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Mechanism of Dimethylallyltryptophan Synthase: Evidence for a Dimethylallyl Cation Intermediate in an Aromatic Prenyltransferase Reaction

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Dimethylallyltryptophan (DMAT) synthase is a prenyltransferase that catalyzes an electrophilic aromatic substitution reaction (Figure 1).[1] The reaction involves the transfer of the dimethylallyl moiety from dimethylallyl diphosphate (DMAPP) to the C-4 position of L-tryptophan. DMAT synthase is found in a variety of fungi, where it catalyzes the first committed step in the biosynthesis of the ergot alkaloids (e.g., ergotamine, lysergic acid).[2]

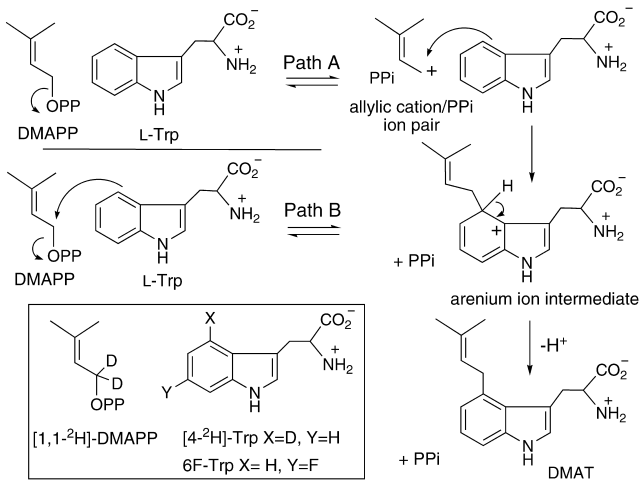

Figure 1. Proposed mechanisms for the reaction catalyzed by dimethylallyltryptophan synthase.

The prenyltransferases are a large and diverse family of enzymes that catalyze the transfer of prenyl groups to a variety of acceptors.[3] The isoprenyl diphosphate synthases (such as farnesyl diphosphate synthase) and the terpenoid cyclases catalyze the transfer of prenyl groups to non-nucleophilic alkene carbons. These enzymes are thought to employ dissociative mechanisms involving discrete allylic carbocation intermediates.[4] The protein prenyltransferases (such as protein farnesyltransferase) catalyze the transfer of prenyl groups to nucleophilic cysteines on protein substrates. These enzymes are thought to employ associative mechanisms involving a direct displacement of pyrophosphate by a thiolate.[5] A further group of prenyltransferases, including the enzymes DMAT synthase, NphB, NovQ, and CloQ, catalyze electrophilic aromatic substitution reactions involving electron-rich aromatics.[6] Since indoles and phenolic rings are much more nucleophilic than alkenes,[7] the possibility of either reaction mechanism exists for these systems.

Potential mechanisms for the DMAT synthase reaction are shown in Figure 1. The dissociative (S_N1) pathway involves an initial ionization of DMAPP to form a dimethylallyl cation/pyrophosphate ion pair (path A). The C-4 position of tryptophan then adds to the carbocation to generate an arenium ion intermediate, and a final deprotonation at C-4 causes re-aromatization. The associative (S_N2) pathway involves a nucleophilic attack of the aromatic ring onto DMAPP with a concerted displacement of pyrophosphate to directly form the arenium ion intermediate (path B). Previous studies have shown that the reaction proceeds with an inversion of stereochemistry at the allylic position of DMAPP; however, this observation does not rule out either mechanism in an enzyme-controlled process.[1c] Studies that employed substrate analogues bearing electron-donating/withdrawing groups have been forwarded as evidence in support of an electrophilic mechanism.[1b] The results showed that electron-withdrawing groups on either substrate slowed the reaction considerably, as expected for either a dissociative mechanism involving a carbocation intermediate or an associative mechanism involving an "exploded" transition state with considerable carbocation character.[8]

In this Communication we present studies that strongly support the dissociative mechanism for the DMAT synthase reaction. The observation of positional isotope exchange (PIX) in isotopically labeled DMAPP provides evidence for the existence of a dimethylallyl carbocation intermediate.

The *Aspergillus fumigatus* DMAT synthase was overexpressed in *Escherichia coli* as a C-terminal hexahistidine-tagged enzyme. The activity was found to be comparable to that reported in the literature ($k_{cat} = 0.27 \pm 0.05\ s^{-1}$, $K_{M,DMAPP} = 11 \pm 4\ \mu M$, $K_{M,L\text{-}Trp} = 10 \pm 4\ \mu M$).[2b] Likewise, the enzyme did not require exogenous divalent metal ions for activity and showed only a 2.0-fold reduction in rate when assayed in the presence of 3 mM EDTA.[2b]

In order to test for the formation of a dimethylallyl cation during catalysis, a PIX experiment was performed.[9] A sample of labeled [1-^{18}O]-DMAPP bearing a 63% enrichment of ^{18}O-isotope (Figure 2A) was incubated with DMAT synthase and 0.8 equiv of L-tryptophan in the presence of 3 mM EDTA, and the reaction was allowed to proceed to near completion. The remaining ^{18}O-labeled DMAPP was analyzed for the presence of isotopic scrambling using ^{31}P NMR spectroscopy. It is well established that a ^{31}P signal will be shifted slightly upfield by an attached ^{18}O-isotope, and that the magnitude of this shift is dependent on the P−O bond order.[10] A ^{31}P NMR spectrum taken before the reaction shows two doublets at −9.28 and −9.30 ppm, corresponding to the α-phosphorus of the unlabeled and labeled starting material, respectively (Figure 2B, before rxn.). A spectrum of the DMAPP remaining after the enzymatic reaction shows the presence of a new signal shifted further upfield, at −9.31 ppm, that accounts for 15% of the labeled material (Figure 2B, L-Trp rxn.).[11] This peak is attributed to DMAPP in which the isotopic label has scrambled from a bridging position (bond order of 1) to a nonbridging position (bond order of 1.5, Figure 2A). Control experiments showed that no scrambling occurs in the absence of tryptophan. This result demonstrates that the bond between the pyrophosphate and the dimethylallyl moieties is reversibly broken during catalysis.

