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Yeast Protein Farnesyltransferase. Binding of *S*-Alkyl Peptides and Related Analogues

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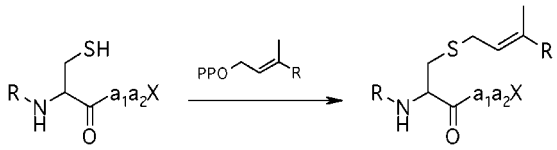
ABSTRACT



Protein farnesyltransferase (PFTase) catalyzes alkylation of cysteine residues by farnesyl diphosphate (FPP). The dissociation constants for the PFTase·peptide analogue complexes for the series of analogues fl-RTRC(X)VIA (X = H, methyl, dodecyl, farnesyl) were measured by fluorescence anisotropy. The results indicate that an ionizable sulfhydryl moiety is important for substrate binding and the farnesyl group in the product facilitates binding.

Protein farnesyltransferase (PFTase) catalyzes the post-translational modification of proteins and peptides with carboxyl-terminal Ca_1a_2X motifs, where C is cysteine, a_1 and a_2 are typically small hydrophobic amino acids, and X is typically serine, alanine, glutamine, or methionine.[1] The modification facilitates association of proteins in eukaryotic cells with membrane and is required for their biological activity. Among the proteins modified by farnesyl residues are the oncogenic Ras proteins that have been implicated in approximately 30% of human cancers.[2] Inhibitors of PFTase are being actively studied as anticancer drugs.[3,4]

PFTase is a zinc metalloprotein, and recent work indicates that the peptide substrate is bound to the enzyme as a Zn^{2+} thiolate.[5,6] This interaction is thought to enhance both the binding of the protein substrates and the nucleophilicity of the sulfhydryl moiety for the alkylation reaction.[7,8] Ca_1a_2X is a sufficient recognition motif for PFTase, and the enzyme farnesylates small peptides with catalytic efficiencies similar to those seen for normal protein substrates (see Scheme 1). We now report the results of binding studies with fluorescent peptides that establish the importance of an ionizable sulfhydryl moiety in the peptide/protein substrates and provide important insights about substrate and product binding.

flRTRC(H)VIA was prepared as previously described.[8] The fluorescent peptide is a substrate for yeast PFTase.[8] The related *S*-alkyl derivatives flRTRC(methyl)VIA, flRTRC-(dodecyl)VIA, and flRTRC(farnesyl)VIA were prepared by treatment of the peptide with methyl iodide, dodecyl iodide, and farnesyl bromide, respectively. In a typical experiment, flRTRC(H)VIA (2 μmol) was dissolved in 100 μL of DMF and solid $NaHCO_3$ (2 μmol) was added. Alkyl halide (2 μmol) was added, and the reaction was allowed to proceed for 24 h at room temperature with occasional swirling. The reaction was quenched by addition of 1 mL of deionized water, and the resulting mixture was frozen and lyophilized.

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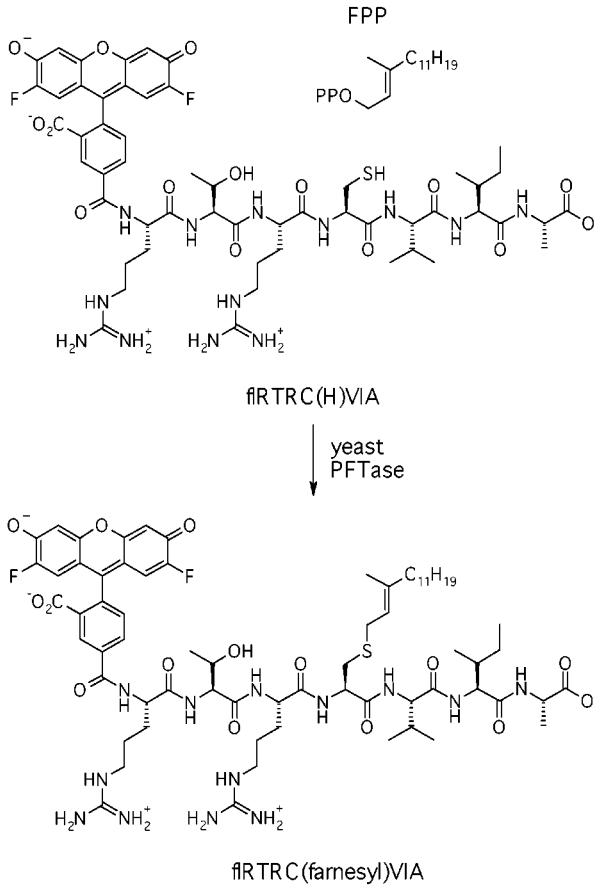
Scheme 1. Alkylation of 2′,7′-Difluoro-5-(*N*-(arginyl-threonyl-arginyl-cysteinyl-valinyl-isoleucinyl-alanine)carboxamide)fluorescein (flRTRC(H)VIA) by FPP Catalyzed by Yeast PFTase



The residue was dissolved in 100:1 acetonitrile/TFA, and the alkylated peptide was purified by reverse-phase HPLC on a C-18 column using a gradient of 40% 100:1 acetonitrile/TFA and 60% water to 100% 100:1 acetonitrile/TFA. Structures for the purified peptides were confirmed by electrospray mass spectrometry.

