Transition State Analysis of Model and Enzymatic Prenylation Reactions

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Protein prenylation involves the attachment of C_{15} (farnesyl) or C_{20} (geranylgeranyl) groups to proteins and is catalyzed by a class of enzymes known as prenyltransferases.[1] The observation that inhibition of Ras farnesylation arrests the growth of tumor cells has been the motivating factor in developing inhibitors of prenyltransferases that can serve as anticancer drugs; currently, several candidates are in Phase 3 clinical trials.[2] Mechanistic analysis of enzymatic reactions can provide insights that are potentially useful in drug design. Since enzymes must bind to the transition state (TS) with greater affinity than the ground state, molecules that mimic the structure of the TS will have the highest possible affinity for the enzyme. Enzyme inhibitors based on such principles can manifest extraordinary affinity and selectivity;[3] accordingly, we are interested in determining the TS structure for the reaction catalyzed by protein farnesyltransferase. Moreover, the detailed knowledge gained in these experiments should increase our understanding of how enzymes activate isoprenoid diphosphates for subsequent reaction. Such knowledge could be particularly useful for manipulating the reactivity of prenyltransferases and the closely related terpene cyclases for biotechnology purposes.

At present, the most reliable method to determine TS structure is through the use of computational methods in conjunction with experimentally measured kinetic isotope effect (KIE) measurements.[4] While a large body of literature exists for KIE measurements performed on benzylic systems, reports for related allylic systems are sparse. Thus, as a prelude to enzymatic measurements, it was decided to investigate several model reactions, shown in Figure 1, first. Results from such experiments would provide KIE values for limiting associative (S_N2) and dissociative (S_N1) mechanisms and allow us to validate the computational methods that would be used in the subsequent determination of the enzymatic TS. For a model substrate, dimethylallyl chloride (**1**) was chosen. Solvolysis of **1** in benzyl alcohol (**2**) was used as a dissociative model while displacement with triphenylphosphine (**5**) was employed as an associative model.[5] Kinetic analysis of these reactions revealed that the solvolysis reaction was first order in **1** and zero order in **2**, whereas the other reaction was first order in both **1** and **5**. To measure the ^{13}C KIEs for the reaction, an NMR method was employed based on the work of Singleton[6] and others; that approach involves the integration of ^{13}C NMR spectra obtained at natural abundance of reactant obtained prior to reaction and after substantial conversion. In the work reported here, the model reactions were performed, monitored by GC, and terminated by vacuum distillation to recover the remaining starting material which was then derivatized by reaction with dimethylmalonate (**7**) and the resulting product (**8**) purified by flash chromatography. ^{13}C NMR spectra were obtained and integrated using C-4 as an internal standard. ^{13}C KIEs were calculated from the ratios of peak areas determined from samples of the derivatized starting material (**8**) before and after reaction. A significant primary ^{13}C KIE at C-1 was measured in the S_N2 model reaction (1.040 ± 0.003), whereas a value near unity (0.997 ± 0.003) was observed in the S_N1 model reaction.
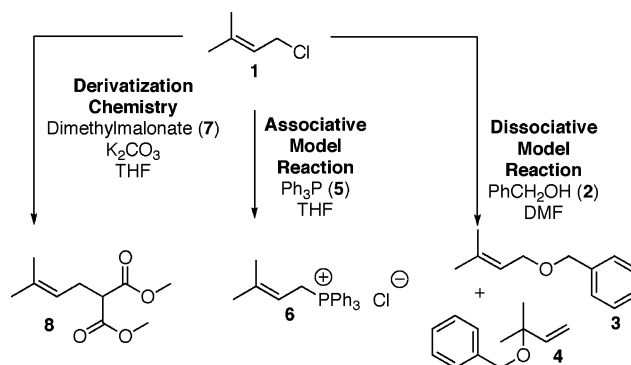

Figure 1. Model reactions and derivatization chemistry for S_N1 and S_N2 prenylation reactions.

The reactants and TS structures for the S_N1 and S_N2 model reactions were determined at the density functional level of electronic structure theory with the 6-31+G(d) basis set.[7] Analytic vibrational frequencies were computed for each stationary point, and KIEs were predicted from the usual transition state theory approach.[8] The calculated primary ^{13}C KIEs are 1.040 for the S_N2 model reaction and 1.001 for the S_N1 model reaction, which are within experimental error of the measured values, suggesting that the theoretical model should be useful for related reactions including those involving isoprenoid diphosphates.

