Yeast Protein Farnesyltransferase. pK_as of Peptide Substrates Bound as Zinc Thiolates†

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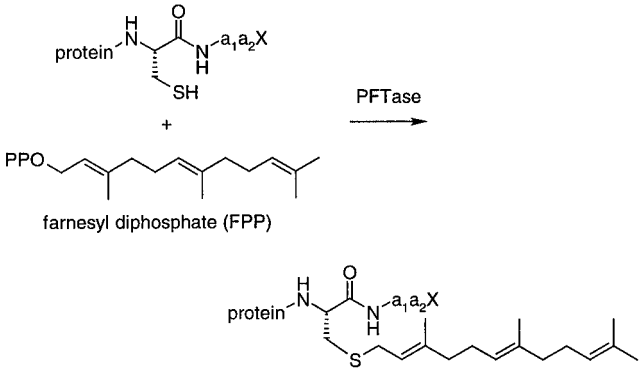
ABSTRACT: Protein farnesyltransferase (PFTase) is a zinc metalloenzyme that catalyzes the posttranslational alkylation of the cysteine in C-terminal $-Ca_1a_2X$ sequences by a 15-carbon farnesyl residue, where C is cysteine, a_1 and a_2 are normally aliphatic amino acids, and X is an amino acid that specifies selectivity for the farnesyl moiety. Formation of a Zn^{2+} thiolate in the PFTase•peptide complex was detected by the appearance of an absorbance at 236 nm ($\epsilon = 15\,000\ M^{-1}cm^{-1}$), which was dependent on the concentration of peptide, in a UV difference spectrum in a solution of PFTase and the peptide substrate RTRCVIA. We developed a fluorescence anisotropy binding assay to measure the dissociation constants as a function of pH for peptide analogues by appending a 2′,7′-difluorofluorescein to their N-terminus. The electron-withdrawing fluorine atoms allowed us to measure peptide binding down to pH 5.5 without having to correct for the changes in fluorescence intensity that accompany protonation of the fluorophore. Measurements of the pK_as for thiol groups in free and bound peptide indicate that peptide binding is accompanied by formation of a zinc thiolate and that binding to PFTase lowers the pK of the peptide thiol by 3 units. In similar studies with the βY310F mutant, the pK_a of the thiol moiety was lowered by 2 units upon binding, indicating that the hydroxyl group in the conserved tyrosine helps stabilize the bound thiolate.

Protein farnesyltransferase (PFTase)[1] catalyzes the alkylation of a cysteine residue in a substantial number of proteins found in eukaryotic cells by farnesyl diphosphate (FPP), as illustrated in Scheme 1 (*1*). This reaction is the first step in a series of posttranslational modifications that enhance association of the proteins with membrane and are required for their proper biological function. The Ras G-proteins (pRas) are prominent among those modified with farnesyl moieties. Mutations in pRas are implicated in approximately 30% of human cancers (*2*), and it has been shown that membrane association is required for oncogenic forms of pRas to transform cells (*3*). Because of the specific link between farnesylation and the ability of oncogenic pRas to transform cells, inhibitors of PFTase are under intense investigation as antitumor agents (*4*).

The protein substrates for PFTase have a carboxy-terminal Ca_1a_2X motif, where C is cysteine, a_1 and a_2 are usually aliphatic amino acids and X selects for farnesylation when methionine, serine, glutamine, or alanine (*5*). Although most substrates for PFTase in vivo are macromolecules, peptides containing only the terminal four residues are farnesylated efficiently by the enzyme (*6*, *7*). As a consequence, much of the research on the mechanism of PFTase has been conducted with small peptide analogues of the natural protein substrates.

Scheme 1: Farnesylation of Cysteine in Ca_1a_2X-Containing Peptides by Farnesyl Diphosphate



Two additional protein prenyltransferases catalyze the addition of the C_{20} geranylgeranyl moiety to cysteines near the carboxy terminus of peptides. A protein geranylgeranyltransferase type I (PGGTase-I) alkylates proteins with a carboxy-terminal Ca_1a_2X motif where X is leucine (*8*), and a protein geranylgeranyltransferase type II (PGGTase-II) alkylates both cysteine residues in proteins with XXCC, XCXC, and CCXX carboxy-terminal motifs (*9*). PFTase and both PGGTases are $\alpha\beta$ heterodimers. The α-subunits of

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[1] Abbreviations: dansylGCVIA, dansyl-glycyl-cysteinyl-valinyl-isoleucinyl-alanine; DTT, dithiothreitol; FCH_2PP, (*E*,*E*)-4,8,12-trimethyl-3,7,11-dodecatrien-1-yl phosphonophosphate; flRTRCVIA, 2′,7′-difluorofluorescein-5-carboxyl-arginyl-threonyl-arginyl-cysteinyl-valinyl-isoleucinyl-alanine; FPP, farnesyl diphosphate; GST, glutathione transferase; LFE, linear free energy; MeIm, *N*-methylimidazole; MES, 2-(*N*-morpholino)ethanesulfonic acid; 3-MMB-VIA, 3-mercapto-3-methylbutanoyl-valinyl-isoleucinyl-alanine; MP-3-VIA, 3-mercaptopropanoyl-valinyl-isoleucinyl-alanine; PFTase, protein farnesyltransferase; PhS^-, benzenethiolate; TFA, trifluoroacetic acid; TAPSO, 3-[*N*-(tris(hydroxymethyl)methyl)amino]-2-hydroxypropanesulfonic acid.

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PFTase and PGGTase-I are identical and show a high degree of similarity to the corresponding subunit in GGTase-II (*10*−*12*). Across different species, the β-subunits for each of these enzymes are different but have overall amino acid similarity of approximately 30% (*1*).

The β-subunit plays an important role in substrate binding and catalysis by PFTase. PFTase is a metalloenzyme that tightly binds 1 equiv of Zn^{2+}. Crystal structures of mammalian PFTases contain Zn^{2+} coordinated to three amino acids in the β-subunit, which correspond to residues βAsp307, βCys309, and βHis363 in the yeast enzyme (*13*, *14*). In addition to the residues involved in Zn^{2+} binding, site-directed mutagenesis and X-ray crystallography suggest that several additional highly conserved amino acids in the β-subunit play important roles in the binding and catalysis (*15*, *16*). An X-ray crystal structure with a bound molecule of FPP shows that the majority of these residues, βHis258, βArg301, βTyr310, and βLys304 in yeast PFTase, are within hydrogen-bonding distance of the diphosphate moiety. The nonpolar farnesyl chain is located in an α−α-barrel of the β-subunit.

