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A Cope Rearrangement in the Reaction Catalyzed by Dimethylallyltryptophan Synthase?

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Supporting Information

ABSTRACT: The enzyme dimethylallyltryptophan synthase catalyzes the "normal" prenylation of Trp at C-4 in the first step of ergot alkaloid biosynthesis. The Lys174Ala mutant is found to produce a hexahydropyrroloindole alkaloid that is "reverse-prenylated" at C-3 as its major product. This is interpreted as evidence in support of a mechanism that involves an initial "reverse-prenylation" at C-3, followed by a Cope rearrangement and rearomatization.

Prenylated indole alkaloids are a large family of natural products derived primarily from tryptophan, other amino acids, and dimethylallyl diphosphate (DMAPP).[1,2] These alkaloids display a wide variety of biological activities and include such compounds as the ergot alkaloids, the brevianamides, echinulin, and roquefortine. Prenylation occurs via an electrophilic aromatic substitution reaction in which a dimethylallyl carbocation intermediate is added onto the indole ring. The prenyltransferases that catalyze this process direct the substitution to a variety of different positions on the indole core, and may involve either "normal" prenylation (attack at the primary center of the dimethylallyl carbocation) or "reverse" prenylation (attack at the tertiary center) depending on the alkaloid being generated. Recently, the genes encoding for many of the prenyltransferases have been identified, and it is now possible to study the enzymology of these systems.[3]

One such enzyme is dimethylallyltryptophan synthase (DMAT synthase or FgaPT2) from the fungus *Aspergillus fumigatus*.[4−6] This enzyme catalyzes a "normal" prenylation at the C-4 position of tryptophan in the first step of the biosynthesis of the ergot alkaloids (Figure 1). It belongs to a recently discovered family of fungal indole prenyltransferases that do not require metal cations for activity.[1,7] A mechanism that has been proposed for this reaction involves the initial dissociation of DMAPP into a dimethylallyl cation/pyrophosphate ion pair.[8−11] An attack from the C-4 position of the indole ring onto the primary carbon of the allylic carbocation gives an arenium intermediate. A subsequent deprotonation at C-4 causes rearomatization and DMAT formation. Implicit in this mechanism is the notion that the enzyme directs alkylation to the poorly nucleophilic C-4 position of the indole instead of the more highly nucleophilic C-2 and C-3 positions.[12,13] Previous mechanistic studies on this enzyme have shown that the reaction proceeds with an inversion of configuration at the electrophilic carbon of DMAPP and that the reaction is highly electrophilic in nature (the rate is dramatically slowed by fluorination of either substrate).[10,11] More recently, a positional isotope exchange (PIX) experiment showed that an ^{18}O-isotopic label in the bridging position of [1-^{18}O]-DMAPP partially scrambles into the nonbridging position of recovered starting material during catalysis (see darkened atoms in Figure 1).[9] This indicates that the cleavage of the C−O bond in DMAPP is reversible. When combined with the observation of a normal secondary kinetic isotope effect (KIE) on [1,1-^{2}H]-DMAPP and an inverse secondary KIE on [4-^{2}H]-tryptophan, this was taken as evidence for the reversible formation of an allylic carbocation/pyrophosphate ion pair. Further evidence was obtained when the unreactive substrate 6-fluorotryptophan was employed in the PIX experiment and complete scrambling of the isotopic label was observed. The structure of DMAT synthase in a complex with Trp and the unreactive substrate analogue, dimethylallyl *S*-thiolodiphosphate, has also been recently reported.[8] This provides an excellent model of the Michaelis complex and implicated two active site residues as playing roles in catalysis. Glu89 was found to form an H-bond with the indole amine, and this interaction is presumably important for promoting attack of the indole onto the carbocation either via deprotonation or by stabilization of the resulting arenium intermediate. Lys174 is suitably positioned to act as the base that deprotonates the arenium in the final rearomatization step.

At the onset of this study, we aimed to probe the roles of the active site residues in greater detail using site directed mutagenesis and PIX experiments. In the course of our work, we found that a mutant enzyme produced an alternate tricyclic alkaloid as the major reaction product. This observation, combined with an analysis of the reported structure of the Michaelis-like complex, leads us to consider an alternate reaction mechanism for the DMAT synthase reaction that involves a Cope rearrangement as a key step in catalysis (Figure 2).

