Identification of a Cysteine Residue Essential for Activity of Protein Farnesyltransferase

Cys\(^{299}\) IS EXPOSED ONLY UPON REMOVAL OF ZINC FROM THE ENZYME\(^*\)

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Protein farnesyltransferase (FTase) is a zinc metalloenzyme that performs a post-translational modification on many proteins that is critical for their function. The importance of cysteine residues in FTase activity was investigated using cysteine-specific reagents. Zinc-depleted FTase (apo-FTase), but not the holoenzyme, was completely inactivated by treatment with N-ethylmaleimide (NEM). Similar effects were detected after treatment of the enzyme with iodoacetamide. The addition of zinc to apo-FTase protects it from inactivation by NEM. These findings indicated the presence of specific cysteine residues(s), potentially located at the zinc binding site, that are required for FTase activity. We performed a selective labeling strategy whereby the cysteine residues exposed upon removal of zinc from the enzyme were modified with \(^{3H}\)NEM. The enzyme so modified was digested with trypsin, and four labeled peptides were identified and sequenced, one peptide being the major site of labeling and the remaining three labeled to lesser extents. The major labeled peptide contained a radiolabeled cysteine residue, Cys\(^{299}\), that is in the \(\beta\) subunit of FTase and is conserved in all known protein prenyltransferases. This cysteine residue was changed to both alanine and serine by site-directed mutagenesis, and the mutant proteins were produced in Escherichia coli and purified. While both mutant proteins retained the ability to bind farnesyl diphosphate, they were found to have lost essentially all catalytic activity and ability to bind zinc. These results indicate that the Cys\(^{299}\) in the \(\beta\) subunit of FTase plays a critical role in catalysis by the enzyme and is likely to be one of the residues that directly coordinate the zinc atom in this enzyme.

Protein farnesyltransferase (FTase)\(^1\) catalyzes the transfer of the 15-carbon isoprenoid from farnesyl diphosphate (FPF) to a conserved cysteine residue of protein acceptors containing the carboxyl-terminal CaaX motif, where C is cysteine, X is generally an aliphatic amino acid, and X is methionine, serine, glutamine, or alanine (1, 2). A number of farnesylated proteins have been identified in eukaryotes, including Ras proteins, nuclear lamins, and at least two \(\gamma\) subunits of heterotrimeric G proteins (3–5). Through the hydrophobic nature of the farnesyl group, this modification mediates protein-membrane, and possibly protein-protein, interactions that play important roles in proper subcellular localization and function of these proteins, most of which are involved in signal transduction.

The properties of FTase are similar to those of a related enzyme, protein geranylgeranyltransferase Type I (GGTase I), which also recognizes and prenylates proteins with the carboxyl-terminal CaaX motif. However, GGTase I transfers the geranylgeranyl isoprenoid and prefers proteins with leucine at the X position of the CaaX motif (1, 2). Known substrates for GGTase I include many Ras-related proteins, such as Rac and Rho, and most \(\gamma\) subunits of heterotrimeric G proteins (3, 4). Since both FTase and GGTase I recognize proteins and short peptides containing appropriate CaaX motifs at their carboxyl terminus, they are also termed CaaX prenyltransferases (6). Geranylgeranyltransferase type II (GGTase II, also known as Rab geranylgeranyltransferase), on the other hand, catalyzes geranylgeranylation of proteins that terminate in CC or CXC motifs at their carboxyl terminus (6). Target proteins of GGTase II belong to the Rab protein family, members of which are involved in protein secretion and endocytosis (2, 4).

Purified FTase is a heterodimer consisting of 48-kDa \(\alpha\) and 45-kDa \(\beta\) subunit polypeptides (7). Like FTase, GGTase I is also composed of two nonidentical subunits, these being a 48-kDa \(\alpha\) and a 43-kDa \(\beta\) subunit (8). cDNA clones for the subunits of both enzymes have been isolated from rat and human cDNA libraries (9–12). Immunological evidence and sequence comparison showed that these two enzymes share the same \(\alpha\) subunit and that significant sequence similarity is found between the two \(\beta\) subunits (12, 13). Since both enzymes show specificity toward their protein and isoprenoid substrates, the unique \(\beta\) subunits of these enzymes are thought to be responsible for the recognition and binding of both substrates. Consistent with this idea are studies showing that photoreactive isoprenoid analogues cross-link to the \(\beta\) subunits of both FTase and GGTase I (11, 14, 15), and that both protein and peptide substrates are cross-linked to the \(\beta\) subunit of FTase (16, 17). Both FTase and GGTase I are zinc metalloenzymes (8, 16). Atomic absorption analysis of purified recombinant enzymes

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\(^1\)The abbreviations used are: FTase, protein farnesyltransferase; GGTase, protein geranylgeranyltransferase; G protein, GTP-binding regulatory protein; FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate; CaaX, a sequence motif of proteins consisting of an invariant Cys residue fourth from the C terminus; Ras-CVLL, Ha-Ras protein with a Leu-for-Ser substitution at the C terminus; NEM, N-ethylmaleimide; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; PCR, polymerase chain reaction.
shows that the metal in these enzymes is important in their interaction with substrates.

