Measurement of the α-Secondary Kinetic Isotope Effect for a Prenyltransferase by MALDI Mass Spectrometry

Valerie A. Weller and Mark D. Distefano*

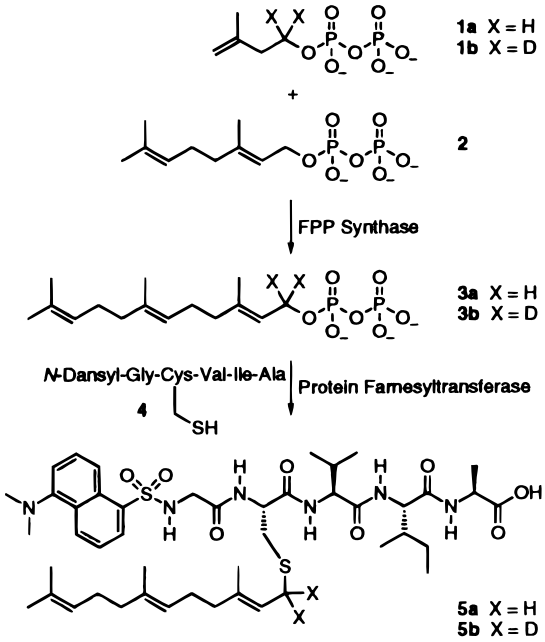
Department of Chemistry, University of Minnesota Minneapolis, Minnesota 55455

Received February 2, 1998

Prenyltransferases catalyze alkylation reactions between prenyl diphosphates and a variety of nucleophiles including nucleic acids, cell wall components, proteins, and smaller cofactors.[1] Recently, considerable interest in understanding protein prenyltransferases has developed due to the discovery that many important proteins are prenylated within the cell; these modifications include farnesylation (C_{15}) and geranylgeranylation (C_{20}).[2] The finding that the Ras protein is farnesylated and that inhibition of this process in a variety of mutant Ras-induced cancer models arrests the growth of tumor cells has resulted in intense efforts to develop inhibitors that prevent farnesylation.[3]

Protein farnesyltransferase (PFTase) catalyzes the nucleophilic substitution reaction between farnesyl diphosphate (FPP) and a protein-derived cysteine residue, and is hence the target for inhibitors of Ras farnesylation.[4] Since information concerning the chemical mechanism of an enzyme-catalyzed reaction can be useful for the development of selective inhibitors, there is considerable interest in defining the mechanism of PFTase. Mu et al. have demonstrated that PFTase catalyzes farnesylation with inversion of configuration at the C-1 isoprenoid center.[5] While these data suggest an associative mechanism for the alkylation reaction, they cannot be used to rule out a dissociative mechanism involving a tight ion pair between a putative allylic carbocation and the departing diphosphate leaving group. An ion pair within the active site of the enzyme may prevent racemization and control the possible trajectory of approach for the incoming nucleophile. Elegant experiments performed by Poulter and colleagues using farnesyl diphosphate analogues containing fluorine substitutions at C-13 indicate that the introduction of electron withdrawing groups decreases the rate of reaction (6.7-fold for CH_2F, 770-fold for CF_3).[6] These data suggest that farnesylation proceeds with some ionic character. However, the magnitude of the substituent effects are significantly smaller than those observed for FPP synthase (10^3-fold for CH_2F, 10^8-fold for CF_3).[7] Efforts to prepare mechanism-based inactivators containing vinyl or cyclopropyl groups that act to trap cationic intermediates have not been successful.[8] Moreover, work by Casey and co-workers indicates that the nucleophile in the PFTase-catalyzed reaction is likely to be a protein-derived thiolate.[9] This suggests that the change in nucleophilicity from an alkene (FPP synthase) to a thiolate (PFTase) could result in a change in transition-state structure from one involving only C−O bond cleavage to one involving both C−O bond cleavage and C−S bond formation. To address this mechanistic issue, we sought to investigate the effect of deuterium substitution at C-1 of FPP on the rate of PFTase-catalyzed farnesylation. Secondary kinetic isotope effect (KIE) measurements such as these have been used extensively to analyze the mechanisms of nucleophilic substitution reactions.[10] In general, reactions that proceed via dissociative pathways manifest normal α-secondary KIEs with k_H/k_D values near 1.2, while associative mechanisms yield α-secondary KIE values close to unity or slightly inverse.[11]

