H-Ras Peptide and Protein Substrates Bind Protein Farnesyltransferase as an Ionized Thiolate†

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ABSTRACT: The zinc metalloenzyme protein farnesyltransferase (FTase) catalyzes the alkylation of a cysteine residue of protein substrates with a 15 carbon farnesyl group. We have developed fluorescence assays to directly measure the affinity of the enzyme for peptide and protein (Ras) substrates. A peptide corresponding to the carboxyl terminus of H-Ras binds to FTase in the μM range (K_D = 4 μM) at physiological pH; however, the peptide affinity is enhanced approximately 70-fold in a ternary complex with an enzyme-bound farnesyl diphosphate (FPP) analogue, indicating that the two substrates bind synergistically. The pH dependence of substrate binding was also investigated, and two ionizations were observed: for the ternary complex, the pK_a values are 8.1, reflecting ionization of the thiol of the free peptide, and 6.4. The pH dependence of the ligand−metal charge-transfer band in the optical absorption spectra of a Co^{2+}-substituted FTase ternary complex suggests that a metal-coordinated thiol ionizes with a pK_a of 6.3. These data indicate that metal coordination of the peptide sulfur with the zinc ion in FTase lowers the pK_a of the thiol resulting in formation of a bound thiolate at physiological pH.

Protein farnesyltransferase (FTase)[1] catalyzes the transfer of a 15 carbon farnesyl group from farnesyl diphosphate (FPP) to substrates such as Ras, nuclear lamins, and several proteins involved in visual signaling. The farnesyl group forms a thioether with a cysteine residue fourth from the carboxyl terminus which is part of the "CaaX" recognition motif (*1, 2, 3*). Since prenylation of Ras is critical for membrane integration and the subsequent transformation of normal cells into cancerous cells by mutant forms of Ras (*4, 5, 6*), compounds that inhibit FTase are being investigated as possible antitumor agents (for reviews, see refs *7, 8*).

FTase is a metalloenzyme that contains one Zn^{2+} per α/β heterodimer (*9*). The Zn^{2+} is required for catalytic activity as well as binding of the protein, but not the isoprenoid, substrate (*9, 10*). Recent evidence suggests that the Zn^{2+} may be directly involved in the catalytic mechanism of the enzyme. In the crystal structure of rat FTase (*11*), both the isoprenoid and protein substrates can be modeled into the structure near the Zn^{2+}, indicating that this region probably contains the active site. In addition, one of the Zn^{2+} ligands is a water molecule, a hallmark of catalytic Zn^{2+} sites (*12*). Spectroscopic analysis of a ternary complex containing Co^{2+}-substituted FTase, FPP, and peptide has shown that the sulfur atom of the peptide cysteine interacts directly with the metal (*13*). FTase, and the related enzyme protein geranylgeranyltransferase type I (GGTase-I), may utilize Zn^{2+}-mediated activation of a thiolate as part of the catalytic mechanism for alkylation of the cysteine. Similar Zn^{2+}-bound thiolates have been proposed for model compounds (*14*−*17*) and for the alkylation reactions catalyzed by methylcobamide:coenzyme M methyltransferase (*18*), cobalamin-dependent (*19, 20*) and cobalamin-independent methionine synthase (*21*), and the *Escherichia coli* DNA repair protein Ada (*22*−*25*).

To further clarify the mechanism and substrate selectivity of FTase, we have directly measured the affinity of the enzyme for peptide and protein substrates. Thermodynamic determinations of binding affinity are essential since the steady-state kinetic parameter, K_M, does not reflect K_D for this enzyme (Huang, C.-c., Hightower, K. E., and Fierke, C. A., unpublished experiments, and refs *26* and *27*). These studies reveal that the two FTase substrates bind synergistically; the affinity of FTase for the peptide substrate, GCVLS, increases approximately 70-fold in the presence of a bound FPP analogue. Furthermore, measurement of the pH dependence of peptide binding indicates that at neutral pH the peptide substrate binds to FTase with the cysteine ionized to a thiolate, consistent with the spectral studies indicating

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[1] Abbreviations: FTase, protein farnesyltransferase; FPP, farnesyl diphosphate; GGTase-I, protein geranylgeranyltransferase type I; TCEP, tris(2-carboxyethyl)phosphine hydrochloride; Dns-GCVLS, dansylated pentapeptide Gly-Cys-Val-Leu-Ser; acrylodan, 6-acryloyl-2-dimethylaminonaphthalene; Mops, 3-[*N*-morpholino]propanesulfonic acid; Taps, *N*-[tris(hydroxymethyl)methyl)-3-aminopropanesulfonic acid; Dns-GAVLS, dansylated pentapeptide Gly-Ala-Val-Leu-Ser; *I,* {(E,E)-2-[2-oxo-2-[[(3,7,11-trimethyl-2,6,10-dodecatrienyl)oxy]amino]ethyl]phosphonic acid, sodium}; Mes, 2-[*N*-morpholino]ethanesulfonic acid; Bes, *N*,*N*-bis[2-hydroxyethyl]-2-aminoehtanesulfonic acid; Heppso, *N*-[2-hydroxyethyl]piperazine-*N*′-[2-hydroxypropanesulfonic acid]; LMCT, ligand−metal charge transfer.

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sulfur−metal coordination (*13*). Additionally, the pK_a of the cysteine thiol is lowered from 8.3 in solution to ≤7 when it is bound to the enzyme. Consistent with this, the pH dependence of the cobalt−sulfur charge-transfer bands in the absorption spectra of a Co^{2+}-substituted FTase ternary complex indicates that a metal-bound thiol group ionizes with a pK_a of 6.3. The pK_a of the bound cysteine thiol is consistent with weak Zn^{2+}-thiolate coordination, which is expected if the cysteine thiolate functions as a nucleophile in the chemical step of the reaction.