While it is clear that the zinc in CaaX prenyltransferases is critical for their activity, the precise role that this metal plays is not yet known. The zinc could be acting catalytically, for example by increasing the nucleophilicity of the cysteine residue in the CaaX motif of protein substrate and/or activating the diphosphate leaving group, or structurally, perhaps by stabilizing the heterodimeric form of the enzyme. In order to clarify the role of the zinc atom in FTase, we first set out to identify residues that might serve as the ligands of the metal. A common metal ligand in many zinc enzymes is the thiol group of a cysteine residue that might serve as the ligands of the metal. A common metal ligand in many zinc enzymes is the thiol group of a cysteine residue.

**EXPERIMENTAL PROCEDURES**

**Determination of Enzymatic Activity and Isoprenoid Binding—FTase activity was determined by quantitating the amount of H transferred from [3H]FPP into the Ha-Ras protein as described previously (24). The standard reaction mixture contained the following components in a final volume of 50 μl: 50 mM Tris-HCl, pH 7.7, 20 mM KCl, 5 mM MgCl2, 5 μM ZnCl2, 2 mM dithiothreitol (DTT), 8 μM Ha-Ras, 1 μM [3H]FPP (8 Ci/mmol), and 50 ng of FTase. Assays were conducted for 15 min at 37 °C, and the amount of trichloroacetic acid-precipitable [3H]farnesylated Ha-Ras was determined as described (18, 19); both enzymes were recombinant proteins produced by infection of Sf9 cells with recombinant baculoviruses and purified from cell extracts as described previously (18, 19); both enzymes were >95% pure as judged by SDS-PAGE analysis. To prepare the apo forms of the two enzymes, the appropriate enzyme (about 1 mg) was dialyzed first for 24 h at 4 °C against 1 liter of 20 mM Tris-HCl, pH 7.7, 1 mM DTT, and 5 mM EDTA and then for another 24 h against the same buffer except that the EDTA concentration was raised to 1 mM. Removal of zinc from FTase was confirmed by assessing the zinc dependence of their activities (8).**

**Inactivation of FTase and GGTase I by Sulfhydryl-specific Reagents—Native and zinc-depleted FTase were chromatographed through a 50 Sephadex spin columns that had been equilibrated at 4 °C with 20 mM Tris-HCl, pH 7.7, containing 5 μM ZnCl2 (for native enzyme) or 20 mM Tris-HCl, pH 7.7, containing 0.1 mM EDTA (for zinc-depleted enzyme) to remove DTT. The DTT-free enzyme (designated as a zinc-depleted enzyme) was incubated with a concentration of 14 μM with 20 mM Tris-HCl, pH 7.7, in the presence or absence of 67 μM FPP. After a 5-min preincubation at room temperature, the inactivation reactions were initiated by the addition of either 250 μM N-ethylmaleimide (NEM) or 2 mM iodoacetamide. At various times, aliquots of the reaction mixture were withdrawn, and reactions were stopped by the addition of 10 mM DTT. For FTase, NEM or iodoacetamide was preincubated with DTT for 1 min before adding aliquots of the preincubated enzyme solution. Treatment of GGTase I by NEM was performed by the same procedure as that of FTase. To prepare NEM-modified FTase for subsequent radio labeling experiments, DTT-free holo-FTase (20 μg) was incubated with 250 μM NEM in 20 mM Tris-HCl, pH 7.7, at room temperature for 20 min. The reaction was quenched with 10 mM DTT. The DTT-free enzyme (designated as holo-NEM-FTase). To assess the ability of added zinc to protect apo-FTase from inactivation, the DTT-free enzyme (~12 μM) was preincubated with increasing concentration of ZnCl2 (0–100 μM) in 20 mM Tris-HCl, pH 7.7, containing 67 μM EDTA, at room temperature for 5 min. Reactions were initiated by the addition of 250 μM NEM and quenched with 10 mM DTT.**