Two scenarios could account for the observed PIX process. The reaction could follow a dissociative mechanism (Figure 1, path A), in which a dimethylallyl cation/pyrophosphate ion pair is generated

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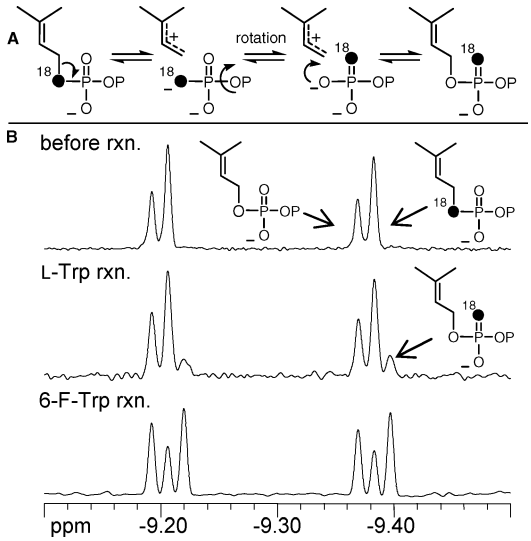

Figure 2. ^{31}P NMR analysis of [1-^{18}O]-dimethylallyl diphosphate in the reaction catalyzed by dimethylallyltryptophan synthase.

and the lifetime of these intermediates is long enough for rotation of the pyrophosphate to occur (Figure 2A). If collapse of the ion pair is competitive with product formation, isotopic scrambling would be observed. The reaction could also follow an associative mechanism with a reversible first step (Figure 1, path B). This could occur if a subsequent step, such as deprotonation of the arenium ion intermediate or product release, were rate-determining. In order to address these possibilities, the nature of the rate-determining step(s) was investigated using kinetic isotope effect (KIE) measurements. When [1,1-^{2}H]-DMAPP (Figure 1, inset) was used as a substrate, a secondary KIE on k_{cat}/K_M of $k_H/k_D = 1.16 \pm 0.03$ was observed. This indicates that cleavage of the C−O bond of DMAPP is a partially rate-determining step of catalysis and that the transition state has considerable carbocation character.[12] When [4-^{2}H]-tryptophan (Figure 1, inset) was used as a substrate, an inverse secondary KIE on k_{cat}/K_M of $k_H/k_D = 0.81 \pm 0.03$ was observed. This indicates that C−C bond formation is also partially rate-determining and involves a transition state in which the hybridization at the C-4 of tryptophan changes from sp^2 to sp^3.[13] The absence of a primary KIE with this substrate indicates that the deprotonation of the arenium ion intermediate is not rate-determining.[14] Since the chemical steps involving C−O bond cleavage and C−C bond formation are rate-determining, we can rule out an associative mechanism with reversible formation of the arenium ion intermediate. Instead, the PIX can best be explained by a reversible formation of a dimethylallyl cation/pyrophosphate ion pair (Figure 2A).

To further test this theory, an unreactive substrate analogue, 6-fluorotryptophan (Figure 1, inset), was used in the PIX experiment. Previous studies showed that 6-methyltryptophan serves as a substrate, indicating that the low reactivity of 6-fluorotryptophan is due to the electron-withdrawing property of fluorine.[1a] When 6-fluorotryptophan and ^{18}O-labeled DMAPP were incubated with DMAT synthase, no products were formed; however, a ^{31}P NMR analysis (Figure 2B, 6F-Trp rxn.) clearly shows that complete PIX had occurred (the ratio of upfield peaks is 2:1). This observation is inconsistent with an associative mechanism, since the fluorine substituent should raise the barrier to arenium ion formation and render the step irreversible. Instead, it strongly supports a dissociative mechanism in which a dimethylallyl cation/pyrophosphate ion pair is formed and collapses at a rate that is much faster than attack by the electron-deficient fluorotryptophan. No scrambling of the isotopic label from the α- to the β-phosphorus of DMAPP could be detected, indicating that pyrophosphate could not tumble within the active site and was not released/re-bound during the lifetime of the carbocation intermediate (Figure S5, Supporting Information).

To our knowledge, this is the first observation of PIX catalyzed by a prenyltransferase. While many of these enzymes are thought to generate cationic intermediates, most are metal-dependent, and it is likely that the metal chelates the phosphate groups of the pyrophosphate intermediate and prevents bond rotations that could lead to PIX.[15] Since DMAT synthase does not require a divalent cation for catalysis, it is possible to observe the scrambling phenomenon. Perhaps the closest precedence to this study can be found in the enzyme farnesyl diphosphate isomerase, where PIX was used to establish the existence of a carbocation intermediate.[16]

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Supporting Information Available: Full experimental details outlining PIX experiments and kinetic studies. This material is available free of charge via the Internet at http://pubs.acs.org.

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