EXPERIMENTAL PROCEDURES

Preparation of FTase and Substrates. Recombinant rat FTase was overexpressed in Sf9 cells, isolated, and purified as described previously (*28*) or overexpressed and purified from *E. coli* (*29*). The enzyme was stored at −80 °C in 50 mM Tris-HCl (pH 7.8 at 25 °C), 5 μM $ZnCl_2$, and 1 mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP). Recombinant H-Ras and H-Ras-CVLL [H-Ras with a C-terminal leucine residue which is a very poor substrate for FTase (*30*)] were prepared as described previously (*1*, *30*). Protein concentrations were determined by the Bradford assay (*31*) using a commercially available dye (Bio-Rad). Peptides (≥95% pure) were synthesized and HPLC-purified by Applied Analytical Industries (Chapel Hill, NC). The concentrations of the peptides were determined by reaction of the cysteine with 5,5′-dithio-bis(2-nitrobenzoic acid) (*32*) using an extinction coefficient of 14 150 M^{-1} cm^{-1} (*33*) or by weight. The peptides were stored at −20 °C in 5 mM Tris-HCl (pH 7 at 25 °C; passed through a 1.5 × 3 cm column of Chelex 100) with (Dns peptides) or without 30% DMSO.

pK_a of Free Peptide Cysteine. The reactivity of the cysteine residue of GCVLS and dansyl (Dns)-GCVLS (Figure 1) was determined from the time-dependent increase in the fluorescence of 6-acryloyl-2-dimethylaminonaphthalene (acrylodan; Molecular Probes, Eugene, OR) that results from alkylation of the cysteine (excitation at 391 nm, emission at 530 nm). Fluorescence measurements were performed at 25 °C on a SLM·Aminco·Bowman Series 2 Luminescence Spectrometer. The assay solution contained 50 mM buffer, either Mops-NaOH (pH 7.45), Taps-NaOH (pH 7.9−9.1), or H_3BO_3−NaOH (pH 9.4), with the ionic strength maintained at 0.1 M with NaCl and 1 mM $MgCl_2$. The reaction was initiated by the addition of 3 μM acrylodan and 0.1 μM peptide to buffer in a disposable fluorescence cuvette. Peptides containing Ala-for-Cys substitutions, GAVLS and Dns-GAVLS, were used as controls for any interaction of the acrylodan with the buffer or with other residues in the peptide. Alkylation of the thiol group in the peptide could not be observed over background changes in the acrylodan fluorescence at pH values below 7.4. The rate constant for modification of the cysteine was calculated using a weighted fit (Kaleidograph, Synergy Software) to eq 1:

$$\Delta FL = EP(1 - e^{(-kt)}) + IF \quad (1)$$

where ΔFL is the fluorescence at time *t* due to alkylation of the cysteine, EP is the fluorescence endpoint, and IF is the initial fluorescence. The pK_a of the free cysteine was determined from the pH dependence of the observed rate constants using a weighted fit to eq 2:

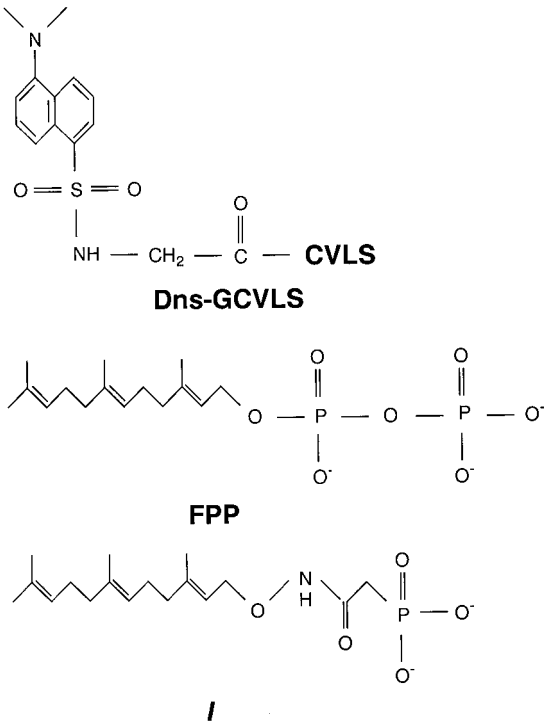


FIGURE 1: Structures of substrates and analogues. Dns-GCVLS is a pentapeptide, based on the carboxyl terminus of H-Ras, with a dansyl group attached to the amino terminus. FPP is an FTase isoprenoid substrate. *I* is an inhibitory FPP analogue (35).

$$k_{obs} = \frac{k}{(1 + 10^{(pK_a - pH)})} \quad (2)$$

Substrate-Binding Assays. Dissociation constants for Dns-GCVLS were determined from the increase in fluorescence of the Dns group following binding (*34*) to FTase or FTase·*I*, a binary complex formed with {(E,E)-2-[2-oxo-2-[[(3,7,-11-trimethyl-2,6,10-dodecatrienyl)oxy]amino]ethyl]phosphonic acid, sodium} (designated *I*, see Figure 1; Calbiochem, San Diego, CA), an inactive FPP analogue (*35*). The fluorescence of bound Dns was monitored at 496 nm after energy transfer from tryptophan residues on FTase (excitation at 280 nm). The assay solution contained 50 mM buffer, either Mes-NaOH (pH 5.6−6.5), Bes-NaOH (pH 6.5−7.2), Mops-NaOH (formation of FTase·Dns-GCVLS; pH 6.5−7.6), or Heppso-NaOH (pH 7.2−8.5), with the ionic strength maintained at 0.1 M with NaCl, 1 mM $MgCl_2$, 2 mM TCEP, and 0.01 μM EDTA. The dissociation constants are not dependent on the concentration of buffer (5−50 mM), NaCl (0−0.09 M), or $MgCl_2$ (0−10 mM). FTase incubated from pH 5.6 to 9.6 for 1 h under the binding assay conditions retains 60%−80% of its catalytic activity when subsequently assayed at pH 7.7 (data not shown), indicating that the enzyme is stable under these conditions. The enzyme (0.02 μM) was preincubated in the stirred assay solution (3 mL total, 25 °C) with or without 0.04 μM *I* for 5 min before titration with the Dns peptides. The Dns fluorescence was measured after a 5 min incubation. The fluorescence signal is stable for at least 15 min after addition of the peptide. The titration range used in these experiments (0−10 μM) reflects peptide concentrations at least 5-fold above and below the K_D at each pH value. Dns-GAVLS was used as a control for background fluorescence. The presence of higher concentrations of *I* did not affect the peptide K_D.