**Selective Labeling of FTase with [3H]NEM and Analysis of Labeling Sites—For the labeling studies, 120 μl of 5 mM unlabeled NEM in 20 mM Tris-HCl, pH 7.7, was mixed prior to use with 1 ml of 18 μM [3H]NEM in pentane, and the mixture was placed on ice under a flow of N2 to evaporate the pentane. DTT-free FTase preparations were diluted to 2 μM with 20 mM Tris-HCl, pH 7.7, containing 0.1 mM EDTA (for the apoenzyme) or 20 mM Tris-HCl, pH 7.7, containing 5 μM ZnCl2 (for the holoenzyme). The pentane-free [3H]NEM solution (4.5 ml, 3.0 Ci/mmol) was mixed with each enzyme solution to give a final NEM concentration of 150 μM. These reaction mixtures were incubated for 30 min at room temperature, stopped by the addition of 10 mM DTT, concentrated by CF-25 Centricon membrane cones (Amicon, Beverly, MA), and then exchanged into buffer containing 20 mM Tris-HCl, pH 8.2, and 50 mM NaCl by G-50 Sephadex spin chromatography; this treatment also served to remove the unreacted [3H]NEM. The amount of [3H]NEM incorporation into the subunits of FTase was determined by resolving the subunits by SDS-PAGE and subjecting the excised bands to scintillation spectrometry.**

**Analysis of Isoprenoid Substrates—FTase complexes formed were measured by filter binding as described (25). One of the peptides purified by HPLC (designated under “Results” as peak I) was further subjected to cyanogen bromide cleavage and then rechromatographed in 70% formic acid as described (26) prior to rechromatography in the acetonitrile/ammonium acetate system.**

**Preparation of Native and Metal-depleted FTase and GGTase I—Except for the mutagenesis studies, which utilize bacterially produced enzymes (see below), the FTase and GGTase I used in this study were recombinant baculovirus proteins produced by infection of Sf9 cells with recombinant baculoviruses and purified from cell extracts as described previously (18, 19); both enzymes were >95% pure as judged by SDS-PAGE analysis. To prepare the apo forms of the two enzymes, the appropriate enzyme (about 1 mg) was dialyzed first for 24 h at 4 °C against 1 liter of 20 mM Tris-HCl, pH 7.7, 1 mM DTT, and 5 mM EDTA and then for another 24 h against the same buffer except that the EDTA concentration was raised to 1 mM. Removal of zinc from FTase was confirmed by assessing the zinc dependence of their activities (8).**
GGGA3-G and FTa-B (5'-CTGTCCTGTACACGACATGATGGG-3') probes were synthesized and immobilized on a nitrocellulose filter. High molecular weight DNA was extracted from 250 μl of rat FTase, respectively, were used in a PCR reaction with 12.5 ng of plasmid pUC13-FTB encoding the α subunit of rat FTase. The resulting PCR product was digested with EcoRI-BsrGI and ligated back into the EcoRI-BsrGI-digested pUC13-FTA. For the β subunit, PCR primers designated FTβ-5'-N (5'-AAAAAGTCTTAGGCTGTTCTGCTCTTCATCTCATT-3') and FTβ-B (5'-CTGTCAGGATCTAGTACAAGCT-CA-3') were used to amplify the sequences of the β subunit of rat FTase, respectively, were used in a PCR reaction with 12.5 ng of plasmid pUC13-FTB encoding the α subunit of rat FTase. The resulting PCR product was digested with HindIII-BamHI and ligated back into the HindIII-BamHI-digested pUC13-FTB. These resulting plasmids, designated as pUC13-PCRFα and pUC13-PCRFβ, for the α and β subunits of FTase, respectively, were confirmed by DNA sequencing. A NdeI-HindIII fragment from pUC13-PCRFα that contained the coding region of the α subunit of FTase was ligated into a NdeI-HindIII-digested pET28a vector (Novagen, Madison, WI). A NcoI fragment from pUC13-PCRFβ that contained the coding region of the β subunit of FTase was ligated into a NcoI-digested pAlteX2 promoter (Promega, Madison, WI). The resulting plasmids, designated as pET7α-PCRFα and pET7β-PCRFβ, were co-transformed into BL21(DE3) E. coli cells; expression of the pET7α-PCRFα plasmid yielded the α subunit of FTase with an N-terminal extension containing a hexahistidine motif.