βTyr310 is completely conserved in PFTases. The βY310F mutant of the yeast enzyme has a K_M^{Pep} that is 10-fold less than wild-type, a K_M^{FPP} that is unchanged, and a k_{cat} that is 200-fold less than wild-type (*17*). This substantial loss of catalytic efficiency suggests that the tyrosine hydroxyl plays an important role in the mechanism of the enzyme. The corresponding mammalian mutant only shows a 5-fold reduction in k_{cat} (*18*). This difference can reconciled by the observation that product release is clearly rate limiting for the mammalian enzyme (*19*), while chemistry is partially rate limiting for the yeast enzyme (*17*). As a result yeast PFTase is more sensitive to changes in the chemical step than the mammalian enzyme.

Several pieces of evidence suggest that the cysteine residue in the peptide substrate for PFTase is bound as a Zn^{2+} thiolate. Zn^{2+} is required for binding of the peptide substrate and turnover (*20*). Replacement of the cysteine in the CaaX motif with serine results in peptide that is neither a substrate nor an inhibitor (*21*). Many of the known inhibitors that are competitive with peptide substrate contain a thiol group (*22*). The results are consistent with the known affinity between sulfur and Zn^{2+} (*23*). In addition, replacement of Zn^{2+} with Cd^{2+} results in an enzyme with activity that is 50% of the Zn^{2+}-containing enzyme (*24*). The most compelling evidence for a Zn^{2+}−thiolate interaction comes from a study of cobalt-substituted PFTase (*25*). Addition of peptide and an unreactive FPP analogue to Co^{2+}−PFTase resulted in an increase in the intensity of the ligand−metal charge-transfer band at 320 nm, which is indicative of the formation of a Co^{2+}−thiolate bond. Measurements of cobalt−thiolate absorbance as a function of pH indicated that the pK_a of the peptide thiol was lowered by 2 pK units upon binding to mammalian PFTase (*26*). We now present evidence for formation of a Zn^{2+}−thiolate complex between yeast PFTase and a peptide substrate and determine the pK_a of the sulfhydryl group in the bound substrate.

MATERIALS AND METHODS

Materials. Farnesyl diphosphate (FPP) was synthesized by the method of Davisson et al (*27*). 3-Mercaptopropionic acid and dimethyl disulfide were purchased from Acros (Pittsburgh, PA). Benzylmercaptan and 3,3-dimethylacrylic acid were purchased from Aldrich (Milwaukee, WI). Peptides were synthesized at the Core facility for DNA and peptide synthesis at the University of Utah using standard solid-phase FMOC peptide coupling chemistry. 2′,7′-Difluorofluorescein (Oregon Green 488) 5-succinimidyl ester was purchased from Molecular Probes (Eugene, OR). Dialysis tubing, 10 mm flat width 12−14 kDa cutoff, was purchased from Spectrapor (Gardena, CA). Reverse phase HPLC separations were performed using a C-18, 250 × 4.6 mm, Magellan column (Phenomenex; Torrance, CA). Fluorescence measurements were performed by a Spex FluoroMax spectrofluorometer (Edison, NJ). UV spectroscopy was conducted by a Cary 4 UV−visible spectrophotometer (Sugarland, TX). The orange glass filter used for fluorescein measurements was purchased from Edmund Scientific (Barrington, NJ).

Methods. Recombinant wild-type (wt) PFTase and βY310F PFTase were obtained by the method of Dolence et al (*16*). Fluorescence assays using dansyl-GCVIA and FPP were performed according to the procedure of Cassidy et al. (*28*). Radioisotopic assays were performed with FPP and RTRCVIA according to the procedure of Roskoski et al. (*29*). A constant ionic strength tribuffer containing TAPSO, MES, and diethanolamine was used for pH studies according to the procedure of Ellis and Morrison (*30*). Difluorofluorescein−RTRCVIA concentrations were measured by UV absorbance at 495 nm (ϵ = 76 000 $M^{-1}cm^{-1}$) (*31*). Concentrations of nonfluorophore-labeled peptides were determined using Ellman's reagent (*32*). Farnesyl phosphonophosphate (FCH_2-PP) was synthesized according to Valentijn et al. (*33*). Data fitting was performed by the program GraFit 3.01, Erithacus Software Ltd.

UV-Difference Spectroscopy. A 2.5 mL solution containing 0.6 mg of recombinant yeast PFTase was dialyzed against 100 mL of 26 mM MES, 26 mM TAPSO, and 50 mM diethanolamine pH 6.0−7.5 tribuffer with 5 mM $MgCl_2$ for 2 h. The PFTase solution and the dialysis solution were filtered through a 0.4 μm syringe filter and placed in 1 × 1 cm quartz cuvettes. FCH_2PP was then added to the PFTase solution to a concentration of 10 μM. The absorbance of the PFTase solution was measured from 220 to 300 nm at a scan rate of 1 nm/s on a Cary 4 UV spectrophotometer using the dialysis solution as a reference. All spectra were the average of three measurements. A solution of RTRCVIA (2−3 μL, 0.02 μmol buffered to pH with 5 mM potassium phosphate buffer) was added to the PFTase and reference samples. The absorbance of the PFTase-peptide solution was then measured as described above, before addition of the peptide. The absorbance for the peptide−PFTase complex was determined by subtracting the spectrum of PFTase in the absence of peptide from the spectrum of PFTase in the presence of peptide.

The dissociation constant for PFTase−RTRCVIA was calculated from the absorbance at 236 nm for the binary complex at different peptide concentrations according to eq 1, where ΔA_{bound} is the absorbance of PFTase−peptide

$$\Delta A_{236} = \frac{\Delta A_{bound}{}^{2}[\text{RTRCVIA}]}{[\text{PFTase}](K_D + [\text{RTRCVIA}])} \qquad (1)$$

complex at infinite peptide concentration, A_{236} is the absor-

bance at 236 nm for a given concentration of peptide, and K_D is the dissociation constant between peptide and PFTase. The extinction coefficient for the PFTase−peptide complex was calculated by dividing ΔA_{bound} by the concentration of PFTase.

Synthesis of Methyl Sulfide 3-Mercaptopropionic Acid Mixed Disulfide. To a solution of 0.5 g (4.5 mmol) of 3-mercaptopropionic acid in 10 mL of tetrahydrofuran was added 9 g (45 mmol) of methyl disulfide and 5 mL of triethylamine. The reaction was stirred for 48 h, and the solvent was removed by rotary evaporation. The resulting solid was dissolved in 20 mL of diethyl ether and extracted with 3 × 50 mL of 100 mM HCl. The ether solution was dried by addition of 5 g of anhydrous Na_2SO_4, and solvent was removed to leave a white solid, which was crystallized from 5 mL of diethyl ether to give 0.2 g (21%) of mixed disulfide: ^{1}H NMR (300 MHz, $CDCl_3$) 2.42 (3 H, s, SCH_3), 2.80−2.97 (4 H, m, CH_2CH_2).