Dissociation constants for recombinant yeast PFTase·peptide complexes (K_D)

$$\text{PFTase·flRTRC(X)VIA} \rightleftarrows \text{PFTase} + \text{flRTRC(X)VIA}$$

were measured by fluorescence anisotropy,[8] and the data are presented in Table 1. flRTRC(H)VIA was the most tightly bound peptide of the analogues studied. Methylation of the sulfhydryl moiety increased K_D by more than 100-fold. We attribute this reduction in binding affinity primarily to loss of the Zn^{2+}−thiolate interactions in the enzyme·peptide complex. When longer chain hydrocarbon moieties were attached to sulfur, the binding affinity increased. The K_D value for flRTRC(farnesyl)VIA was only 2-fold higher than that of the peptide substrate itself. Yeast PFTase was not highly selective for the farnesyl residue. K_D only increased 1.5-fold when the farnesyl group was substituted by a dodecyl chain. Other enzymes that use allylic isoprenoid diphosphates as substrates are typically substantially more discriminating toward the allylic hydrocarbon moiety.[9]

Table 1. Dissociation Constants for Yeast PFTase·FlRTRC(X)VIA Complexes

peptide analog	K_D (μM)
flRTRC(H)VIA	19 ± 3[a]
flRTRV(H)VIA + F(CH_2)PP	12 ± 2[a]
flRTRC(methyl)VIA	>1000[b]
flRTRC(dodecyl)VIA	30 ± 4[b]
flRTRC(farnesyl)VIA	22 ± 2[b]

[a] Reference 8. [b] 50 mM HEPES buffer pH 7.0 containing 5 mM $MgCl_2$ and 5 mM dithiothreitol.

flRTRC(farnesyl)VIA was not displaced from the enzyme·peptide complex by 50 μM FPP (K_D = 0.075 nM[10]) or 50 μM RTRC(H)VIA (K_D = 10 μM[8]). However, flRTRC(farnesyl)VIA was displaced when F(CH_2)PP (Figure 1),[11]

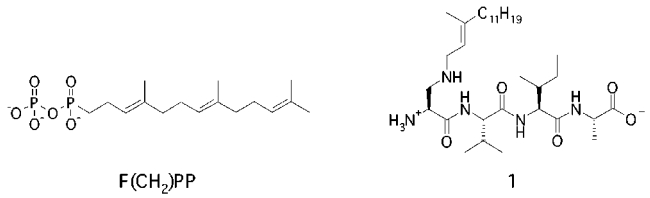


Figure 1. Structures of F(CH_2)PP and **1**.

a nonreactive analogue of FPP where the bridging oxygen between C1 and P1 was replaced by a methylene group, in combination with RTRC(H)VIA. As shown in Table 1, F(CH_2)PP produced only a modest enhancement in flRTRC(H)VIA binding. flRTRC(farnesyl)VIA was also displaced by farnesylated peptide **1**, a transition state analogue for the reaction,[12] with $K_D^{\mathbf{1}}$ = 16 μM. These results indicate that both the peptide and hydrocarbon moieties contribute substantially to the binding of flRTRC(farnesyl)VIA.

It was recently reported that K_D = 1 μM for PFTase·C(farnesyl)VIM when the complex was formed by addition of synthetic peptide to PFTase but that the radiolabeled product in PFTase·C(farnesyl)VIM formed in situ from [^{3}H]-FPP and CVIM did not exchange with unlabeled synthetic C(farnesyl)VIM over a period of 10 min.[13] These results imply an extraordinarily strong binding of the enzyme·product complex when formed in situ, and the authors proposed a substrate-induced displacement of the farnesylated product from the active site of the enzyme to account for the turnover rate for the enzyme during catalysis. We

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generated yeast PFTase·flRTRC(farnesyl)VIA in situ by incubation of enzyme (4 μM), FPP (2 μM), and flRTRC-(H)VIA (2 μM) in 50 mM HEPES buffer, pH 7.0, containing 5 mM $MgCl_2$ and 5 mM dithiothreitol (DTT) at 30 °C for 10 min, and the dissociation constant for the enzyme·product complex, $K_D = 10$ μM, was measured by fluorescence anisotropy within 20 min of initiating the reaction. As previously seen for synthetic flRTRC(farnesyl)VIA, addition of $F(CH_2)PP$ or RTRC(H)VIA did not significantly alter K_D for PFTase·flRTRC(farnesyl)VIA formed in situ.

In summary, replacing the cysteine thiol group in peptide substrates for PFTase by a thiomethyl moiety substantially reduces peptide binding, presumably by removing a favorable Zn^{2+}−thiolate interaction in the enzyme·substrate complex. However, the normal farnesylated product and the dodecyl analogue bind to PFTase almost as tightly as the peptide substrate itself. flRTRC(farnesyl)VIA is not displaced by either of the individual substrates but is displaced by the peptide substrate in combination with $F(CH_2)PP$ or by **1**, a transition state analogue containing structural elements of both substrates. In contrast to a previous report,[13] we saw no difference between binding of synthetic flRTRC(farnesyl)-VIA or product formed in situ.

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