To perform these experiments, a synthesis of [1-$^2H_2$]FPP was required. Since we planned to prepare a number of isotopically substituted forms of FPP, we elected to employ a chemoenzymatic route.[12] Dideuterated isopentenyl diphosphate ([1-$^2H_2$]IPP, **1b**) was first prepared in six steps from methyl allyl bromide;[13] the deuterium atoms were introduced by $LiAl^2H_4$ reduction of methyl isopentenoate to [1-$^2H_2$]isopentenyl alcohol. The desired diphosphate was obtained by tosylation of the alcohol followed by pyrophosphorylation.[14] [1-$^2H_2$]IPP (**1b**) was then combined with geranyl diphosphate (**2**) and FPP synthase to produce [1-$^2H_2$]FPP (**3b**), which was subsequently purified by reversed-phase HPLC.[15] Unlabeled FPP was synthesized and purified in a similar manner.



Kinetic isotope effect experiments were performed by incubating a mixture of [1-H_2]FPP (**3a**) and [1-$^2H_2$]FPP (**3b**) with *N*-dansyl-GCVIA (**4**) and yeast PFTase at 30 °C for 240 min followed by fractionation on a Sep-Pak C_{18} reversed-phase

(1) Goldstein, J.; Brown, M. S. *Nature* **1990**, *343*, 425−430.
(2) Zhang, F. L.; Casey, P. J. *Annu. Rev. Biochem.* **1996**, *65*, 241−269.
(3) (a) Omer, C. A.; Kohl, N. E. *Trends Pharm. Sci.* **1997**, *18*, 437−444. (b) Buss, J. E.; Marsters, J. C. *Chem. Biol.* **1995**, *2*, 787−791.
(4) For a review, see: Leonard, D. M. *J. Med. Chem.* **1997**, *40*, 2971−2990.
(5) (a) Gibbs, R. A.; Mu, Y.-Q.; Wang, F. *Bioorg. Med. Chem. Lett.* **1993**, *3*, 281−284. (b) Mu, Y. Q.; Omer, C. A.; Gibbs, R. A. *J. Am. Chem. Soc.* **1996**, *118*, 117−123.
(6) Dolence, J. M.; Poulter, C. D. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 5008−5011.
(7) Poulter, C. D.; Wiggins, P. L.; Le, A. T. *J. Am. Chem. Soc.* **1981**, *103*, 3926−3927.
(8) (a) Gibbs, R. A.; Krishnan, U.; Dolence, J. M.; Poulter, C. D. *J. Org. Chem.* **1995**, *60*, 7821−7829. (b) Mu, Y.-Q.; Gibbs, R. A.; Eubanks, L. M.; Poulter, C. D. *J. Org. Chem.* **1996**, *61*, 8010−8015.
(9) Huang, C.-C.; Casey, P. J.; Fierke, C. A. *J. Biol. Chem.* **1997**, *272*, 20−23.
(10) (a) Anderson, V. E. *Cur. Opin. Struct. Biol.* **1992**, *2*, 757−764. (b) Kirsch, J. F. *Secondary Kinetic Isotope Effects*; Kirsch, J. F., Ed.; University Park Press: Baltimore, MD, 1977; pp 100−121. (c) Simon, H.; Palm, D. *Angew. Chem., Int. Ed. Engl.* **1966**, *5*, 920−933.
(11) For studies of solvolysis reactions, see: (a) Harris, J. M. *Prog. Phys. Org. Chem.* **1974**, *11*, 89−173. For a discussion of S_N2 reactions, see: (b) Seltzer, S.; Zavitsas, A. A. *Can. J. Chem.* **1967**, *45*, 2023−2030. (c) Lee, C. C.; Wong, E. W. C. *Tetrahedron* **1965**, *21*, 539−545. (d) Leffek, K. T.; Llewellyn, J. A.; Robertson, R. E. *Can. J. Chem.* **1960**, *38*, 1505−1510. (e) Shiner, V. J. J.; Buddenbaum, W. E.; Murr, B. L.; Lamaty, G. *J. Am. Chem. Soc.* **1968**, *90*, 418−426.
(12) Christensen, D. J.; Poulter, C. D. *Bioorg. Med. Chem.* **1994**, *2*, 631−637.
(13) Wagner, R. B. *J. Am. Chem. Soc.* **1949**, *71*, 3214−3218.
(14) Davisson, V. J.; Woodside, A. B.; Poulter, C. D. *Methods Enzymol.* **1985**, *110*, 130−144.
(15) Zhang, D.; Poulter, C. D. *Anal. Biochem.* **1993**, *213*, 356−361.