A 3-liter culture of E. coli BL21(DE3) cells harboring the appropriate FTase plasmids was grown to an optical density of 0.6, and FTase expression was induced by the addition of 0.5 mM isopropyl-β-D-galactoside; 0.5 mM ZnSO4 was also added at this time to ensure zinc availability. After a 4-h induction at 37°C, cells were harvested and washed with phosphate-buffered saline. The cell pellet was resuspended in 40 ml of buffer containing 20 mM Tris-HCl, pH 7.7, 5 mM imidazole, 1 mM β-mercaptoethanol, and a protease inhibitor mix. The cells were lysed by two passes through a French press. The sample was diluted to 80 ml, NaCl was added to a final concentration of 300 mM, and the extract was centrifuged at 30,000 × g for 1 h at 4°C. The supernatant was subjected to Ni2+-NTA affinity chromatography under the conditions recommended by the manufacturer (Qiagen, Chatsworth, CA). The resin was eluted with a 5-100 mM imidazole gradient in 20 mM Tris-HCl, pH 7.7, 300 mM NaCl, 1 mM β-mercaptoethanol, and the protease inhibitor mix. Peak fractions, analyzed by assessing their FTase activity, were pooled and dialyzed twice for 1 h each against 1 liter each of 50 mM Tris- HCl, pH 7.7, 5 μM ZnCl2, and 1 mM DTT. The column was washed with the equilibration buffer and then eluted with a linear gradient to 500 mM NaCl in the same buffer. Peak fractions were pooled and concentrated with buffer exchange into the starting buffer using a Centricon 30 concentrator (Amicon, Beverly, MA) to a protein concentration of ~1 mg/ml, flash-frozen in aliquots, and stored at ~80°C until use. For the mutant enzymes, the purification procedure was the same except that the Mono Q HR/5/5 column (Pharmacia) was used instead of the Q-HP column, and peak fractions were determined by immunoblot analysis and FFP binding rather than by enzymatic activity.

Determination of Zinc Binding Activity—An aliquot of each of the three purified mutant FTases (~20 μmol, based on FFP binding) was diluted to 60 μl with 20 mM Tris-HCl, pH 7.1, 1 mM DTT, and 10 μM EDTA. Samples were chromatographed through G-50 Sephadex spin columns that had been equilibrated at 4°C with 20 mM Tris-HCl, pH 7.7, 1 mM DTT, 100 mM NaCl, and 1 μM EDTA and incubated at 30°C for 1 h. A 14 μM solution of 65ZnCl2 (30 Ci/mmol), prepared by diluting the stock reagent with 200 mM Tris-HCl, pH 8.5, was added to each of the enzyme samples so that the final concentration of 65ZnCl2 was 2 μM. Following an incubation for 3 h at room temperature and overnight at 0°C, the incubation mixtures were supplemented with FFP to a final concentration of 5 μM (to prevent dissociation of bound zinc) and held for 15 min at room temperature. One-fifth of each reaction mixture was loaded onto a 10% semi-denaturing native polyacrylamide gel prepared in 90 mM Tris-HCl (pH 8.2), 80 mM boric acid, and 2.5 mM EDTA, and electrophoresis was performed in 90 mM Tris-HCl, pH 8.2, 80 mM boric acid, and 2.5 mM EDTA for 1 h at 200 V. The gel was washed with electrophoresis buffer for 15 min, wrapped in cellophane, and exposed to film.

Miscellaneous Methods—Site-specific mutations were introduced into FTase using the Altered Sites Mutagenesis system (Promega, Madison, WI). Three mutant enzymes were produced, these being FTαC341A with a mutation in the α subunit of FTase and FTβC299A with a mutation in the β subunit of FTase, respectively. The resulting cDNAs was digested with restriction enzymes, and the mutation was confirmed by DNA sequence analysis. Each was then subcloned into the expression vector pET28a (for the α subunit mutant) or pAlteX2 (for the β subunit mutants). Standard molecular biology methods for DNA sequencing and manipulation were used (28). DNA sequencing was performed using the Sequencing version 2.0 DNA sequencing kit (U.S. Biochemical Corp.), except that the DNA plasmid pUC13-PCRFTα was sequenced at the University of North Carolina (Chapel Hill) Automated DNA Sequencing Facility on a model 373A DNA Sequencer (Applied Biosystems). Radiolabeled peptides were sequenced by automated Edman degradation on an amino acid sequencer (Applied Biosystems) by C. R. Moomaw at the University of Texas Southwestern Medical Center (Dallas). SDS-PAGE and immunoblot analysis were performed as described previously (18). Antiserum p538, a polyclonal rabbit antibody directed against a peptide corresponding to residues 359–370 of rat FTase-α, was used to detect the α subunit of FTase; antiserum p12I, a polyclonal rabbit antibody directed against holo-FTase, was used to detect the β subunit of FTase. Both antisera were affinity-purified on Protein A-Sepharose prior to use. Protein concentration was routinely analyzed by the Bradford method using a commercial dye (Bio-Rad) and bovine serum albumin as standard.