Synthesis of 3-Mercaptopropanoyl-valinyl-isoleucinyl-alanine (3-MP-VIA). The mixed disulfide of methyl sulfide and 3-mercaptopropionic acid was coupled by solid-phase synthesis to valinylisoleucinylalanine at the Core facility for DNA and peptide synthesis at the University of Utah. The methyl sulfide group was then removed by treatment with 10 mM dithiothreitol in 100 mM TRIS−HCl buffer pH 8. The resulting peptide was then purified by C-18 reverse phase HPLC using a linear gradient from 80% water/0.1% (v/v) TFA to 100% acetonitrile/0.1% (v/v) TFA to give 3-MP-VIA in >95% yield. Negative ion FABMS, $C_{17}H_{31}N_3O_5S^+ - H^+$: calcd *m*/*z* 417; obsd *m*/*z* 417.

Synthesis of 3-Mercapto-3-methylbutanoyl-valinyl-isoleucinyl-alanine (3-MMB-VIA). 3-(Benzylthio)-3-methylbutanoic acid was synthesized from benzylmercaptan and 3,3-dimethylacrylic acid according to the method of Pattenden and Shuker (*34*). The benzyl-protected thiol was then coupled to VIA at the Core facility for DNA and peptide synthesis. The resulting peptide, 3-(benzylmercapto)-3-methylbutanoyl-VIA (20 mg), was reduced with 100 mg of sodium in 5 mL of liquid ammonia according to the procedure of Corie et al. (*35*). The product was purified by reverse-phase HPLC using a linear gradient from 80% water/0.1% (v/v) TFA to 100% acetonitrile/0.1% (v/v) TFA gradient to give 2 mg (10%) of 3-MMB-VIA. Positive ion FABMS, $C_{17}H_{31}N_5O_3S + H^+$: calcd *m*/*z* 390; obsd *m*/*z* 390.

Synthesis of Difluorocarboxyfluorescein-RTRCVIA (flRTRCVIA). To a solution of 10 mg of RTRCVIA (0.012 mmol) in 2 mL of 100 mM aqueous $NaHCO_3$ was added 1 mg (2 μmol) of 2′,7′-difluorofluorescein succinimidyl ester in 1 mL of dimethylformamide, which had been dried over 4 Å activated molecular sieves. The solution was stirred for 16 h before addition of 10 mL of water. The solution was then filtered through a 0.4 μm syringe filter, frozen, and lyophilized. The resulting solid was purified by C-18 reverse-phase HPLC chromatography using a linear gradient of 100% water/0.1% (v/v) TFA to 100% acetonitrile/0.1% (v/v) TFA to give 1 mg (24%) of a yellow solid. Positive ion FABMS, $C_{54}F_2H_{71}N_{13}O_{15}S + H^+$: calcd *m*/*z* 2114; obsd *m*/*z* 2114.

Fluorescence Anisotropy Binding Assay. A 100−200 μL solution of 0.5 mg of recombinant PFTase (30 μM) was dialyzed for 2 h against 1 L of 0.1 mM TRIS−HCl buffer pH 7.5. This solution was used to prepare 25 μL samples containing 0−24 μM PFTase in 26 mM MES, 26 mM TAPSO, and 50 mM diethanolamine pH 5.5−9.0 buffer containing 10 μM FCH_2PP, 1 μM flRTRCVIA, 0 or 5 mM $MgCl_2$, and 5 mM DTT. The enzyme solutions were then added to 1 × 1 mm quartz fluorescence cuvettes. The samples were excited at 495 nm through a polarizing filter using 1 nm slit widths, and emission was measured through a 515 nm cutoff glass filter (OG515, Schott). A second polarizing filter was arranged perpendicular or parallel to the excitation filter, and intensities for parallel and perpendicular orientations were measured. Fluorescence anisotropy (A) was calculated according to eq 2 for each concentration

$$A = \frac{I_{||} - I_{\perp}}{I_{||} + 2I_{\perp}} \tag{2}$$

of enzyme. A plot of $A - A_{free}$ as a function of [PFTase] was fit to eq 3, where A_{bound} is the anisotropy for fully bound

$$A - A_{free} = \frac{(A^{bound} - A_{free})^2[\text{PFTase}]}{[\text{flRTRCVIA}](K_D^{\text{flRTRCVIA}} + [\text{PFTase}])} \tag{3}$$

peptide, A_{free} is the anisotropy of the peptide in the absence of enzyme, [flRTRCVIA] is the concentration of difluorfluorescein−RTRCVIA, and $K_D^{\text{flRTRCVIA}}$ is the dissociation constant of the fluorescein−peptide·PFTase complex. Optimal fits were obtained for $A_{free} = 0.020$ and $A_{bound} = 0.074$.

Competitive Binding Assay. A 100−200 μL solution containing 0.5 mg of recombinant yeast PFTase (30 μM) was dialyzed for 2 h against 1 L of 0.5 mM TRIS−HCl buffer pH 7.5. The solution was used to prepare a 40 μL sample containing 38 μM PFTase in 33 mM MES, 33 mM TAPSO, and 63 mM diethanolamine pH 5.5−9.0 buffer containing 13 μM FCH_2PP, 1.3 μM flRTRCVIA, 0 or 6.3 mM $MgCl_2$, and 6.3 mM DTT. The solution was divided into two 20 μL portions, and 5 μL of water or a nonfluorescein-labeled peptide solution was added to make 25 μL solutions with 0−30 μM PFTase in 26 mM MES, 26 mM TAPSO, and 50 mM diethanolamine pH 5.5−9.0 buffer containing 10 μM FCH_2PP, 1 μM flRTRCVIA, 5 mM $MgCl_2$, 5 mM DTT, and 5−25 μM of the peptide analogue. The solutions were then placed in 1 × 1 mm quartz cuvettes, and the anisotropy of the flRTRCVIA was measured. The concentration of enzyme was calculated from the anisotropy of the solution in the absence of the CVIA analogue according to eq 4.

$$[\text{PFTase}] = \frac{(A - A_F)(K_D)}{(A_B - A_F) - (A - A_F)} \tag{4}$$

K_D for the unlabeled peptide was calculated according to eq 4, which was derived from the competitive binding equation developed by Weinhold and Knowles (*36*),

$$K_D = \frac{[\text{Pep}]}{\left(\frac{[\text{PFTase}](A_B - A)}{K_{DF}(A - A_F)}\right) - \left(\frac{[\text{flRTRCVIA}](A_B - A)}{K_{DF}(A_B - A_F)}\right) + 1} \tag{5}$$

where K_D is the dissociation constant between nonlabeled peptide and PFTase, $K_D$flRTRCVIA is the dissociation

constant between flRTRCVIA and PFTase, and [Pep] is the concentration of unlabeled peptide.