Dissociation constants were determined from a weighed fit of the data to eq 3 (K_D > 3[FTase]) or eq 4 (K_D < 3[FTase]) (*36*):

$$\Delta FL = \frac{EP}{(1 + K_D/[Pep])} + IF \quad (3)$$

$$\Delta FL = IF + \frac{(EP - IF)}{2[FTase]}\left(([FTase] + [Pep] + K_D) - \sqrt{([FTase] + [Pep] + K_D)^2 - 4[FTase][Pep]}\right) \quad (4)$$

where ΔFL is the observed fluorescence corrected for background, EP is the fluorescence endpoint, IF is the initial fluorescence, [Pep] is the concentration of Dns-GCVLS, and K_D is the dissociation constant for Dns-GCVLS.

The pK_a values associated with interaction of the peptide cysteine with FTase and FTase·*I* were calculated from the pH dependence of the observed K_D using a weighted fit of eq 5:

$$K_D^{app} = \frac{K_D(1 + 10^{(pH - pK_{a1})})}{(1 + 10^{(pH - pK_{a2})})} \quad (5)$$

Dissociation constants for GCVLS and H-Ras were determined by competition assays in which the fluorescence of the FTase·Dns-GCVLS or FTase·*I*·Dns-GCVLS complex decreases as the concentration of the competitive peptide or protein increases due to formation of the nonfluorescent species, FTase·GCVLS, FTase·*I*·GCVLS, or FTase·*I*·H-Ras. The assay conditions were the same as those used in the Dns-GCVLS binding assays described above. Increasing concentrations of GCVLS, GAVLS, H-Ras, or H-Ras-CVLL were titrated into a solution containing 0.02 μM FTase, with or without 0.04 μM *I*, that had been preincubated for 10 min with a concentration of Dns-GCVLS equal to the K_D. The resulting decrease in the Dns fluorescence was fit to eq 6:

$$\Delta FL = \frac{IF}{1 + (K_D/[Pep])(1 + [Sub]/K_S)} + EP \quad (6)$$

where ΔFL is the observed fluorescence corrected for background, IF is the initial fluorescence, EP is the fluorescence endpoint, K_D is the dissociation constant for Dns-GCVLS determined previously, [Pep] is the concentration of Dns-GCVLS, [Sub] is the concentration of added peptide or protein substrate, and K_S is the dissociation constant for GCVLS or H-Ras. The concentration of H-Ras reflects the amount that could be farnesylated (73% of total protein) rather than the total protein concentration (*37*).

pH Titration of the Optical Absorption Spectra of Co^{2+}-Substituted FTase·I·GCVLS. Apo-FTase was prepared and reconstituted with cobalt(II) as described previously (*13*). Optical absorption spectra of Co^{2+}-substituted FTase were recorded on a Uvikon 9410 UV/VIS double-beam spectrophotometer using a 120 μL cuvette. The Co^{2+}-FTase·*I*·GCVLS ternary complex was formed in 50 mM buffer, either Heppso-NaOH (pH 7.8) or Mes-NaOH (pH 5.9), 1 mM TCEP, and 5 mM $MgCl_2$, by adding a 1:1 stoichiometry of *I* and GCVLS to Co^{2+}-FTase (35−60 μM) where [Co^{2+}-FTase] ≫ K_D. A sample of Zn^{2+}-FTase·*I*·GCVLS at an identical concentration was used in the reference cuvette.

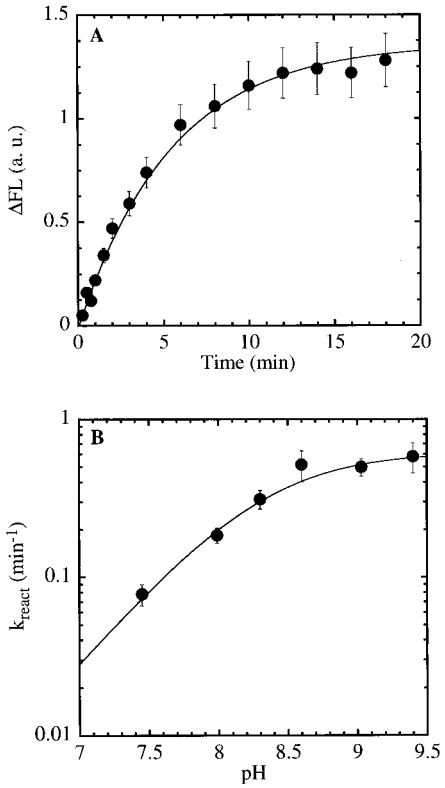


FIGURE 2: pK_a of the cysteine thiol in the free peptide. (A) The fluorescence increase associated with the alkylation of the free peptide cysteine by acrylodan was used to determine the reactivity of the cysteine as described under "Experimental Procedures". The plot shows a time course for the reaction of 0.1 μM Dns-GCVLS with 3 μM acrylodan at pH 7.99, 25 °C, where $k = 0.18 \pm 0.02$ min^{-1}. (B) The pK_a of the free peptide thiol, 8.3 ± 0.1 for Dns-GCVLS (shown) and 8.6 ± 0.1 for GCVLS, was calculated from the pH dependence of reactivity (eq 1) assuming that the reactivity of the protonated thiol is negligible.