Materials—Recombinant Ha-Ras and Ras-CVLL proteins were purified from bacterial expression systems as described (24). Plasmids pRI-EcoRI-digested pUC13-FTB. These resulting ⌟-I fragment from pUC13-PCRFTa and pUC13-FTB were digested with restriction enzymes, and the mutation was sequenced by automated Edman degradation on an amino acid sequencer and manipulated (28). DNA sequencing was sequenced at the University of North Carolina (Chapel Hill) Automated DNA Sequencing Facility on a model 373A DNA Sequencer (Applied Biosystems). For the α subunit mutant or pAlteX2 (for the β subunit mutants). Standard molecular biology methods for DNA sequencing and manipulation were used (28). DNA sequencing was performed using the Sequencing version 2.0 DNA sequencing kit (U.S. Biochemical Corp.), except that the DNA plasmid pUC13-PCRFTα was sequenced at the University of North Carolina (Chapel Hill) Automated DNA Sequencing Facility on a model 373A DNA Sequencer (Applied Biosystems). Radiolabeled peptides were sequenced by automated Edman degradation on an amino acid sequencer (Applied Biosystems) by C. R. Moomaw at the University of Texas Southwestern Medical Center (Dallas). SDS-PAGE and immunoblot analysis were performed as described previously (18). Antiserum p538, a polyclonal rabbit antibody directed against a peptide corresponding to residues 359–370 of rat FTase-α, was used to detect the α subunit of FTase; antiserum p12I, a polyclonal rabbit antibody directed against holo-FTase, was used to detect the β subunit of FTase. Both antisera were affinity-purified on Protein A-Sepharose prior to use. Protein concentration was routinely analyzed by the Bradford method using a commercial dye (Bio-Rad) and bovine serum albumin as standard.

RESULTS

Inactivation of Zinc-depleted FTase by Sulfhydryl-specific Reagents—To determine whether cysteine residues play an important role in FTase activity, cysteine-specific alkylating reagents were tested for their ability to inactivate the enzyme. Treatment of zinc-depleted (apo-) FTase with 250 μM NEM resulted in a loss of 90% of its activity within 2 min and almost total loss of activity after 10 min. In contrast, the holoenzyme was quite resistant to inactivation by the same treatment (Fig. 1A). The addition of Zn2+ to apo-FTase completely protected it from inactivation by NEM (Fig. 1B). Interestingly, preincubation of apo-FTase with FPP, the isoprenoid substrate of FTase, was also found to protect against NEM inactivation (Fig. 1A). When the other CaaX prenyltransferase, GGTTase I, was treated with 250 μM NEM, the apoenzyme was inactivated in a time-dependent manner similar to that of apo-FTase, and preincubation of apo-GGTTase I with GGPP also prevented inactivation of the enzyme (data not shown).

To confirm these findings, we repeated these studies with another cysteine-specific alkylating reagent, iodoacetamide. Again, treatment of apo-FTase with iodoacetamide resulted in essentially complete inactivation in a time-dependent fashion, whereas the holoenzyme retained its activity even after a 1-h modification (Fig. 1C). As observed with NEM inactivation, inclusion of FPP prevented inactivation of apo-FTase by iodoacetamide (data not shown). Taken together, these results indicated the presence of specific cysteine residue(s) required for the activity of the CaaX prenyltransferases and, furthermore, that such cysteine residue(s) might be ligands that coordinate the zinc atom. We therefore designed a strategy, using FTase as the model enzyme, to identify the cysteine residue(s) involved and assess their influence on enzyme activity. Changes in the cysteine residue(s) in FTase were produced, these being Cys299 was Essential for FTase Activity
ment of the viability of the approach, we examined whether the NEM modification of FTase was a homogenous event. We first assessed whether NEM-modified holo-FTase that was subsequently subjected to zinc depletion behaved in a similar manner as unmodified FTase. We found that the NEM-modified enzyme was catalytically inactive after zinc depletion and that the activity could be completely restored by the addition of zinc to the enzyme (data not shown). These findings indicated that holo-NEM-modified FTase was quite similar to its unmodified counterpart, the only difference detected being a slightly reduced catalytic ability of the NEM-modified enzyme (see Fig. 1A). Since this reduction in activity is not seen with the enzyme modified by iodoacetamide (Fig. 1, compare A and C), it is likely that the reduced activity of the NEM-modified enzyme compared with that modified by iodoacetamide is due to some type of steric hindrance by the larger size of the former alkylating agent. In support of this assessment that NEM modification of holo-FTase did not significantly affect the protein's structure or function, the FPP binding ability of both holo-FTase and holo-NEM-modified FTase was found to be essentially identical; analysis of the binding data showed that the stoichiometry of FPP binding by NEM-modified FTase was on average 10% less than that of the unmodified enzyme (data not shown).