Assay of PFTase Activity with Nonfluorescent CVIA Analogues. A TLC assay was based on the method developed by Goldstein et al. (*21*). A mixture of 50 mM TRIS−HCl buffer pH 7.0, 5 mM $MgCl_2$, 5 mM DTT, 9 μM [^{3}H]FPP (80 μCi/μmol), 10 μM of CVIA analogue, and 116 nM PFTase in a total volume of 25 μL was incubated at 25 °C for 30 min. The entire mixture was spotted onto a glass-backed silica gel TLC plate and placed into a tank containing *n*-propanol, NH_4OH, and water (6:3:1, v/v/v). The plate was developed for 4 h, after which time 1 cm length sections were scraped into 25 mL scintillation vials. Cytoscint (ICN) (10 mL) was added, and the radioactivity was measured. Unreacted FPP remained at the baseline while the farnesylated peptides moved with the solvent front.

Determination of the pK_a of Free Thiols. Samples (2.5 mL) of 5.2 mM MES, 5.2 mM TAPSO, and 10 mM diethanolamine tribuffer pH 5.2 buffer containing 0 or 5 μM peptide were prepared and placed in 1 × 1 cm quartz cuvettes. The absorbance of the samples was measured at 240 nm using the buffer as reference. Afterward, 4 μL of 1 M NaOH was added to both the reference and peptide samples, and the absorbance of the sample was measured again. The cycle of NaOH addition and UV absorbance measurements was repeated 15 times (total volume of 1 M NaOH added was 60 μL). To determine the pH of the solutions during titration, a 2.5 mL sample of 5.2 mM MES, 5.2 mM TAPSO, and 10 mM diethanolamine pH 5.2 buffer was prepared. Additions of 1 M NaOH were made, just as before for the absorbance measurements, and the pH of the solution was measured after each addition. The fraction of thiolate, Φ, at each pH was calculated from the ratio $(A - A_{min})/(A_{max} - A_{min})$, where A is the absorbance at a given pH, A_{min} is the minimum absorbance measured, and A_{max} is the maximum absorbance measured. A plot of Φ as a function of pH was fit to the relationship $10^{(pH-pK_a)}/(10^{(pH-pK_a)} + 1)$ by GraFit to determine the pK_a of the thiol. In the case of H_2NCVIA, eq 6, which

$$\Phi = \frac{\frac{K_a}{K_b} + \frac{K_d}{[H^+]}}{\frac{[H^+]}{K_b} + \frac{K_a}{K_b} + \frac{K_d}{[H^+]} + 1} \quad (6)$$

accounts for the ionization of the amino terminus, was used to fit the data (*37*), where K_a is the ionization constant for the thiol of the ammonium peptide, K_b is the ionization constant for the amine, and K_d is the ionization constant for the thiol of the amine peptide.

RESULTS

UV Difference Spectroscopy of wt and βY310F PFTase Indicates That the Peptide Substrate is bound as a Zn^{2+} Thiolate. Thiolates and Zn^{2+} thiolates both absorb in the ultraviolet between 230 and 240 nm; however, the extinction coefficients for Zn^{2+} thiolates ($\epsilon \sim 15\,000$ $M^{-1}cm^{-1}$) are approximately four times larger that those of thiolates (*38*, *39*). In contrast, thiols do not absorb in this region. The thiolate bands are difficult to detect in the presence of proteins because of interference from carbonyl and aromatic groups. However, the UV spectrum of the bound substrate can be determined by subtraction when a thiol-containing substrate is bound as a Zn^{2+} thiolate. This is accomplished by measuring the UV spectra of the protein in the absence of the substrate and the substrate in the absence of protein. These spectra are then subtracted from the spectrum of a sample containing both protein and substrate. Using this approach, the UV spectra measured at pH 7.0 for PFTase and its peptide substrate RTRCVIA were subtracted from the spectrum of a sample containing both PFTase and RTRCVIA. To minimize errors due to slight differences from sample to sample, only samples prepared at the same time were subtracted from one another, the samples were extensively dialyzed, and the dialysis solution was used as the reference sample. An absorbance at 236 nm was observed in the UV difference spectrum upon addition of the peptide substrate RTRCVIA to PFTase (Figure 1a). A plot of the extinction coefficient at 236 nm as a function of [RTRCVIA] gave a hyperbolic curve typically seen for reversible binding. The data were fit to eq 1 to give a maximum extinction coefficient, $\epsilon = 15\,000 \pm 2000$ M^{-1} cm^{-1}, and $K_D^{RTRCVIA} \sim 8$ μM (Figure 1b). In comparison, $K_D^{RTRCVIA} = 9.5 \pm 0.6$ μM was calculated from the association/dissociation rate constants of PFTase·RTRCVIA measured by rapid quench techniques (*17*). These data suggest that the band in the difference spectrum arises from the Zn^{2+} thiolate formed upon binding of the peptide to PFTase.

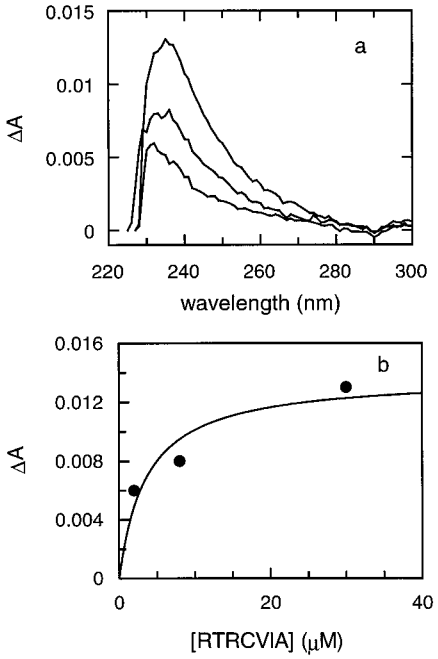
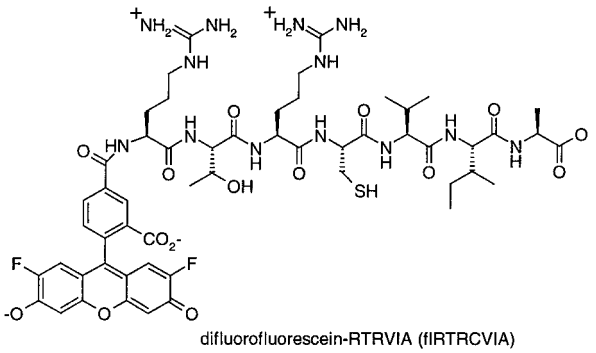
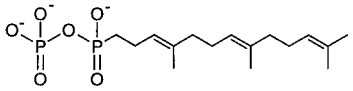


FIGURE 1: (a) UV difference spectra showing the zinc thiolate absorbance at 236 nm for the PFTase·RTRCVIA binary complex. RTRCVIA was added to 2 μM PFTase to give final peptide concentrations of 2, 8, and 30 μM. (b) Plot of absorbance at 236 nm for each addition of peptide versus [RTRCVIA]. The data were fit to eq 1 to calculate the dissociation constant for E·RTRCVIA ($K_D = 8 \pm 1$ μM) and the extinction coefficient of the binary complex ($\epsilon = 15\,000 \pm 2000$ M^{-1} cm^{-1}).