The optical absorption spectra of Co^{2+}-FTase·*I*·GCVLS were recorded from pH 5.9 to 7.5 by adding 1 M Heppso (pH 7.8) or from pH 7.8 to 6.0 by adding 1 M Mes (pH 5.5) in small aliquots to both the sample and the reference cuvettes. The pH dependence of the extinction coefficient of the ligand−metal charge-transfer (LMCT) band was fit to eq 7:

$$\epsilon_{obs} = \frac{\epsilon}{(1 + 10^{(pK_a - pH)})} + EP \quad (7)$$

where ϵ_{obs} is the observed extinction coefficient at 340 nm and EP is the low pH endpoint.

RESULTS

pK_a of Peptide Thiol. Acrylodan reacts with thiolates to form a complex with a stable thioether bond (*38*). The fluorescence quantum yield of the thioether product is much higher than that of unreacted acrylodan, allowing it to be used to measure the reactivity of the peptide cysteine residue at relatively low concentrations of peptide (0.1 μM) (see Figure 2A). Since the nucleophilicity of thiolates is much higher than that of thiols, the pK_a of the peptide thiol in solution can be calculated from the pH dependence of the rate constant of alkylation (*39*). Using this method, the pK_a of the cysteine thiol was measured as 8.3 ± 0.1 for Dns-GCVLS (Figure 2B) and 8.6 ± 0.1 for GCVLS (data not

shown). Furthermore, the pH-independent second-order rate constant for the reaction of the thiolate with acrylodan under these conditions was determined to be $3.4 \pm 0.4 \times 10^3$ M^{-1} s^{-1} for Dns-GCVLS and $2.4 \pm 0.3 \times 10^3$ M^{-1} s^{-1} for GCVLS.

Affinity of Dns Peptides. We developed a fluorescent assay using dansylated substrates to directly measure the affinity of FTase for peptides. Dansylated peptides corresponding to the C-terminus of H-Ras (CVLS) were originally constructed for use in continuous, nonradioactive FTase activity assays (*40*, *41*). The fluorescence of the dansyl (Dns) group is enhanced 13-fold (at 505 nm), and the peak of the Dns emission spectrum is blue shifted for the farnesylated product (515 nm) relative to the free substrate (565 nm). Similar fluorescence changes were observed upon binding of Dns-GCVLL to GGTase-I and were used to measure dissociation constants for this peptide with apo- ($K_D > 13$ μM) and Zn^{2+}-GGTase-I ($K_D = 0.11$ μM) (*34*). However, direct excitation of the Dns moiety at 340 nm causes significant background fluorescence from the free Dns-GCVLL, requiring the use of high enzyme concentrations to obtain a good signal. To enhance the sensitivity of these binding assays, we quantified the FTase·Dns-GCVLS and FTase·*I*·Dns-GCVLS complexes using fluorescence resonance energy transfer (*42*) from excited tryptophan residues in FTase to the bound Dns group. Since the fluorescence emission of Dns excited at 280 nm is very low (Figure 3A) and resonance energy transfer is highly distance dependent (*42*), only the Dns group in the bound peptide is excited and thus the background signal from the free peptide is significantly decreased. Therefore, this method increases the sensitivity of the assay, allowing the concentration of FTase in the binding assay to be lower than the peptide dissociation constant so that the data can be fit using a single binding isotherm (eq 3). A typical K_D determination is shown in Figure 3B. Peptides lacking a thiol moiety (Dns-GAVLS and GAVLS) bind weakly under all conditions and are used to assay nonspecific changes in the fluorescence, such as the inner filter effect (*42*).

pH Dependence of Peptide Binding. The pH dependence of peptide binding to the FTase·*I* complex reflects changes in the ionization state of the free peptide, the FTase·*I* binary complex, and the FTase·*I*·peptide ternary complex. In particular, ionization of the free peptide thiol ($pK_a = 8.3$) could affect the apparent peptide affinity. On the basis of the model in Scheme 1A, if FTase·*I* binds the thiol (Pep-SH) and thiolate (Pep-S^-) forms of the peptide equally well, the peptide K_D will not change with varying pH (see Figure 4A). If the enzyme only binds the thiolate form of the peptide, the K_D will increase with decreasing pH as the concentration of thiolate decreases. If the enzyme binds both forms of the peptide but has greater affinity for the thiolate, the pH dependence will be characterized by plateaus at both high and low pH with tighter binding at high pH.

