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Measurement of the α-Secondary Kinetic Isotope Effect for the Reaction Catalyzed by Mammalian Protein Farnesyltransferase

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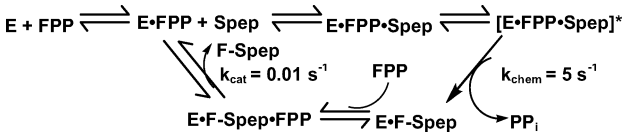
The posttranslational prenylation of proteins is catalyzed by the protein prenyltransferases, including protein farnesyltransferase (FTase) and protein geranylgeranyltransferase type I (GGTase I), that belong to the class of zinc enzymes that catalyze sulfur alkylation. Farnesylation is required for the membrane association and proper biological function of many important signaling proteins, including Ras which is mutated in 30% of all human cancers.[1] FTase inhibitors are currently in phase III clinical trials in the treatment of cancer, while GGTase I inhibitors show promise in the treatment of cardiovascular disease.[2] Protein and peptide substrates for both enzymes contain a C-terminal CaaX sequence, where C is a conserved cysteine, a is generally any amino acid, and X confers preference for reaction with FTase or GGTase I.[3]

The FTase bisubstrate reaction is functionally ordered, with FPP binding prior to peptide (Scheme 1).[4] On the basis of structural and mutagenesis studies, a conformational rearrangement of FPP has been proposed, in which the first two isoprene units of FPP rotate to bring the C1 of FPP within reacting distance of the peptide thiolate.[5] The diphosphate dissociation rate constant is as fast or faster than the farnesylation rate constant, and the dissociation of farnesylated product is the slowest step, so that the steady-state k_{cat} value reflects the product release rate constant.[6,7] In addition, a second molecule of FPP binds to the E·product complex to facilitate efficient product dissociation (Scheme 1).[8] Single turnover kinetics with limiting FPP (relative to [E]) isolate the farnesylation step from the product release step.[10,11] However, the observed rate constant for formation of the farnesylated product includes both the conformational rearrangement and the chemical steps. No information is yet known about the kinetics or thermodynamics of the conformational step. To date, the chemical step has been assumed to be the main rate-contributing step under single turnover conditions.

Scheme 1. Kinetic Mechanism of Mammalian FTase for Farnesylation of GCVLS[4,6−9]



The structure of the transition state for the reaction catalyzed by FTase has yet to be clearly defined. Evidence for an associative mechanism includes sensitivity to the reactivity of the thiolate nucleophile and inversion of stereochemistry during the reaction.[12] In contrast, fluorine substitution at the C4 methyl of FPP decreases k_{chem}, consistent with carbocation character in the transition state.[11,13] Together, these observations suggest that the FTase-catalyzed reaction proceeds via a concerted mechanism with dissociative character (Figure 1).[11,13] An increased knowledge of the geometry and electrostatic features of the TS may provide a blueprint for the design of potent FTase inhibitors.

Given the sensitivity of transition state energies to structural perturbations, the best method for investigating the structure of the transition state in FTase is heavy atom kinetic isotope effects (KIEs).[14] The α-secondary deuterium KIE for yeast FTase was measured under steady-state conditions and determined to be slightly inverse (0.977).[15] However, the KIE measured under steady-state conditions is complicated by the rate-limiting product release and high commitment factors for both substrate binding and product formation, and probably does not reflect the intrinsic KIE for either the rat or yeast FTases.[4,7,10,11] Therefore, we have measured the intrinsic KIE at the sensitive C1 position of FPP under single turnover conditions so that the step that undergoes isotopic discrimination is the chemical step. A combination of the competitive method for KIE measurement and transient kinetics has been used to measure the intrinsic KIEs for purine nucleoside phosphorylase and glutamate mutase.[16,17]

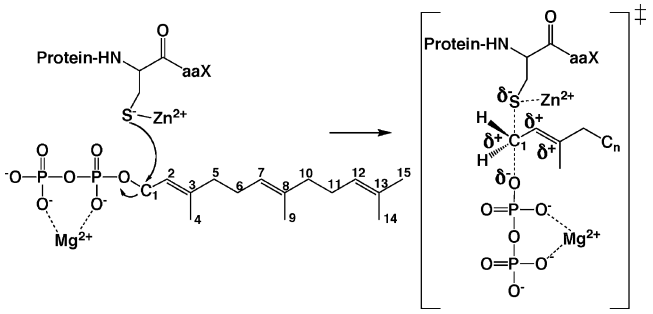


Figure 1. Transition state model for FTase.[11]

FPP with radiolabels at both the sensitive (C1) and remote (C11) positions were synthesized using the precursors isopentenyl diphosphate and geranyl diphosphate or dimethylallyl diphosphate labeled in the appropriate positions, as dictated by the FPP synthase reaction.[18] To measure the α-secondary KIE, saturating FTase was incubated with a substoichiometric concentration of a mixture of [1-^{3}H] FPP and [11-^{14}C] FPP, mixed with saturating peptide (TKCVIF) and stopped by an acid quench. Substrate and farnesylated product were separated using TLC and quantified by scintillation counting. The rate constant for farnesylation (k_{chem}) was calculated from a single-exponential fit of the fraction product versus time and the k_{chem} ratio was used to calculate an approximate KIE (Figure 2A). To measure the exact KIE, the ratio of products at 20−50% and 100% reaction was determined to calculate the apparent KIE (eq 1) and the KIE extrapolated to 0% conversion to obtain the actual KIE (eq 2).[19] Time courses were also done to establish that the observed KIE decreases as a function of fractional conversion to product (f), according to eq 2.