A similar set of UV-difference experiments was conducted with βY310F PFTase in parallel to those for wt enzyme (data not shown). The βY310F mutant also gave a difference spectrum characteristic for formation of a Zn^{2+} thiolate upon binding RTRCVIA at pH 7.0 with λ^{Max} at 236 nm ($\epsilon = 10\,000 \pm 2000$ M^{-1} cm^{-1}). The dissociation constant

Chart 1





calculated from the UV absorbances, $K_D = 8 \pm 1$ μM, was similar to that of wt PFTase. Thus, replacement of the hydroxyl group in Y310 by a hydrogen atom does not significantly alter the stability of the peptide·enzyme complex at pH 7.

Measurements of K_D for Enzyme−Peptide Complexes by Fluorescence Anisotropy. We used a fluorescence anisotropy binding assay (*40*, *41*) to measure K_D for a series of PFTase·peptide complexes. A fluorescent peptide, flRTRCVIA (Chart 1), was synthesized by appending a 2′,7′-difluorofluorescein moiety to the amino-terminal arginine. The molecule was an excellent alternate substrate for yeast PFTase, giving steady-state kinetic constants similar to those measured for RTRCVIA. Excitation of the fluorescein fluorophore in flRTRCVIA at 495 nm resulted in an intense emission at 526 nm. The difluoro derivative was chosen in order to extend the binding measurements to pH < 6. The fluorescence form of the fluorescein moiety has a deprotonated phenolic hydroxyl group. The pK_a of fluorescein itself is 6.7, while the difluoro derivative has a pK_a of 4.7 (*31*). The decrease in pK_a extends the useful range of the measurements by two pK units. The arginine residues of flRTRCVIA allow us to use the filter paper assay originally developed for RTRCVIA to measure the kinetic constants for the fluorescent derivative (*29*). flRTRCVIA has kinetic parameters ($K_M^{Pep} = 2.0 \pm 0.6$ μM, $k_{cat} = 0.6 \pm 0.1$/s) similar to that measured for the unlabeled substrate RTRCVIA ($K_M^{Pep} = 3.1 \pm 0.2$ μM, $k_{cat} = 2.6 \pm 0.1$/s) (*17*).

PFTase binds substrates by an ordered mechanism where FPP binds first, followed by the peptide (*17*). Since we could not measure K_D^{Pep} in the presence of FPP, we substituted the unreactive phosphonophosphate analogue FCH_2PP. The structure of FCH_2PP is identical to FPP except that a methylene group has replaced the oxygen atom attached to the farnesyl moiety. FCH_2PP was an inhibitor of yeast PFTase with $IC_{50} \sim 1$ μM, similar to the value reported for the mammalian enzyme (*42*).

Dissociation constants for PFTase·peptide and PFTase·peptide·FCH_2PP complexes were calculated from fluorescence anisotropies (*A*). For the fluorescent substrate flRTRCVIA, *A* was measured for samples containing a fixed concentration of peptide and varied concentrations of PFTase.

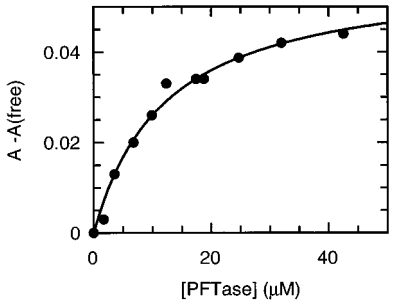


FIGURE 2: Plot of fluorescence anisotropy for FlRTRCVIA versus [PFTase]. Enzyme was added to a solution of 1 mM of flRTRCVIA, 5 mM $MgCl_2$, and 10 μM FCH_2PP at pH 7.0. Fluorescence intensities were measured in the parallel and perpendicular directions, and the fluorescence anisotropy was calculated according to eq 2. The data were fit to eq 3 to give $K_D^{RTRCVIA} = 12 \pm 2$ μM.

Table 1: Dissociation Constants for wt PFTase[a]

sample	K_D, μM
$FCH_2PP + MgCl_2$	12 ± 2
FCH_2PP	29 ± 2
$MgCl_2$	19 ± 3
no additives	19 ± 3

[a] 26 mM MES, 26 mM TAPSO, 50 mM diethanolamine, pH 7.0, and 5 mM DTT.

$K_D^{flRTRCVIA}$ was calculated by fitting the data from a plot of $A - A_{free}$ as a function of [PFTase] (see Figure 2) to eq 3. The dissociation constants were measured for release of flRTRCVIA from PFTase·flRTRCVIA and PFTase·flRTRCVIA·FCH_2PP complexes with and without added Mg^{2+}. The results are given in Table 1. K_D for the peptide was 19 μM. Addition of Mg^{2+} to the buffer did not alter the dissociation constant. In the presence of 10 μM FCH_2PP under conditions where a ternary PFTase·flRTRCVIA·FCH_2-PP complex is formed, but without Mg^{2+} in the buffer, K_D only increased slightly. However, addition of both FCH_2PP and Mg^{2+} lowered K_D slightly to 12 μM. All of our subsequent measurements of K_D for peptides were conducted in buffer containing FCH_2PP and Mg^{2+}.

Replacement of the hydroxyl group at βTyr310 by a hydrogen atom did not change the dissociation constant for the PFTase mutant and flRTRCVIA at pH 7.0, $K_D = 11 \pm 2$ μM. These observations are consistent with our measurements of thiolate formation by UV-difference spectroscopy, which showed that both wt and βY310F PFTase formed Zn^{2+} thiolates with equal affinity for RTRCVIA at pH 7.0.

Measurement of K_D as a Function of pH. Our UV-difference experiments indicated that RTRCVIA binds to PFTase as a Zn^{2+} thiolate at pH 7.0. Since the pK_a for a peptidyl cysteine thiol in solution is typically ~8.5, we suspected that the pK_a of the thiol in RTRCVIA was lowered upon binding to the enzyme. This was confirmed by measuring the pK_a of the thiol group in RTRCVIA (8.47 ± 0.01) by changes in the absorbance at 240 nm upon titration of a solution of the peptide (*37*).

Several lines of evidence, including the observation that peptides where serine has replaced cysteine do not bind tightly to PFTase (*21*), suggest that the thiol form of the peptide binds less tightly that the thiolate. If this were the case, the pK_a of the bound thiolate can be determined from measurements of K_D as a function of pH (see Figures 3 and

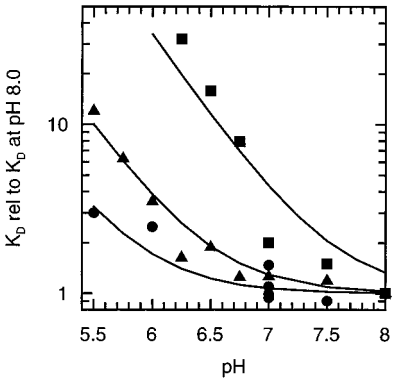


FIGURE 3: Plot of dissociation constants for wt PFTase and CVIA peptide analogs versus pH. Key: ■, 3-MMB-VIA; ▲, 3-MP-VIA; ●, AcCVIA. The curves were generated by fitting the data to eq 7.