The results described above suggested a selective radiolabeling strategy that we felt would lead to the identification of the critical cysteine residue(s) required for FTase activity. Native FTase was first treated with unlabeled NEM to alkylate accessible cysteine residues, and then the enzyme so modified was treated with EDTA to remove zinc. This apo-NEM-modified FTase was then further treated with [3H]NEM to label the cysteine residues exposed upon the removal of zinc. As a control, NEM-modified FTase not subjected to EDTA treatment was also treated with [3H]NEM. A diagram summarizing this selective labeling scheme is shown in Fig. 2. The stoichiometry of [3H]NEM incorporation into FTase subjected to these two treatments was determined by gel slice assay. For holo-FTase, approximately 0.1 and 0.3 mol of [3H]NEM/mol of protein was incorporated into the α and β subunits, respectively, while incorporation into apo-FTase was substantially greater, with stoichiometries of 1.0 and 3.6 mol of [3H]NEM/mol of protein into the α and β subunits, respectively (Fig. 3). Since the total incorporation of radiolabel into holo-FTase was only one-tenth of that into apo-FTase, it seemed likely that there were indeed specific cysteine residues protected by zinc in the enzyme.

The modified enzymes were subjected to trypsin digestion, and the peptides produced were isolated by reverse-phase
HPLC. The comparison of the elution patterns of the peptides derived from the two samples is shown in Fig. 4, A and B. Rov revealed that the chromatograms for both holo- and apo-FTase were essentially indistinguishable. However, analysis of the radioactivity in the profile revealed that four major radioactive peaks were obtained from apo-FTase but not from holo-FTase (Fig. 4C). Since this profile was still somewhat complex, the material eluting in each of these peaks was collected and further separated by reverse-phase HPLC using a second solvent system. Rechromatography of the material in peak IV (the major radiolabeled species) yielded one major radiolabeled product that eluted from the second chromatographic step as a single, well defined peak of both absorbance and radioactivity (Fig. 5, A and B). Sequencing of this peptide yielded a radiolabeled cysteine derivative that was released at cycle 5 (Fig. 5C). This labeled peptide, whose sequence is shown in Fig. 5C, is in the β subunit of FTase. The cysteine residue in this peptide is conserved in all known FTases and is also conserved in the X. laevis CaaX prenyltransferase (GGTase II) (Table I).

In all, four [3H]labeled polypeptides, i.e. one from each of the peaks in the first HPLC profile, were obtained and identified by sequence analysis (Table I). Only one of the additional three peptides appeared interesting. This peptide, whose sequence is shown in Table I, was isolated from the peak I material in the original chromatography. The labeled cysteine residue identified was in the α subunit of FTase in a position that is conserved in mammalian FTase and GGTase I and II (Table I).

Site-directed Mutagenesis of Cysteine Residues—To determine if either or both of the conserved cysteine residues identified through the selective labeling approach (i.e. Cys299 in the β subunit and Cys341 in the α subunit) were in fact essential for FTase activity, site-directed mutagenesis and analysis of the mutant enzymes were undertaken. Cys299, the major [3H]NEM-labeled cysteine residue that is in the β subunit of FTase, was changed to both alanine and serine; these mutants were designated FTβC299A and FTβC299S, respectively. Cys341 in the α subunit was changed to alanine to produce mutant FTαC341A. These three mutant proteins were produced by expression in E. coli and purified by chromatography over the Ni2+ -NTA affinity and Mono Q resins as described under “Experimental Procedures.” Fractions obtained from the

Fig. 3. Stoichiometry of [3H]NEM incorporation into FTase. Three μg of each of the final preparations of holo-FTase (closed bars) and apo-FTase (hatched bars) obtained from the [3H]NEM labeling strategy summarized in Fig. 2 were subjected to SDS-PAGE, and the α and β subunits were visualized by staining with Coomassie Blue. The region of the gel corresponding to each subunit was excised and subjected to an overnight digestion in an aqueous solution containing 20% H2O2 and 20% HClO4, at 60 °C, and the radioactivity in each sample was determined. The results from three independent experiments were pooled, and means and standard deviations are shown.