S0002-7863(98)00353-9 CCC: $15.00
Published on Web 07/23/1998

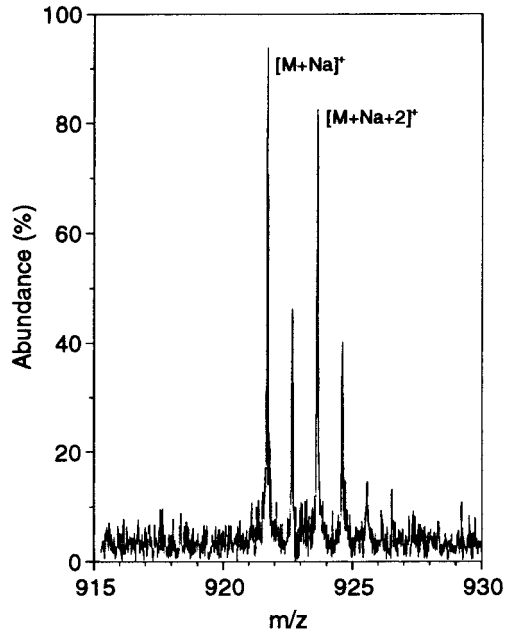


Figure 1. MALDI−ICR mass spectrum of a mixture of **5a** and **5b** obtained from a reaction between a mixture of **3a**, **3b**, and **4** catalyzed by yeast PFTase. The major peaks contributed from **5a** ($[M + Na]^+$) and **5b** ($[M + Na + 2]^+$) are indicated.

cartridge to desalt and concentrate the products (*N*-dansyl-GC-(S-farnesyl)VIA, **5a** and **5b**). Reactions were performed under two types of conditions to determine the KIE. The first condition consisted of an excess of the labeled FPP mixture (**3a** and **3b**, 2.5 μM) relative to *N*-dansyl-GCVIA (**4**, 0.5 μM), while the second contained the same FPP concentration with the peptide in excess (3.0 μM). This experimental design allowed the isotopic composition of the products to be examined at low levels of FPP conversion from reactions performed under the first type of condition, while the isotopic composition analysis of the products at complete FPP conversion allowed for determination of the actual isotopic composition present in the FPP starting material. The isotopic composition of each sample was determined by mass spectrometric analysis using matrix-assisted laser desorption ionization (MALDI) in conjunction with ion cyclotron resonance (ICR) detection.[16] The ratio of **5a**/**5b** in each reaction was calculated by deconvolution of the intensity pattern from the isotopic distribution observed for the $[M + Na]^+$ product ion peaks ($[M + Na]^+$, $[M + Na + 1]^+$, $[M + Na + 2]^+$, $[M + Na + 3]^+$, and $[M + Na + 4]^+$).[17] A representative mass spectrum containing a mixture of **5a** and **5b** is shown in Figure 1. The ratio of the products **5a**/**5b** obtained at partial conversion divided by the product ratio obtained at total conversion gives the isotope effect on $^DV/K_{[FPP]}$.[18]

(16) (a) Castro, J. A.; Koster, C.; Wilkins, C. *Rap. Commun. Mass Spec.* **1992**, *6*, 239−241. For a review, see: (b) Dienes, T.; Pastor, S. J.; Schurch, S.; Scott, J. R.; Yao, J.; Cui, S.; Wilkins, C. L. *Mass Spec. Rev.* **1996**, *15*, 163−211.

(17) Isotope ratios were calculated from peak intensity data. Deconvolution of percent abundance data was completed using a program developed by Dr. E. Larka (Department of Chemistry, University of Minnesota). A standard curve was constructed from a set of samples similar to the total conversion samples discussed in the text, where the ratio of **3a** to **3b** was varied from 1.0:1.1 to 1.0:1.7. The data plotted as premixed ratio versus calculated ratio fit a linear equation with a correlation coefficient of 0.992.