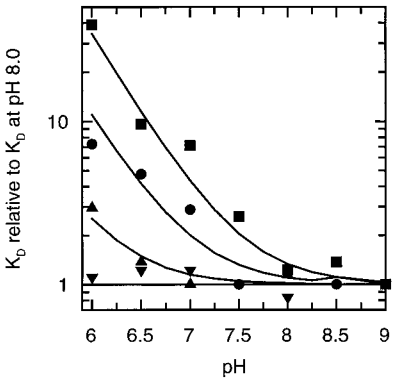


FIGURE 4: Plot of dissociation constants for βY310F PFTase and CVIA peptide analogs versus pH. Key: ■, 3-MP-VIA; ●, AcCVIA; ▲, flRTRCVIA; ▼, H_2NCVIA. The curves were generated by fitting the data to eq 7.

4). For yeast PFTase, measurements are limited to 5.5 < pH < 9 because the enzyme is irreversibly denatured outside of these limits. K_D for flRTRCVIA and wt PFTase in buffer containing FCH_2PP and Mg^{2+} did not change between pH 5.5 and 9. Presumably the pK_a of the peptide thiol in the PFTase·FCH_2PP·flRTRCVIA complex is <5.5. In contrast, K_D for flRTRCVIA and βY310F PFTase, measured under similar conditions, increased noticeably at lower pH (see Figure 4) as expected upon protonation of the thiolate. The difference between wt and βY310F PFTase suggests that the hydroxyl group in the tyrosine residue plays an important role in lowering the pK_a of the peptide thiol upon binding.

The range of pHs over which K_D can be measured for wt and βY310F PFTase is too narrow to measure pK_as for bound flRTRCVIA. Thus, we were not able to determine the extent to which the pK_a for the thiol moiety is lowered when bound to the enzyme. To answer this question, we synthesized a series of CVIA analogues (see Table 2) whose pK_as varied over a range of approximately two pK units. We assumed that changes in the pK_as of the free thiols would produce corresponding changes in the pK_as of the bound thiols. The pK_as of the thiols were altered by making substitutions N-terminal to the α-carbon of the cysteine residue in RTRCVIA. Replacement of the flRTR moiety with dansylglycyl (dansylG, pK_a = 8.40 ± 0.02) or an acetyl (Ac, pK_a = 8.88 ± 0.02) groups produced modest changes in the measured K_Ds. Removal of flRTR gave CVIA with a free amino terminus. In this case we measured two thiol pK_as, one at 7.8 when the amino group was protonated and another at 9.4 for the free amine. We also replaced cysteine with 3-mercaptopropionic acid (3-MP-VIA, pK_a = 9.7 ± 0.02) and 3-methyl-3-mercaptobutyric acid (3-MMB-VIA, pK_a = 9.9 ± 0.1) to more basic peptide analogues.

Table 2: pK_as for Free and Bound Peptide Thiols

		pK_a bound (ΔpK_a)[a]	
peptide	pK_a free	wt PFTase	βY310F PFTase
H_3N^+CVIA[b]	7.8 ± 0.1	n.d.	n.d.
H_2NCVIA[b]	9.4 ± 0.1	n.d.	n.d.
dansylGCVIA	8.4 ± 0.02	n.d.	6.4 ± 0.02 (2.0)[c]
flRTRCVIA	8.47 ± 0.01	n.d.	6.3 ± 0.04 (2.2)
AcCVIA	8.88 ± 0.02	5.85 ± 0.06 (3.0)	6.9 ± 0.05 (2.0)
3-MP-VIA	9.7 ± 0.02	6.51 ± 0.02 (3.2)	7.6 ± 0.04 (2.1)
3-MMB-VIA	9.9 ± 0.02	7.7 ± 0.03 (2.2)	>9.0

[a] $\Delta pK_a = (pK_a^{free} - pK_a^{bound})$. [b] The p$K_a$ for the amine is 6.7. [c] Estimated from a plot of K_M^{Pep} versus pH.

Peptides AcCVIA, 2-MP-VIA, and 3-MMB-VIA were tested as alternate substrates for yeast PFtase using the TLC procedure developed by Goldstein et al. (*21*). Both of the primary thiols AcCVIA and 3-MP−CVIA were alkylated by FPP, but tertiary thiol 3-MMB-VIA was not a substrate for the enzyme. Goldstein et al. (*21*) had reported that 3-mercaptopropionic acid-derived peptides were substrates for mammalian PFTase. They also found that a peptide containing the penicillamine moiety bearing a tertiary thiol was not a substrate but was an inhibitor (*21*).

K_Ds for the nonfluorescent peptide analogues were determined from competition experiments on the basis of their ability to displace flRTRCVIA from PFTase as described in Materials and Methods. As seen in Figure 4, the pH dependence of K_D for (βY310F PFTase)·FCH2PP·peptide complexes correlates directly with the pK_a of the free thiol. The pK_as of the bound thiolates (Table 2) were determined by fitting the data shown in Figures 3 and 4 to eq 7, where

$$K_D^{(rel)} = 1 + (K_a^{(B)}/[H^+]) \quad (7)$$

$K_D^{(rel)}$ is the peptide dissociation constant at a specific H^+ concentration divided by the dissociation constant measured at pH 8.0 and $K_a^{(B)}$ is the K_a of the bound thiolate. Equation 7 assumes that PFTase does not bind detectable amounts of the thiol substrates under the conditions of our experiments. On the basis of the pK_as in Table 2 for free thiol and bound thiolates, we calculate that βY310F PFTase lowers the pK_a of the thiol groups in CAAX peptides by 2 pK units upon binding.

A similar set of studies was conducted for wt PFTase. In contrast to the βY310F mutant, a dependence of K_D on pH was only observed for the three least acidic peptides, AcCVIA, 3-MP-VIA, and 3-MMB-VIA, among those we studied (see Figure 3). pK_as for AcCVIA, 3-MP-VIA, and 3-MMB-VIA bound to wt PFTase were determined as described above for the βY310F mutant and are presented in Table 2. From the data presented in Table 2, it appears that the pK_as for peptides AcCVIA and 3-MP-VIA are lowered by approximately 3 pK units in the (wt PFTase)·FCH_2PP·peptide complex relative to free peptide. In contrast, the pK_a of 3-MMB-VIA is only lowered by 2 pK units. We attribute the difference in ΔpK_as between the primary and tertiary peptide analogues to steric shielding of the tertiary thiol in 3-MMB-VIA. We were unable to go to sufficiently

high pHs to measure the pK_a of the thiol moiety in the (βY310F PFTase)·FCH_2PP·3-MMB-VIA complex without denaturing the enzyme. A comparison of ΔpK_as for AcCVIA and 3-MP-VIA in wt and βY310F PFTase complexes indicates that the tyrosine hydroxyl group lowers the pK_a of the thiol group in the bound peptides by approximately 1 pK_a unit.