To investigate possible ionizations that affect peptide affinity, the peptide dissociation constant was determined over a pH range (from 5.6 to 8.5) where the enzyme is stable. The binding of Dns-GCVLS to FTase·*I* is dependent on pH (Figure 4B) and the data are best fit to a curve (eq 5) describing two pK_a values, $pK_{a1} = 8.1 \pm 0.2$ and $pK_{a2} = 6.4 \pm 0.1$ for FTase·*I* [similar pK_a values are obtained using Dns-GCVLS and FTase (see Figure 4B)]. For the higher

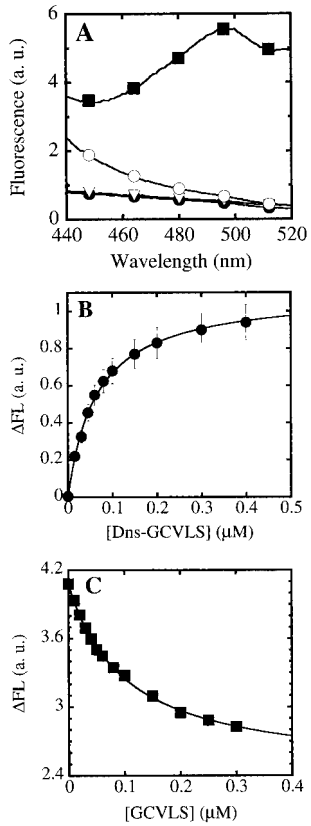
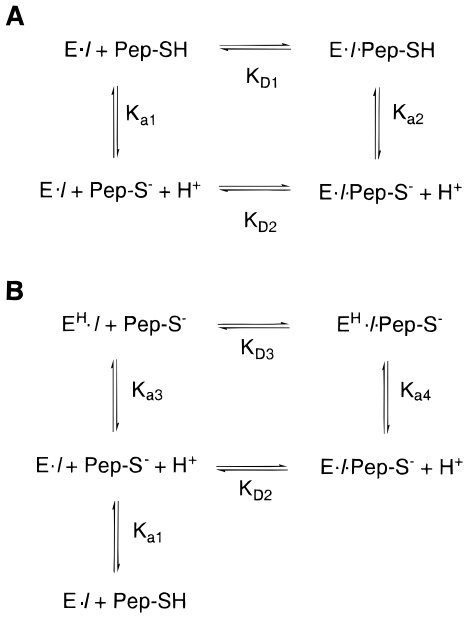


FIGURE 3: Peptide binding assays. (A) Emission spectra. Emission spectra (excitation at 280 nm, emission at 496 nm) were recorded at pH 7.7 for the following: buffer only (50 mM Heppso-NaOH, ionic strength maintained at 0.1 M with NaCl, 2 mM TCEP, 1 mM $MgCl_2$, and 0.01 μM EDTA) (●); 0.3 μM Dns-GCVLS (▽); 0.02 μM FTase (○); and 0.02 μM FTase + 0.3 μM Dns-GCVLS (■). (B) Dns peptide binding assay. Dns peptides were titrated into assay solution (pH 7.7) containing 0.02 μM FTase and 0.04 μM *I*. The fluorescence of the Dns group was monitored at 496 nm after excitation by energy transfer from tryptophan (tryptophan excitation at 280 nm). The Dns-GCVLS fluorescence was corrected by subtraction of FTase·*I* + Dns-GAVLS background and was then fit to eq 3 (see Table 1). (C) Competition binding assay. A ternary FTase·*I*·Dns-GCVLS complex was formed by addition of 0.06 μM Dns-GCVLS to 0.02 μM FTase that had been preincubated with 0.04 μM *I* at pH 7.7. GCVLS or GAVLS was then titrated into the solution, and the fluorescence was measured. The K_D for GCVLS was calculated using eq 6 (see Table 1).

pK_a, the affinity of the peptide decreases as the pH is lowered indicating that addition of a proton weakens binding. This pK_a reflects ionization of the cysteine thiol in the unbound peptide, since this pK_a is nearly identical to the pK_a obtained for the free peptide (8.3 ± 0.1). These data indicate that the peptide thiolate binds to FTase·*I* more tightly than the peptide thiol does (Scheme 1A). Therefore, at neutral pH the bound peptide is ionized to the thiolate, consistent with spectral studies suggestive of thiolate-metal coordination (*13*). However, if deprotonation of the free peptide were the only important ionization, the $K_D^{peptide}$ should continue to increase as the pH is lowered (see Figure 4A). Instead, Figure 4B shows that at low pH the peptide affinity becomes pH-independent and is tighter than would be predicted from a single ionization. Therefore, a second protonation occurs that stabilizes the bound complex relative to the free enzyme and peptide. The simplest mechanism to account for this second pK_a is protonation of the substrate cysteine thiolate in the bound complex (Scheme 1A). For this mechanism,

Scheme 1. Models for Peptide Binding to FTase·*I*[a]



[a] (A) Peptide binding is described by two ionizations: K_{a1}, deprotonation of the cysteine thiol on the free peptide, and K_{a2}, deprotonation of the cysteine thiol on the enzyme-bound peptide, where $K_D^{app} = [K_{D2}(1 + [H^+]/K_{a1})]/(1 + [H^+]/K_{a2})$. (B) Ionization of a second group on the enzyme, denoted by $E^H{\cdot}I$, increases the affinity of the enzyme for the peptide so that pK_{a1} describes the ionization of the peptide thiol, pK_{a3} describes the ionization on the enzyme, pK_{a4} describes the ionization of the $E^H{\cdot}I$·peptide complex, and $K_D^{app} = [K_{D2}(1 + [H^+]/K_{a3})(1 + [H^+]/K_{a1})]/(1 + [H^+]/K_{a4})$.

the pH-independent $K_D^{peptide}$ at low and high pH directly measures the affinity of FTase·*I* for Pep-SH (K_{D1}) and Pep-S^- (K_{D2}), respectively, and the two observed pK_a values directly reflect ionization of the free and bound peptide. Thus, these data suggest both that the peptide thiolate binds 16-fold more tightly than the peptide thiol and that the pK_a of the peptide thiol is decreased by 1−2 units upon binding to FTase·*I*.