Table 1. Values for $^DV/K_{[FPP]}$

experiment	KIE	no. of samples
1	0.965 ± 0.086	3
2	0.968 ± 0.040	4
3	1.041 ± 0.054	4
av	0.991 ± 0.033	

Table 1 gives the values for the KIE obtained in three sets of experiments; the average value for the α-secondary KIE in the yeast PFTase catalyzed reaction is 0.991 ± 0.033. By itself, this low value suggests an associative mechanism for PFTase. However, some care is necessary in interpreting these results. First, given the ordered sequential kinetic mechanism followed by PFTase,[19] the relationship between $^DV/K_{[FPP]}$ and the intrinsic KIE (k_H/k_D) is $^DV/K_{[FPP]} = (k_H/k_D + c_f)/(1 + c_f)$, where c_f is the "forward commitment factor."[20] Thus, the value for the intrinsic KIE may be masked if c_f is large. A recent presteady-state analysis of the yeast PFTase reaction[21] allows the calculation of a value for c_f and permits an estimation of 0.977 for the intrinsic KIE to be made.[22] Such a magnitude for the intrinsic α-secondary KIE is more consistent with an associative mechanism than with a cationic dissociative pathway. However, this value is still greater than some of the inverse effects observed in reactions that proceed via S_N2 mechanisms.[23] Thus, yeast PFTase may catalyze farnesylation with partial, but not complete, S_N2 character; we favor this interpretation since it provides a rationale that is consistent with both the KIE results described here as well as the substituent effects noted above. A complete understanding of the transition-state structure in this reaction will require additional KIE experiments; however, the present study provides compelling evidence for associative character in this reaction. Finally, it should be noted that the use of MALDI mass spectrometry to determine isotopic ratios for use in KIE experiments reported here may greatly facilitate kinetic analysis in other macromolecular systems.

Acknowledgment. The authors thank Dr. C. D. Poulter for the generous gifts of the FPP synthase expression plasmid pMJY18911 and the PFTase expression system *Escherichia coli*. DH5α/pGP114, Dr. J. Dolence for advice in the purification of FPP synthase, Dr. I. Gaon for preparing *N*-dansyl-GCVIA, and Dr. E. Larka for obtaining the mass spectral data. This work was supported by grants from the American Cancer Society (BE-222 and JFRA-584).

Supporting Information Available: Characterization data for the compounds used in this study and descriptions of the procedures used to perform the KIE experiments (9 pages, print/PDF). See any current masthead page for ordering information and Web access instructions.

JA980353M

(18) Values were first corrected for extent of conversion as described by: (a) Bigeleisen, J.; Wolfsberg, M. *Adv. Chem. Phy.* **1958**, 15−53. For definitions and a discussion of $^DV/K$, see: (b) Cleland, W. W. *CRC* **1982**, *13*, 394.

(19) Dolence, J. M.; Cassidy, P. B.; Mathis, J. R.; Poulter, C. D. *Biochemistry* **1995**, *34*, 16687−16694.

(20) (a) Cook, P. F.; Cleland, W. W. *Biochemistry* **1981**, *20*, 1790−1796. (b) Northrop, D. B. *Biochemistry* **1975**, *14*, 2644−2651.

(21) Mathis, J. R.; Poulter, C. D. *Biochemistry* **1997**, *36*, 6367−6376.

(22) For $^DV/K_{[FPP]}$, $c_f = (k_3/k_{-1}k_{-2})(k_{-1} + k_2[\mathbf{4}]) = 1.50$; see ref 18 for definitions and values of the specific rate constants. This value for c_f was calculated using $[\mathbf{4}] = 0.5$ μM.

(23) For an example of an inverse effect in an enzyme-catalyzed reaction, see: (a) Hegazi, M. F.; Borchardt, R. T.; Schowen, R. L. *J. Am. Chem. Soc.* **1978**, *101*, 4359−4365. For an example of a normal effect, see: (b) Dahlquist, F. W.; Rand-Meir, T.; Raftery, M. A. *Biochemistry* **1969**, *8*, 4214−4221.