$$(1)\ \mathrm{KIE}_{\mathrm{apparent}} = \frac{([\mathrm{product}]_{\mathrm{normal}}/[\mathrm{product}]_{\mathrm{heavy}})_{20-50\%}}{([\mathrm{product}]_{\mathrm{normal}}/[\mathrm{product}]_{\mathrm{heavy}})_{100\%}}$$

$$(2)\ \mathrm{KIE}_{\mathrm{actual}} = \frac{\ln(1 - f\mathrm{KIE}_{\mathrm{obs}})}{\ln(1 - f)}$$

10.1021/ja065838m CCC: $33.50

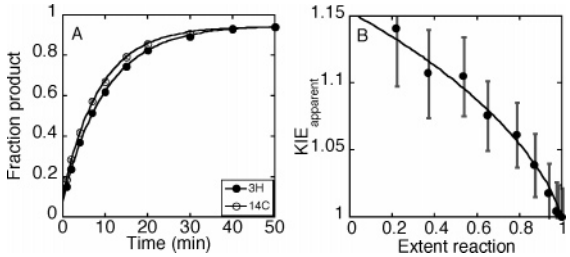


Figure 2. Measurement of α-2^0 ^{3}H KIE for TKCVIF. Final reactions contained 4 μM FTase, 1 μM FPP (900 nM [11-^{14}C]FPP, 100 nM [1-^{3}H]FPP), 100 μM TKCVIF, 50 mM Heppso, pH 7.8, and 2 mM TCEP. (A) Fraction product measured by ^{3}H (●) and ^{14}C (○) counts; (B) KIE_{app} calculated by eq 1 as a function of extent reaction and fit to eq 2.

Isotope experiments have identified concerted mechanisms with dissociative character for many enzymes. The reaction of pertussis toxin, like FTase, has an attacking cysteine thiolate as a nucleophile with a primary ^{14}C KIE of 1.049 ± 0.003 and an α-2^0 ^{3}H KIE of 1.199 ± 0.009.[20] A transition state structure analysis of pertussis toxin reveals a transition state with oxocarbenium ion character due to accumulation of positive charge on the ribosyl ring of NAD^+.[20] A pre-steady-state KIE characterization for purine nucleoside phosphorylase measured an α-2^0 ^{3}H KIE of 1.151 ± 0.004 and a primary ^{14}C KIE of 1.045 ± 0.005, and the transition state was defined as having oxocarbenium ion character and weak participation of the attacking nucleophile.[16] For FTase, the α-2^0 ^{3}H KIE of 1.154 ± 0.006 measured for the peptide TKCVIF is consistent with a concerted mechanism with dissociative character, since it is significantly less than either experimental α-2^0 ^{3}H KIEs or the theoretical equilibrium isotope effect for the formation of a fully dissociated carbocation in the transition state (1.4−1.5).[21] A primary ^{14}C KIE was also measured for FTase; however, the value was too small to quantify using this method (1.03 ± 0.03, data not shown) but is consistent with this proposed transition state structure.

GCVLS corresponds to the C-terminal sequence of H-Ras, and is a well-characterized substrate for FTase. While the α-2^0 ^{3}H KIE measured for a slowly farnesylated peptide, TKCVIF, was measured as 1.154 in the absence of Mg^{2+}, the 2^0 KIE for GCVLS measured under the same conditions was near unity (1.00 ± 0.04), indicating that another step besides the chemical step is most likely obscuring measurement of the intrinsic KIE for this peptide under these conditions. Diphosphate release is rapid relative to the farnesylation rate constant.[6] As a control, inorganic pyrophosphatase was added to the single turnover reaction to rapidly hydrolyze the diphosphate product; the observed KIE was not altered, thereby eliminating the possibility of a reversible reaction or a high reverse commitment factor (data not shown). These data suggest that the rate-limiting step for farnesylation of GCVLS is the conformational rearrangement of FPP prior to the chemical step that obscures the intrinsic KIE (Scheme 1).

Mg^{2+} enhances catalysis by FTase up to 700-fold for GCVLS.[22] In the transition state model, Mg^{2+} has been proposed to stabilize the transition state by coordinating to the diphosphate leaving group (Figure 1).[10] Another role for Mg^{2+} has been proposed in which a high affinity Mg^{2+} binding site has been modeled to accommodate the conformational rearrangement of FPP.[23] Consistent with a catalytic role, Mg^{2+} enhances farnesylation of TKCVIF approximately 100-fold but the α-2^0 ^{3}H KIE is not greatly altered (Table 1). Thus both the peptide sequence and Mg^{2+} concentration may play a crucial role in the kinetics of the conformational rearrangement of FPP, as supported by crystallographic and mutagenesis studies.[5] This step may be important for determining the substrate specificity of FTase.

Table 1. Effect of Mg^{2+} on α-2^0 ^{3}H KIE for reaction with TKCVIF

	0 $MgCl_2$	0.1 mM $MgCl_2$	5 mM $MgCl_2$
k_{chem} (s^{-1})	0.0026 ± 0.0001	0.016 ± 0.001	0.27 ± 0.01
2^0 ^{3}H KIE	1.154 ± 0.006	1.14 ± 0.01	1.13 ± 0.01

The measurement of KIEs using transient kinetics is a powerful technique to overcome large commitments in complex enzymatic reactions, but has only been used for a limited number of systems thus far. In the case of FTase, measurement of intrinsic KIEs are complicated using steady-state analysis, and the α-2^0 ^{3}H KIE measurement reported here provides the first direct evidence of a transition state with dissociative character for this class of enzymes that catalyze zinc-dependent sulfur alkylation. Additional KIE measurements may provide further information about the structure of the transition state, as well as details about the effect of different substrates on the conformational change.

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Supporting Information Available: Experimental procedures for preparation of radiolabeled FPP molecules and KIE measurements. This material is available free of charge via the Internet at http://pubs.acs.org.

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