These pH dependence data are also consistent with a mechanism in which protonation of a second site in the bound complex enhances peptide affinity (Scheme 1B), assuming that the pK_a of the bound peptide thiolate is much lower than the observed pK_a of 6.4. In this mechanism, the two observed pK_a values reflect ionization of the free peptide (K_{a1}) and the enzyme in the $E^H{\cdot}I{\cdot}$Pep-S^- complex (K_{a4}); at low pH the binding reaction can be described as $E^H{\cdot}I$ + Pep-S^- → $E^H{\cdot}I{\cdot}$Pep-S^- which is thermodynamically equivalent to E·*I* + Pep-SH → $E^H{\cdot}I{\cdot}$Pep-S^- (*43*). For this mechanism to fit the observed pH dependence data (Figure 4B), the pK_a of the peptide thiol must be significantly lowered and the pK_a of the enzyme group must be significantly increased in the bound complex. Since parts A and B of Scheme 1 differ mainly by the position of the proton in the bound complex, our thermodynamic binding data are not sufficient to distinguish between these two models (*43*). However, the pH dependence of the absorption spectrum of Co^{2+}-FTase·*I*·GCVLS, described below, is easier to reconcile with Scheme 1A since it limits the ionizations to the metal coordination sphere such as Cys299 (*11, 13, 44*) or the peptide thiolate. Nonetheless, the pK_a of 6.4 represents an upper limit for the pK_a of the substrate thiol bound to the enzyme.

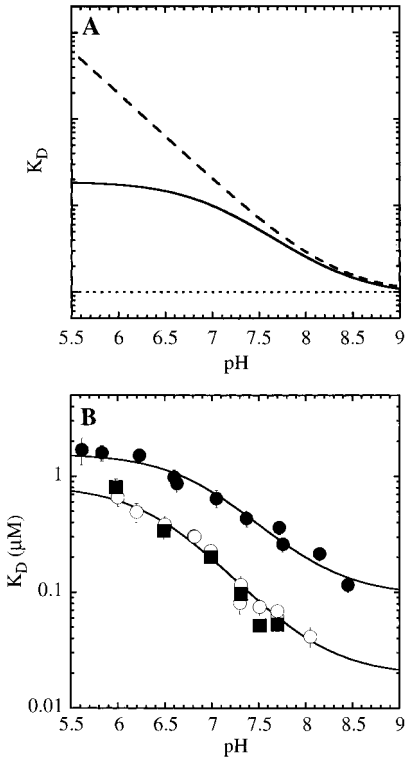


FIGURE 4: pH dependence of peptide binding. (A) (- - -) represents a system where the enzyme binds both R−SH and R−S^- equally well resulting in no pH dependence of peptide binding, (− − −) represents a system that only binds R−S^- and is described by a single pK_a, and (—) represents a system with 2 pK_a values (8.3 and 7, for example) where R−S^- binds better than R−SH (Scheme 1A). (B) Dissociation constants were determined for Dns-GCVLS binding to FTase (●) and FTase·*I* (○) between pH 5.6 and pH 8.5 as described under "Experimental Procedures". The K_D values were fit to eq 5, and the resulting pK_a values are 8.1 ± 0.2 and 6.9 ± 0.1 for FTase·Dns-GCVLS and 8.1 ± 0.2 and 6.4 ± 0.1 for FTase·*I*·Dns-GCVLS. Dissociation constants for GCVLS binding to FTase·*I* (■) are also indicated.

pH Dependence of Co^{2+}-FTase·I·GCVLS Absorption Spectra. The low-wavelength absorbance (below 450 nm) of the spectra of Co^{2+}-substituted enzymes is assigned to a ligand−metal charge-transfer (LMCT) band indicative of sulfur-to-cobalt charge transfer resulting from thiol(ate) coordination (*45, 46*). In the spectra of Co^{2+}-FTase and Co^{2+}-FTase·isoprenoid without added peptide, a LMCT band was observed consistent with a single metal ligand, Cys299 (*11, 13, 44*). For the Co^{2+}-FTase·isoprenoid·peptide ternary complex at pH 7.8, a doubling of the extinction coefficient of the LMCT band was observed, suggestive of a Co^{2+}-thiol(ate) bond between the metal and the substrate sulfur (*13*). In this work, we measured the pH dependence of both the LMCT band and the higher-wavelength ligand field bands in the absorption spectrum of Co^{2+}-FTase·*I*·GCVLS (Figure 5A). Since pH-dependent changes in the absorption spectrum of Co^{2+} implicate ionizations of groups within the coordination sphere of the bound metal (*47*), this method should be useful for further identifying the source of the second pK_a observed in the pH dependence of peptide binding. In particular, alterations in the metal−sulfur coordination should be observable as changes in the LMCT bands. A pH titration of Co^{2+}-FTase·*I*·GCVLS reveals that the shape and extinction coefficient of the ligand field bands in the absorption spectrum are not dependent on pH (Figure