Fig. 4. Analysis of the tryptic digests of [3H]NEM-labeled FTase preparations. [3H]NEM-labeled apo-FTase (1 nmol, panels A and C) and holo-FTase (0.5 nmol, panels B and C), obtained from the labeling approach summarized in Fig. 2, were subjected to trypsin digestion, and resultant digests were processed by reverse-phase HPLC using the acetonitrile/trifluoroacetic acid gradient (dashed line) described under “Experimental Procedures.” The eluent was monitored at 214 nm to detect peptides produced (panels A and B), and aliquots of collected fractions were analyzed for radioactivity (panel C). The four major peaks of radioactivity derived from the digest of apo-FTase (C) are designated I–IV; negligible radioactivity was associated with the digest of the holoenzyme (●).

Cys299 Is Essential for FTase Activity

—Cys299 in the β subunit of FTase was changed to both alanine and serine. These mutants were designated FTβC299A and FTβC299S, respectively. Cys341 in the α subunit was changed to alanine to produce mutant FTαC341A. These three mutant proteins were produced by expression in E. coli and purified by chromatography over the Ni2+ -NTA affinity and Mono Q resins as described under “Experimental Procedures.” Fractions obtained from the...
Cys\textsuperscript{299} Is Essential for FTase Activity

Mono Q chromatographic step for all three mutant enzymes were assayed both for FTase activity and FPP binding, and the results are shown in Fig. 6. The \( \alpha \) subunit mutant, FT\textalpha{}C341A, exhibited properties essentially indistinguishable from the wild-type enzyme and retained both enzymatic activity and the ability to bind FPP. In contrast, both of the proteins that contained the Cys\textsuperscript{299} mutations in the \( \beta \) subunits lost essentially all of their catalytic activity. However, in spite of their inability to catalyze farnesylation, both mutant enzymes retained the ability to bind FPP.

To directly compare the activity of each mutant to the wild-type enzyme, the latter was produced in \textit{E. coli} and purified in a similar manner. As with the mutant enzyme, the wild-type protein eluted from the anion exchange column in a peak centered at 0.3 M NaCl (data not shown). For each of the four enzymes (i.e. the wild-type and the three mutants), the peak fraction from the anion exchange column was selected for analysis of enzyme levels and for catalytic and FPP binding activities. Immunoblot analysis confirmed that both the \( \alpha \) and \( \beta \) subunits were present in all four purified enzymes (Fig. 7). The \( \alpha \) subunits of the enzymes contained the His-tag fusion at their N termini and hence migrated at an apparent molecular mass of 57 kDa in this system. This analysis indicated that all four enzymes were produced in \textit{E. coli} as heterodimers, since the enzyme could be purified through the use of the affinity tag on the \( \alpha \) subunit, and the \( \beta \) subunit could be co-purified. Additionally, the fact that all four enzymes retained the ability to bind FPP indicated that all of these proteins had folded correctly. A comparison of the enzymatic and FPP binding activities of each of the mutant enzymes to wild-type FTase is shown in Table II. The turnover number determined for the wild-type enzyme of 2.6 min\textsuperscript{-1} agrees well with previous studies on this enzyme (11, 21). As noted above, the FT\textalpha{}C341A variant displayed similar enzymatic activities as the wild-type enzyme; in fact, this quantitative analysis indicated that it has a slightly higher turnover number, although this is probably not significant. We were able to detect very low levels of enzymatic activity in mutant FT\textbeta{}C299A, about 2\% that of the wild-type enzyme. No residual enzymatic activity in mutant FT\textbeta{}C299S could be detected. Additionally, increasing the zinc concentration in the assay buffer did not restore FTase activity to either of the two enzymes containing the Cys\textsuperscript{299} mutations (data not shown).

The results detailed above strongly suggested that the loss in activity of the two \( \beta \)-Cys\textsuperscript{299} mutants of FTase was due to an inability to coordinate the zinc atom required for activity of this enzyme. To directly examine this, we developed a \textsuperscript{65}Zn binding assay for the enzyme (see "Experimental Procedures"). Important points in this assay are the binding of FPP to the enzymes after the zinc exchange reaction to prevent dissociation of bound metal and the use of a native gel system in which the FTase dimer migrates as an intact species.\textsuperscript{3} The results of this analysis, shown in Fig. 8, clearly indicate that the FT\textalpha{}C341A mutant retains the ability to bind zinc but that the two enzymes containing the Cys\textsuperscript{299} mutations had lost this ability. Thus, the loss of FTase activity in the two FTase forms containing the Cys\textsuperscript{299} mutations correlates with a loss of zinc binding by these enzymes.