Thiolate Absorbances Are Proportional to K_D Values Measured at Various pH Values. We determined the dissociation constant for 3-MP-VIA complexed with wt and Yβ310F PFTase at pH 6 and 7 using UV difference spectroscopy as described above for RTRCVIA in order to confirm our estimate of the pK_a of the bound thiolate. For both enzymes, the dissociation constants measured at different pHs were the same as measured by fluorescence spectroscopy (Table 2). Furthermore, the extinction coefficients of the bound thiolates in each enzyme·peptide complex were the same, 13 000 $\pm$ 2000 M^{-1} cm^{-1}, at each pH. The fact that the extinction coefficient of the thiolate was independent of pH suggests that the zinc thiolate is the only bound species. One would expect that if bound thiol were generated at low pH, the measured extinction coefficient would decrease. Thus, binding of peptide and deprotonation of thiol are coupled, and the pK_a of the bound thiolate can be estimated by measuring dissociation constants for the enzyme·peptide at different pHs.

The Michaelis Constant Is Proportional to K_D. If we assume that K_M^{Pep} is proportional to the K_D for the enzyme·peptide complex, K_M^{Pep} should also be dependent on pH. K_M^{Pep} for wt yeast PFTase is only 3-fold smaller than K_D^{Pep}, and the rates for chemistry and product release are similar (*17*). K_M^{Pep} for wt PFTase using dansyl-GCVIA as the peptide substrate was constant between pH 5.5 and 9.0. A similar lack of dependence on pH was seen for K_D^{Pep} of PFTase·flRTRCVIA. Although the dansyl chromophore interfered with a direct measurement of danyslGCVIA binding by our competition assay, the pK_as of the thiol moieties in dansylGCVIA and flRTRCVIA, 8.40 and 8.47, respectively, are similar, and one would anticipate a similar dependence on pH. However, one would expect to see a pH dependence for $K_M^{dansylGCVIA}$ on the basis of the K_Ds we measured for βY310F PFTase and flRTRCVIA. In contrast to the behavior of the wt enzyme, $K_M^{dansylGCVIA}$ for βY310F PFTase increased at pH < 6.5 (data not shown). A fit of K_M^{Pep} as a function of pH for βY310F by eq 7, using K_M^{Pep} instead of $K_a^{(B)}$, gave a value for the pK_a of the bound thiolate of 6.4, which represents a decrease of 2 pK units below the pK_a of the free thiol (Table 2).

DISCUSSION

Protein prenyltransferases are members of a much larger family of proteins that catalyze prenyl transfer reactions. In each case the individual enzyme transfers the hydrocarbon moiety in an allylic isoprenoid diphosphate to a molecule containing an electron-rich acceptor. Prenyl transfer is accompanied by formation of PP_i and loss of a proton, typically from the substrate containing the acceptor. A wide variety of electron-rich moieties are prenyl acceptors, ranging from relatively poor nucleophiles such as carbon−carbon double bonds to the potent sulfur nucleophiles encountered in protein prenylation. The available evidence suggests that prenyl transfer reactions are dissociative when the acceptor is a weak nucleophile, for example a carbon−carbon double bond. Product studies with alternate substrates (*43*, *44*) for FPP synthase and linear free energy (LFE) correlations probing substituent effects with the allylic substrates (*45*) indicate that highly electrophilic allylic carbocations are generated during catalysis.

LFE experiments with yeast PFTase also are consistent with an electrophilic mechanism for the alkylation reaction (*46*). However, the correlation shows a substantial attenuation of the substituent effects relative to FPP synthase characteristic of an electrophilic alkylation that proceeds through an associative transition state. Stereochemical studies of prenyltransfer reactions with double bond or with sulfur acceptors show inversion of configuration at the carbon atom that attaches to the acceptor. While consistent with an associative reaction, the stereochemical results are not definitive because the stereochemistry of the reaction may simply reflect the orientation of the substrates in the enzyme·substrate complex rather than stereoelectronic properties of the reaction.

Peptides, where the cysteine has been replaced by serine, are not substrates for PFTase. On the basis of the pK_as we measured for sulfhydryl groups in peptides bound to PFTase, one would anticipate that the corresponding hydroxyl groups in serine analogues would not be ioinized when bound to the enzyme. Thus, serine analogues should bind less tightly than their cysteine counterparts, and the serine hydroxyls should be substantially poorer nucleophiles in an associative reaction. In contrast, even carbon−carbon double bonds are sufficiently nucleophilic to react with allylic carbocations in the dissociative prenyl transfer reaction catalyzed by FPP synthase during isoprenoid chain elongation (*43*−*45*). Since carbon−carbon double bonds are less nucleophilic than hydroxyl groups (*47*), the serine hydroxyl should be sufficiently reactive in a dissociative mechanism that proceeds through an allylic cation−PP_I ion pair.

Most prenyltransferases require Mg^{2+} or Mn^{2+} for activity. These metals do not bind tightly in the absence of diphosphates, and it is generally assumed that the metal salts of the diphosphates are the true substrates. For FPP synthase where an X-ray structure of an enzyme·allylic diphosphate complex has been obtained, Mg^{2+} forms a bridge between the diphosphate residue in the allylic substrate and aspartate residues in the catalytic site. Protein prenyltransferases also require divalent metals for activity. However, these enzymes are Zn^{2+} metalloproteins. The tightly bound Zn^{2+} is required for binding peptide substrate and thiol activation. Mg^{2+} provides added stimulation, presumably by stabilizing the pyrophosphate leaving group. The Zn^{2+} binding pocket in PFTase is formed by three conserved amino acids, which correspond to Asp307, His309, and Cys363 in the β-subunit of the yeast enzyme. In the absence of substrates, the inner coordination sphere of the bound Zn^{2+} consists of two negatively charged ligands, Asp307 and Cys363, and two neutral ligands, His309 and a water molecule.

In the normal catalytic cycle, substrate binding is ordered with FPP binding before peptide. When the peptide binds, the thiol group of the cysteine in the CaaX sequence displaces the water molecule. Substitution of any of the three conserved amino acids in the Zn^{2+} binding pocket by alanine reduces Zn^{2+} binding and severely compromises the catalytic activity

of the enzyme. Huang et al. (*25*) recently reported that a Co^{2+}-substituted form of rat PFTase binds the peptide substrate as a thiolate. They reported that the characteristic UV absorption for the Co^{2+} thiolate complex was only seen for a ternary complex consisting of the enzyme, an unreactive analogue of FPP, and peptide. We used UV-difference spectroscopy to detect a characteristic band for a Zn^{2+}−thiolate interaction in the yeast enzyme. However, the peptide substrate bound to yeast PFTase as Zn^{2+} thiolate in either the presence or absence of FCH_2PP, an FPP analogue. These differences are consistent with observations that FPP enhances the binding of peptide to mammalian PFTase; whereas, peptide binding is not dependent on the presence of FPP for the yeast enzyme.