Protein farnesyltransferase (PFTase) catalyzes the transfer of a prenyl group from farnesyl diphosphate (FPP, **9**) to proteins including Ras that terminate in CVIA and related sequences. Since the enzyme recognizes only the four C-terminal residues, tetrapeptides are efficient substrates for the enzyme with kinetic constants similar to full-length protein substrates.[9] To measure V_{max}/K_M (V/K) KIEs under steady state conditions, the substrate, FPP, must manifest a low commitment factor (c_f).[10] Isotope trapping experiments, using FPP as a substrate, to measure the c_f suggest significant commitment to catalysis.[11] In contrast, similar experiments using the related, weaker binding isoprenoid (Figure 2), geranyl diphosphate (GPP, **10**), gave a low value of c_f ($c_f = 0.057$, data not shown), indicating that KIEs measured using GPP would not be masked by excessive commitment. On the basis of the work described above with the model substitution reactions, it was noted that the primary ^{13}C KIE was a particularly sensitive reporter of the extent of bonding between the electrophilic carbon center and incoming nucleophile. Thus it was decided to measure the primary ^{13}C KIE using [1-^{13}C]-GPP as a substrate. The labeled substrate was prepared in four steps using commercially available [1-^{13}C]-triethylphosphonoacetate. For the KIE measurement, a mixture of [1-^{13}C]-GPP and unlabeled GPP was incubated with the peptide *N*-dansyl-GCVIA (**11**) in the presence of PFTase. Reactions were performed either with limiting peptide or limiting GPP. After enzymatic incorporation, the farnesylated peptide product, **12**, was purified by reversed-phase HPLC and analyzed by ESI-MS (ion trap instrument) operating in the zoom scan mode. The mass spectra of the samples were
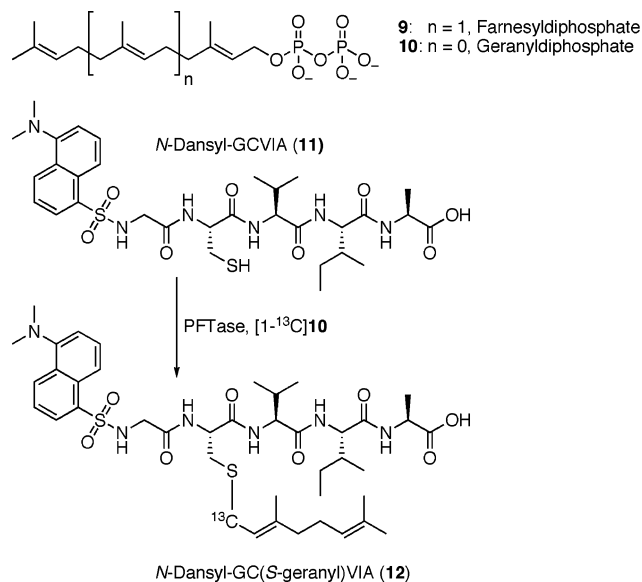
9: n = 1, Farnesyldiphosphate
10: n = 0, Geranyldiphosphate

N-Dansyl-GCVIA (**11**)

PFTase, [1-^{13}C]**10**

N-Dansyl-GC(S-geranyl)VIA (**12**)

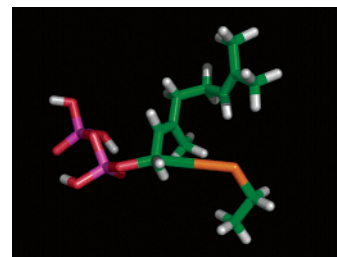**Figure 2.** Isoprenoid substrates and prenylation reaction catalyzed by PFTase studied here.

Figure 3. Proposed TS structure for PFTase-catalyzed reaction based on calculations using GPP and ethane thiolate. The second isoprene unit is omitted for clarity. Colors: S (orange), C (green), and O (red).

deconvoluted into ratios of labeled and unlabeled species using spectra obtained from pure labeled and unlabeled products; the V/K KIE using [1-^{13}C]-GPP as a substrate calculated from that data was found to be 1.039 ± 0.003. A similar approach was used to measure the α-secondary ^{2}H KIE, using [1-^{2}H$_2$]-GPP (prepared from [1-^{2}H$_2$]-geraniol)[12] as a substrate, which was found to be 1.068 ± 0.003. To determine the structural significance of these KIE values, a TS structure for the nonenzymatic reaction between ethane thiolate and GPP via an associative mechanism was computationally determined; a density functional level of electronic structure theory using the mPW1N functional in combination with the 6-31+G(d) basis set was employed for those calculations. An associative model was chosen for the starting point for these calculations since a significant primary ^{13}C KIE was observed only in the S$_N$2 model reaction described above. For the nonenzymatic reaction between GPP and ethane thiolate, the primary ^{13}C KIE was calculated to be 1.067. Interestingly, this value is significantly higher than the experimentally measured value of 1.039 determined for the enzymatic reaction, indicating that the TS structure for the protein-catalyzed process must be somewhat different. Iterative cycles of altering the C–O (leaving group) and C–S (nucleophile) bond lengths and calculation of the resulting primary ^{13}C KIE were used to generate a plot of the variation of the ^{13}C KIE with C–O and C–S bond lengths; a similar plot was created for the α-secondary ^{2}H KIE. Using the two KIEs reported here enabled us to computationally identify a set of C–O (1.69 Å) and C–S (3.70 Å) TS bond lengths that are consistent with both measurements; a proposed structure for the TS is shown in Figure 3. Interestingly, the C–O bond lengths in the model S$_N$2 TS and enzymatic TS are essentially the same. However, the C–S bond lengths are substantially different with the enzymatic TS (3.70 Å), manifesting less bonding to C-1 of GPP than is present in the model system (2.80 Å). This suggests lower electron density at C-1 than is typified by S$_N$2 reactions and provides a rationale for why substrates containing electron-withdrawing substituents decrease the enzymatic reaction rate.[13] It should be noted that the KIEs reported here for yeast PFTase are similar, but not identical, to KIEs recently reported by Pais et al. for a mammalian PFTase.[14] These variations may reflect differences in the TS structures between the yeast and mammalian enzymes that could be exploited for drug design.

In summary, we present here a TS structure for the reaction catalyzed by PFTase that complements X-ray crystallographic studies[15] and provides a clear structural framework for understanding the results of previous mechanistic investigations of this enzyme, including stereochemical[16,17] and kinetic analyses.[14,18] The experiments reported here provide powerful insights into an important class of biological reactions through a combination of model chemistry, computation, and kinetic analysis. Finally, it should be noted that the KIE analysis described here was accomplished through experiments performed with stable isotopes and did not require radiolabeled compounds. It is likely that the role of ESI-MS and NMR in KIE analyses of biological processes will continue to grow in the future.

Supporting Information Available: Details for the synthesis of [1-^{13}C]-GPP, c_f determination, KIE experiments, TS calculations, and data analysis are included. This material is available free of charge via the Internet at http://pubs.acs.org.

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JA069119J