isoprenoid substrate. (*See text* for details.)

proceeds through an $S_N1$ or $S_N2$ mechanism depends on both the stability of the carbocation in the transition state and the strength of the nucleophile *(113)*. When the carbocation intermediate has a long enough lifetime, the reaction can proceed via a stepwise $S_N1$ mechanism. On the contrary, if the lifetime of the intermediate is short, the reaction may occur through an enforced preassociation mechanism, where the reactants are assembled before the first bond-making or -breaking step occurs. This preassociation mechanism could either be concerted with no intermediate, or stepwise with an intermediate.

## 3. CONCLUSIONS

The close similarities in both structure and function of GGTase I and FTase, which include conservation of the zinc-binding residues in the β-subunit of GGTase I and the requirement for this metal for protein substrate binding, make it is very likely that both enzymes use a similar catalytic mechanism. Nonetheless, it will still be important to conduct studies similar to those described previously with GGTase I to confirm this hypothesis; furthermore, it could well turn out that there will be some important differences between these enzymes. Moreover, one must always keep in mind with these enzymes that the actual catalytic step has little influence on their steady-state rate and thus extreme caution must be exercised in the use of steady-state data to draw conclusions on such parameters as specificity in recognition of CAAX sequences, and so forth. For example, a peptide substrate that can be prenylated but is poorly released may exhibit the behavior of an inhibitor of this enzyme. To understand fully the mechanism of substrate specificity and other parameters of these enzymes, examination of the individual steps in the catalytic process, i.e., by presteady-state kinetics, is required.

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