\textsuperscript{3} W. R. Tschantz and P. J. Casey, unpublished observations.
Cys\(^{299}\) Is Essential for FTase Activity

**DISCUSSION**

We have used a selective chemical modification strategy to identify a cysteine residue in FTase that is critical for catalytic activity of the enzyme. This strategy was designed to identify cysteine residues that we felt would be involved in zinc coordination in this metalloenzyme and was initiated upon finding cysteine-specific alkylating agents inactivated FTase only after zinc was removed from the enzyme. This predominant residue labeled in this strategy was Cys\(^{299}\) in the \(\alpha\) subunit of the enzyme, and this residue was found to be conserved in all known protein prenyltransferases. Indeed, replacement of Cys\(^{299}\) with either serine or alanine resulted in production of an altered enzyme that had lost essentially all catalytic activity. The finding that these two mutant forms of FTase were still produced as heterodimers and retained normal binding of FPP in spite of the loss in enzymatic function indicated that Cys\(^{299}\) is truly essential for FTase activity and not simply for correct folding of the enzyme. Additionally, since labeling of Cys\(^{299}\) by \(^{3}H\)NEM only occurred after removal of the zinc and both Cys\(^{299}\) mutants lost the ability to bind this metal, we feel it is likely that this residue is directly involved in binding of zinc by the enzyme and that it is quite possibly one of the metal ligands.

Although several other cysteine residues were identified through this labeling strategy, they were modified to a much lesser degree than Cys\(^{299}\) and were also less conserved in the protein prenyltransferase family. Nonetheless, we did select one of these, that being Cys\(^{341}\) in the \(\alpha\) subunit of the enzyme, for mutagenesis, since its labeling intensity was second only to Cys\(^{299}\) in the \(\alpha\) subunit and it was conserved among mammalian, although not yeast, Caa\(_X\) prenyltransferases. However, expression of the FT\(\alpha\)C341A mutant produced an enzyme that was fully active in both FPP binding and catalytic activity, indicating that this is not an essential residue in the enzyme. It
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seems possible that its labeling is due to some type of conformational change in FTase that occurs upon NEM modification of Cys$^{299}$ that might result in an increased accessibility of this and other cysteine residues for modification. In this regard, the ability to incorporate [3H]NEM into Cys$^{299}$ and Cys$^{366}$ in the β subunit after zinc removal may be significant, although the incorporation stoichiometry was quite low. Specifically, it is intriguing that these two Cys residues are juxtaposed to a histidine residue, His$^{362}$, that is conserved in all β subunits of the protein prenyltransferase family. Moreover, we have found that diethylpyrocarbonate, a histidine-specific modification reagent, can inactivate apo-FTase much more rapidly than holo-FTase. This can be viewed as preliminary evidence for the existence of a histidine residue involved in zinc coordination; histidine residues are in fact the most common zinc ligands found in zinc metalloenzymes (22). Hence, His$^{362}$ in the β subunit may also be one of the zinc ligands in FTase and other protein prenyltransferases.

In this thiol-specific modification study, binding of isoprenoid substrates to both FTase and GGTTase I protected their apoenzymes from inactivation. This observation is consistent with the finding that binding of GGPP to GGTTase I prevented the inactivation of the enzyme by metal chelating reagents (20). It seems likely that the binding site of the isoprenoid substrate is close to that of the zinc atom in these enzymes such that binding of this substrate physically blocks access of the alkylating reagent to the zinc binding site. Alternatively, binding of the isoprenoid substrate could induce a conformational change in the enzymes that results in a shielding of the zinc from molecules as large as the alkylating agents employed. In this regard, a conformational change in FTase upon FPP binding has been observed by fluorescence analysis (29).

The exact function of the critical cysteine residue in FTase-β identified in this study, Cys$^{299}$-FTβ, in catalysis by the enzyme is not yet known. Cysteine residues have been identified as essential components of the active site of many different types of enzymes (30). In addition to their role as metal ligands in many metalloenzymes (22), they can also function directly in catalysis both as nucleophiles and in a general acid/base capacity, since the thiol group can be readily protonated or deprotonated under physiological conditions (30). However, as noted above, our results support the idea that this residue is involved in the coordination of the zinc atom in FTase. If this is the case, the loss in activity observed in the FTβC299A and FTβC299S mutants may be due primarily to a reduced binding of zinc in the mutant enzymes. While this hypothesis is the one that is most consistent with the data, we cannot yet exclude the possibility that Cys$^{299}$ is not actually a zinc ligand but rather is adjacent to the metal, where it plays one of the other above noted roles in catalytic function of the enzyme. A formal answer to this question will await detailed structural information on the enzyme; such information will be required to design mechanistic studies that would provide details of the precise role of this cysteine residue in FTase function.

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