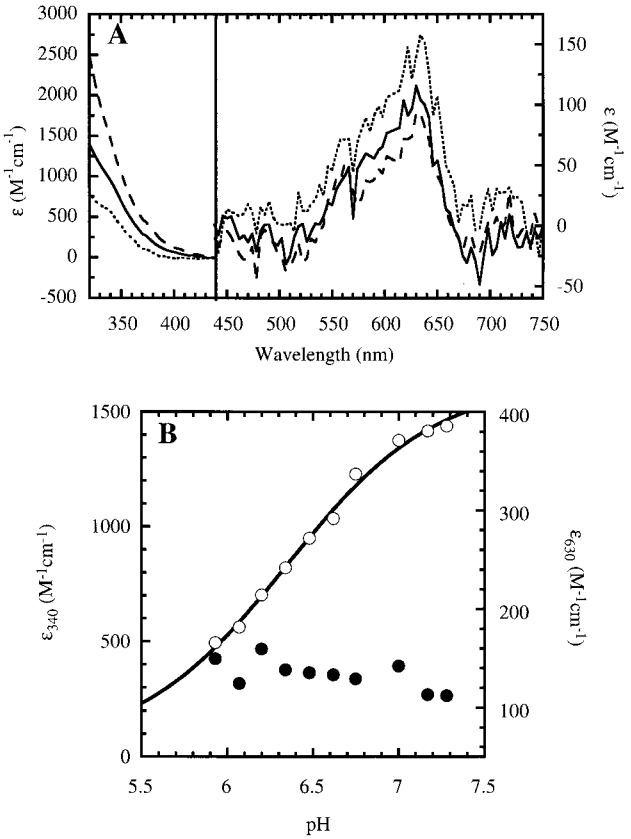


FIGURE 5: pH dependence of Co^{2+}-FTase·*I*·GCVLS absorbance spectra. (A) Absorbance spectra of Co^{2+}-FTase·*I*·GCVLS were measured over a pH range from 5.9 to 7.5, as described under "Experimental Procedures". Spectra at pH 6.07 (- - -), 6.48 (—), and 7.28 (— — —) are shown. The decrease in the ligand field absorption band at 630 nm was probably due to a 10% sample volume increase by the end of the titration. (B) Extinction coefficients were calculated for the peaks at 340 nm (○) and 630 nm (●) corresponding to the LMCT band and the ligand field absorption band, respectively. A fit of the 340 nm extinction coefficients to eq 7 results in a pK_a of 6.3 ± 0.2 and a poorly defined low pH endpoint (33 ± 93 M^{-1} cm^{-1}).

5B), indicating little change in the geometry or metal occupancy of this site. However, the LMCT band (measured at 340 nm) decreases as the pH is lowered and reflects a single ionization with a pK_a of about 6.3. This decrease is not due to significant dissociation of the bound peptide since the enzyme concentration remains higher than the measured K_D even at low pH. Therefore, the observed decrease in the absorbance at low pH indicates a diminution in the cobalt−sulfur coordination due to protonation of a group in the coordination sphere of the metal, probably either the coordinating cysteine side chain or the peptide substrate thiolate. Since the geometry of the site is unchanged, protonation of the peptide substrate thiolate is most likely. Furthermore, the behavior of Co^{2+}-FTase mimics the natural zinc enzyme very well, including the following: (1) the steady-state activity is unchanged (*13*); (2) the individual rate constants for substrate association and the formation of farnesylated peptide are unaffected (Huang, C.-c., and Fierke, C. A., unpublished experiments); and (3) the K_D values for Dns-GCVLS dissociating from the Co^{2+}-FTase·*I*·Dns-GCVLS complex are identical to those measured for the zinc enzyme (pH 6−8, data not shown). Therefore, the pK_a observed in the LMCT band of the Co^{2+}-substituted enzyme likely reflects a similar ionization in the Zn^{2+}-FTase ternary complex which is also observed in the binding of peptide substrates.

Table 1: Affinity of FTase for Peptide and Protein Substrates

FPP analog	substrate	K_D (μM)[a]
none	Dns-GCVLS	0.35 ± 0.06
I	Dns-GCVLS	0.063 ± 0.005
none	GCVLS	4 ± 1
I	GCVLS	0.058 ± 0.005
I	H-Ras	0.5 ± 0.2[b]

[a] The K_D values represent averages of at least 3 different experiments. [b] The K_D for H-Ras was determined in the presence of dithiothreitol (10 μM at the end of the titration).

Affinity of Peptides and H-Ras. Competition assays were used to determine the affinity of FTase both for the peptide GCVLS and for full-length H-Ras. The decrease in the fluorescence emission upon addition of GCVLS or H-Ras in the presence of Dns-GCVLS reflects a decrease in the concentration of the bound Dns-GCVLS complex as the unlabeled substrate competes for this binding site (Figure 3C). The dissociation constants for GCVLS in the ternary complex are similar to the K_D values for Dns-GCVLS over the pH range studied (Figure 4B), indicating that the Dns group does not affect either the affinity or the pH dependence of peptide binding in the ternary complex. However, in the absence of the *I* inhibitor, the affinity of FTase for GCVLS is measured as 4 ± 1 μM at pH 7.7 which is significantly weaker than both the affinity of FTase for Dns-GCVLS (0.35 ± 0.06 μM) and the affinity of FTase·*I* for GCVLS (0.058 ± 0.005 μM) at the same pH (Table 1). Therefore, the presence of *I* increases the GCVLS binding affinity approximately 70-fold, suggesting that there is a significant synergistic effect of substrate binding. The Dns group can also enhance the affinity of FTase for GCVLS if an isoprenoid is not bound. Finally, the affinity of FTase·*I* for H-Ras was also determined. At pH 7.7, the K_D for H-Ras dissociating from the FTase·*I*·H-Ras ternary complex is 0.5 ± 0.2 μM, approximately 10-fold higher than the K_D for GCVLS (0.058 ± 0.005 μM). Therefore, the peptide substrates bind more tightly than recombinant H-Ras.