Site-directed mutagenesis was used to generate E89Q, E89A, K174Q, and K174A, and the kinetic constants of these mutants were determined using an assay for pyrophosphate release (Table 1). The mutation of Glu89 to either Gln or Ala caused a 31-fold or >400-fold reduction in k_{cat}, respectively, consistent with the notion that this residue plays a key role in increasing the nucleophilicity of the indole ring. The mutation of Lys174 to Gln resulted in only a 17% reduction in k_{cat} (consistent with a previous report),[8] whereas conversion of this residue to Ala resulted in a 20-fold reduction of k_{cat}. We interpreted this as being consistent with its role as a general base that deprotonates the arenium intermediate; a Gln residue at this position may still be able to fulfill this role since the proton is quite acidic; however, an Ala residue lacks any potential to act as a base and the rate is more considerably reduced. We also probed the effect of the mutations

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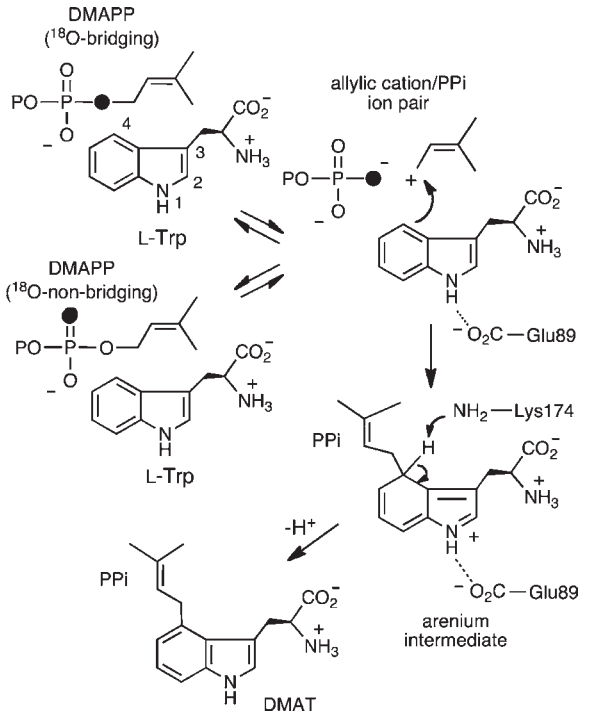

Figure 1. A proposed mechanism for the reaction catalyzed by dimethylallyltryptophan (DMAT) synthase and depiction of the PIX experiment. Darkened atoms represent ^{18}O-isotopic labels.

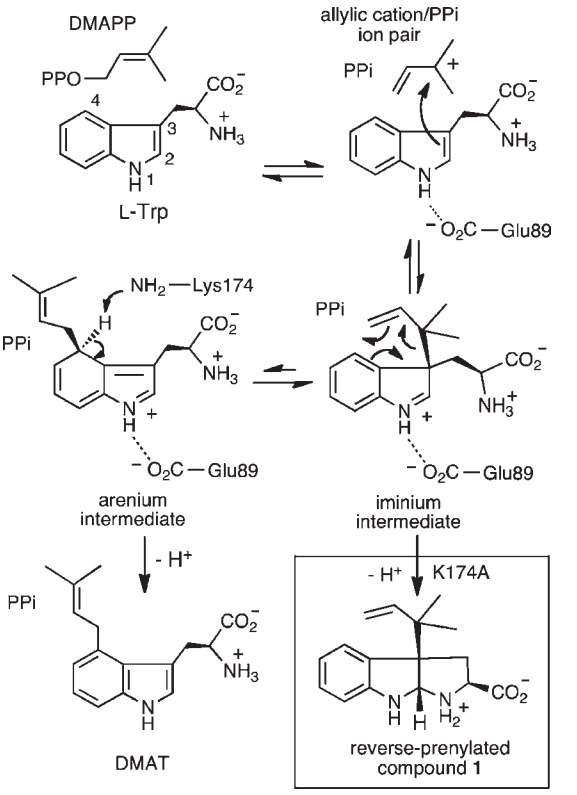

Figure 2. A Cope mechanism for the reaction catalyzed by DMAT synthase and the structure of compound **1** formed by the K174A mutant.

on the extent of isotopic scrambling (PIX) that is observed in the recovered pool of [1-^{18}O]-DMAPP starting material. Both the extent of scrambling and the extent of reaction can be monitored simultaneously using ^{31}P NMR spectroscopy and the ratio v_{PIX}/v_{rxn} can be determined in this fashion. With the wild-type enzyme, the value of v_{PIX}/v_{rxn} was found to be 0.39, indicating that the probability of the carbocation intermediate partitioning forward toward products or backward to reactants is roughly equivalent (Table 1). With each of the mutants, however, the value of v_{PIX}/v_{rxn} was increased and the magnitude of the increase roughly correlated with the decrease in k_{cat}. This is consistent with the notion that the mutations have the largest effect on the barriers of steps that occur subsequent to ion pair formation, so that the commitment to catalysis is decreased and a greater degree of scrambling occurs. In the case of the essentially inactive mutant E89A, it was possible to monitor PIX during extended incubations with large amounts of enzyme, and it was also interesting to note that a significant amount of recovered starting material (9% after 8% consumption) showed DMAPP bearing ^{18}O isotope at the terminal β-position (see Supporting Information). This indicates that a movement of the pyrophosphate moiety relative to that of the dimethylallyl cation occasionally allows the phosphate groups to switch positions.