Considerably more is known about activation of oxygen nucleophiles as Zn^{2+}-bound hydroxides than about sulfur nucleophiles as Zn^{2+}-bound thiolates, primarily through studies of carbonic anhydrase (*48*). However, an example of nucleophilic displacement by a Zn^{2+}-bound thiolate has been reported for the *Escherichia Coli* Ada protein, which removes methyl groups from methylated phosphate residues in DNA by nucleophilic displacement. The nucleophile is a cysteine residue in the protein whose sulfhydryl moiety is activated as a Zn^{2+}-bound thiolate (*49*). Activation of the cysteine thiol by Zn^{2+} in the Ada protein is similar to the enhancement of hydroxide nucleophiles in carbonic anhydrase. In both enzymes, the nucleophile, thiol or water, is complexed with the active site Zn^{2+}. This interaction lowers the pK_a of the nucleophile sufficiently for deprotonation, thereby enhancing its nucleophilicity.

Wilker and Lippard (*50*) studied demethylation of trimethyl phosphate by zinc benzenethiolates as models for DNA repair by the *E. coli* Ada protein. They systematically altered charge in tetracoordiante Zn^{2+} complexes from −2 to 0 by replacing benzenethiolate (PhS^-) ligands with *N*-methylimidazole (MeIm) and found a corresponding decrease in reactivity as the negatively charged thiolates were replaced. $[Zn(PhS^-)_4]^{2-}$ was 10 times more reactive than $[Zn(PhS^-)_3(MeIm)]^-$ and at least 1000 times more reactive than $[Zn(PhS^-)_2(MeIm)_2]$. The authors concluded that benzenethiolate, formed by dissociation of the tetracoordinate complex, was the active nucleophile for $[Zn(PhS^-)_4]^{2-}$. Although they presented evidence for dissociation of $[Zn(PhS^-)_3(MeIm)]^-$ as well, it is unclear if the free or zinc-bound thiolate was the nucleohile in that case. Benzenethiol itself was not reactive. On the basis of these results, the $[Zn(His)(Asp\text{-}CO_2^-)(Cys\text{-}S^-)(peptide\text{-}S^-)]^-$ active site complex in PFTase should be sufficiently reactive for alkylation by the electrophilic farnesyl moiety in FPP. The model studies also raise an interesting question about the mechanism of prenyl transfer reaction. Is the nucleophile a Zn^{2+}-bound thiolate, or does the peptide thiolate dissociate prior to reaction with FPP, as was suggested for the demethylation reaction catalyzed by the *E. coli* Ada protein (*50*)? An X-ray structure of mammalian FPTase containing a bound molecule of FPP places the substrate approximately 7 Å from the anticipated location of the thiolate sulfur. Thus, a conformational change in the enzyme that moves FPP toward the zinc-bound thiolate or a dissociation of the thiolate with concomitant translocation closer to FPP is required.

We estimate that Zn^{2+} increases the acidity of cysteine thiols bound to PFTase by 3 p*K* units; whereas, the acidity of water is enhanced by 5−7 p*K* units by carbonic anhydrase (*51*). These differences are consistent with the differences in charge of the active site $[Zn(His)_3(H_2O)]^{2+}$ and $[Zn(His)(Asp\text{-}CO_2^-)(Cys\text{-}S^-)(peptide\text{-}S^-)]^-$ coordination environments in carbonic anhydrase and PFTase, respectively (*52*). The Lewis acidity of the Zn^{2+} in PFTase is delicately balanced to activate the thiol moiety in bound peptide substrates but not the bound water in the resting enzyme. This could be especially important for the mammalian forms of PFTase, which bind FPP so tightly that the enzyme probably normally "rests" as the E·FPP binary complex rather than free enzyme, by preventing hydrolysis of FPP. Selective deprotonation of the thiol moiety in the peptide should also improve selectivity because of the enhanced binding of the thiolate form of the substrates. In this regard, peptide analogues bearing serine (*21*) or (*S*)-methylcysteine residues (*53*) bind at least 2 orders of magnitude less tightly than those of cysteine-containing substrates. Finally, neutral water should be displaced more easily upon peptide binding than and Zn^{2+} hydroxide as the enzyme turns over.

If PFTase binds the thiolate form of the substrate directly from solution, one would expect that the dissociation constants for the peptides would decrease as the pH of the solution approached the pK_a of the thiol. Our results indicate that the peptide cysteines were ionized in the active site, and their K_Ds did not change significantly as the pH of the buffer was lowered from ∼9 until the bound thiolates were protonated. Although more complex schemes are possible, this behavior can be rationalized by a scenario where PFTase weakly binds the thiol form of the peptide from solution. Subsequent transfer of the thiol proton to a base in the active site then gives the tightly bound peptide thiolate and the conjugate acid of the active site base. One anticipates that the conjugate acid of the base that deprotonates the peptide thiol is stabilized within the E·thiolate complex and is not deprotonated at the higher pHs employed in this study. We also found that the K_Ds for peptide binding to yeast PFTase did not decrease at higher pH, even under conditions where significant amounts of the free thiolates were formed. These results suggest that the basic form of PFTase does not bind the thiolate form of the substrates.

In the Yβ310F mutant of PFTase, the acidity of thiols in bound peptides is only enhanced by 2 p*K* units, as opposed to 3 p*K* units for wt enzyme. Thus, the tyrosine hydroxyl of Tyr310 is directly involved in lowering the pK_a of the bound thiolate. A tyrosine hydroxyl also helps stabilize the thiolate in glutathione bound to glutathione transferase (*54*). In the crystal structure of PFTase·FPP, the hydroxyl of βTyr310 is about 5 Å from the water attached to zinc. This distance is too far for a direct hydrogen bond between the tyrosine hydroxyl and atoms directly attached to the metal. The influence of βY310 might be exerted directly by a conformational change that positions the hydroxyl moiety nearer the Zn^{2+} or through a bound molecule of water.

In conclusion, PFTase uses a tightly bound zinc to enhance binding of the peptide substrate and enhance the nucleophilicity of the cysteine thiol. The enzyme appears to losely bind the peptide in the thiol form before deprotonating the sulfhydryl moiety to give a tightly bound zinc thiolate. The ligands in the β-subunit of PFTase that coordinate zinc delicately balance the acidities of the metal-bound water molecule in the resting enzyme and the peptide thiol in the

active ternary complex so that only the peptide thiol is deprotonated. Thus, the nucleophilicity of the sulfhydryl moiety in the peptide substrate is selectively enhanced for the subsequent prenyl transfer step.

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