DISCUSSION

The specificity of prenyltransferases for modification of protein substrates is essential for the biological function of these enzymes. Previous estimations of peptide/protein affinity and selectivity in mammalian FTase have been derived mainly from competition assays (*48*, *49*) or inferred from the steady-state kinetic parameters K_M and k_{cat}/K_M (*50*−*52*). However, $K_M^{peptide}$ does not directly reflect $K_D^{peptide}$, the true substrate affinity, for this enzyme since the rate constant for the chemical step is faster than either peptide or product dissociation (*26*, *53*). In addition, the pH dependence of the steady-state kinetic parameters, k_{cat}/K_M and k_{cat}, will not provide true thermodynamic measures of important ionizations of the enzyme and substrate since the rate-limiting steps are substrate association and product release, respectively (*26*, *53*); therefore, the observed pK_a values will likely be kinetically perturbed by a change in the rate-limiting step as the pH is altered (*43*, *54*). Thermodynamic pK_a values can be determined most straightforwardly by directly mea-

suring the effect of pH on a single elementary step, such as substrate binding. Therefore, we have developed sensitive fluorescence assays to directly measure the interaction of peptide and protein substrates with FTase and the binary FTase·isoprenoid complex. In the absence of FPP or an FPP analogue, the affinity of FTase for peptide substrates is in the micromolar range. Previous studies have shown that, although FTase can bind either substrate independently, the preferred reaction path is the addition of the protein/peptide substrate to a FTase·FPP complex (*26, 55*). In addition, FPP has been proposed to increase the affinity of FTase for K-Ras (*55*). Indeed, our data demonstrate that binding of the FPP analogue *I* increases the affinity of FTase for GCVLS approximately 70-fold, indicating synergism between the binding of the two substrates, presumably due either to direct contacts between the substrates or to a conformational change induced by binding of the isoprenoid.

Zinc is required for the function of FTase (*9, 10*), and a variety of experimental evidence supports the hypothesis that the Zn^{2+} ion is directly involved in catalysis (*11, 13*). In enzymes that use Zn^{2+} in such a fashion, the Zn^{2+}-bound water can directly participate in catalysis through ionization by the Zn^{2+} or polarization by a base, or the water can be displaced by a substrate (*12*). The latter appears to be the case for FTase, as there is substantial evidence indicating that the Zn^{2+} is involved in the binding of protein/peptide substrates. First, catalytic activity is only observed for Zn^{2+}-FTase or when the Zn^{2+} is substituted by Co^{2+} or Cd^{2+} (*9, 10, 13, 56*). Second, removal of the Zn^{2+} abolishes protein binding without affecting FPP binding (*9, 10*). Finally, direct interaction of the sulfur of the peptide cysteine residue with the Co^{2+}, as evidenced by the enhancement of cobalt−sulfur charge-transfer bands, is observed for ternary complexes of the Co^{2+}-substituted enzyme formed with either FPP or the *I* analogue (*13*). In addition, the metal geometry of these complexes is tetrahedral, suggesting that the water ligand is displaced by the substrate sulfur. These studies indicate that one of the functions of the Zn^{2+} in FTase is to coordinate the sulfur atom of the relevant cysteine in the protein substrate. Such metal−sulfur coordination can provide both a large energetic contribution to binding (a typical Zn−S bond in small molecule complexes is > 4 kcal/mol; *57*) and high selectivity, since similar amino acids such as Ser and Ala are discriminated against (*4, 48*). However, the participation of the Zn^{2+} in protein substrate binding does not exclude the possibility that the metal may also play an important catalytic role.

The pH dependence of peptide binding determined both by direct titration methods (Figure 4B) and by optical absorption spectroscopy of Co^{2+}-substituted FTase·*I* (Figure 5B) indicates that there is an ionization in the ternary complex with a pK_a of about 6.4. These data reveal that this protonation leads to an apparent increase in the affinity of the peptide substrate (versus a single pK_a model, Figure 4A) and a decrease in the observed cobalt−sulfur charge-transfer band in the ternary complex. These spectral data indicate that the ionization occurs within the metal coordination sphere and specifically affects the metal−sulfur coordination. While it is possible that the ionization could be due to another residue on the enzyme (Scheme 1B) such as one of the Zn^{2+} ligands, the fact that the geometry of the metal site is unchanged compared to the E·*I*·peptide ternary complex (*13*) suggests that the pK_a likely represents the protonation of the bound peptide substrate thiolate to a thiol at low pH (Scheme 1A).

Enzyme-mediated lowering of the thiol pK_a of catalytic cysteine residues has been observed in systems such as the glutathione transferases (*58, 59*) and the cysteine proteases (*60*−*62*). For FTase, sulfur−metal coordination stabilizes the thiolate 100-fold relative to the thiol in the ternary complex and lowers the pK_a of this thiol at least 2 pH units. A 100-fold stabilization of a thiolate by metal coordination is modest compared to noncatalytic zinc−sulfur bonds, such as those found in metallothionein (*63*), suggesting that the metal−thiolate bond in FTase may be weaker than normal, and this may be important for maximizing the nucleophilicity of the bound thiolate. Studies of the reactivity of model thiol compounds suggest that decreased basicity correlates with decreased nucleophilicity since the Brønsted correlation between the pK_a of the thiol and the log k_{Nuc} is approximately 0.4 (*39, 64*−*69*). Therefore, to obtain a maximal rate enhancement, the pK_a of the bound cysteine should be just less than the physiological pH to ensure maximal thiolate concentration and reactivity, consistent with the measured pK_a of 6.4.

Several mechanisms have been proposed for the reaction catalyzed by FTase. In one model, catalysis proceeds through an electrophilic mechanism with formation of a carbocation on C1 of FPP (*26, 50, 70, 71*). In an alternate model of catalysis, ionization of the protein substrate "CaaX" cysteine would lead to a nucleophilic attack on C1 of FPP, displacing the pyrophosphate (*10, 26, 50*). It is also possible that FTase utilizes a combination of both models such that an "exploded transition state" (*72*) develops where C1 of FPP has carbocation character and the thiolate functions as a nucleophile. The current studies provide evidence that at neutral pH the peptide binds to the enzyme as the thiolate species. An enzyme thiolate could enhance the catalytic step by providing a better nucleophile, by stabilizing the formation of positive charge on the carbocation at C1 of FPP (*73*), and/or by correctly positioning the sulfur nucleophile. Investigation of the pH dependence of the farnesylation step using transient kinetics (*74*) should provide further insight into the catalytic mechanism of this enzyme.

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