We also considered the possibility that the fate of the dimethylallyl carbocation could be altered by mutation; for example, a reaction with water could form either a tertiary or primary allylic alcohol.[14] We therefore analyzed the products of the mutant reactions using ^{1}H NMR spectroscopy. With E89Q, E89A, and K174Q, the only product observed was DMAT. With K174A, however, we were surprised to find that only 10% of the product was DMAT and 90% of the product was an unexpected compound. The presence of three vinylic protons and two upfield methyl signals clearly indicated that this compound was "reverse"

Table 1. Properties of Wild-Type and Mutant Enzymes

enzyme	k_{cat} (s^{-1})	$K_{M\text{-}L\text{-}Trp}$ (μM)	v_{PIX}/v_{rxn}	products
WT	0.47 ± 0.02	6.7 ± 0.8	0.39	DMAT
E89Q	0.015 ± 0.001	6.8 ± 0.5	1.5	DMAT
E89A	<0.001	–	3.9	DMAT
K174Q	0.39 ± 0.01	8.6 ± 0.5	0.74	DMAT
K174A	0.023 ± 0.001	8.6 ± 1.0	4.3	1:9 DMAT/**1**

prenylated and we began to suspect that it bore the hexahydropyrroloindole structure of compound **1** (Figure 2).[15] A capillary electrophoresis separation of the reaction mixture coupled with UV detection allowed us to collect UV spectra of both the DMAT and the compound **1** produced by K174A (see Supporting Information). The two maxima at 237 and 292 nm of compound **1** were very similar to those observed with the hexahydropyrroloindole alkaloids such as chimonanthine (λ_{max} = 240 and 294 nm, acidic conditions) and clearly showed that the indole chromophore (λ_{max} = 278 nm) had been disrupted.[16,17] In past studies, Danishefsky and co-workers had prepared the methyl ester of compound **1** during their synthesis of amauromine, and this provided us with a way to obtain a sample of this material.[18] We repeated their synthesis and saponified the ester under carefully controlled basic conditions. The spectra of the enzymatically produced and synthetic compounds were very similar, yet small changes in certain chemical shifts and coupling constants were present, presumably due to minor differences in the pH of the solution. Ultimately, a spectrum of a 1:1 mixture of the compounds unambiguously demonstrated that the structure

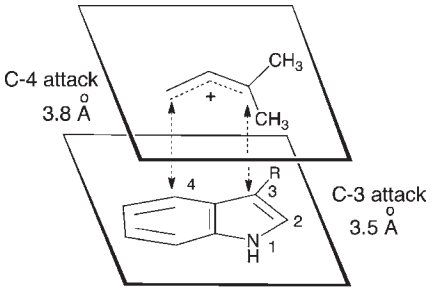

Figure 3. The relative positioning of the indole ring and the dimethylallyl cation in the active site if ionization occurs without significant motion. Distances taken from PDB 3I4X.[8].

and stereochemistry of compound **1** were as depicted in Figure 2 (see Supporting Information).

One possible explanation for the formation of compound **1** is that DMAT synthase normally employs a "Cope mechanism" that was originally suggested by the Arigoni and Wenkert groups.[19−21] This mechanism was forwarded to explain how substitution takes place at the relatively unactivated C-4 position of the indole ring, but was ultimately disfavored by a lack of support from the nonenzymatic reactivity of model compounds (see Supporting Information for further discussion). In this mechanism, the initial step involves an attack from the *Re* face of the indole C-3 onto the tertiary position of the carbocation to give a "reverse"-prenylated iminium intermediate (Figure 2). A reversible Cope rearrangement then interconverts this species with the C-4-"normal"-prenylated arenium intermediate, and a final deprotonation of the latter species gives product. We note that the proposed arenium intermediate bears an extensively conjugated iminium functionality that is very similar in structure to the intermediate formed in acid-catalyzed aza-Claisen rearrangements of *N*-dimethylallyldihydroindoles.[22,23] In the case of the K174A, there is no base present to deprotonate the arenium intermediate and the more thermodynamically stable iminium intermediate is released into solution where it cyclizes to produce compound **1** (this also explains why there was only a 20-fold reduction in k_{cat} with this mutant). Alternative explanations for the formation of compound **1** could be envisioned without invoking such a mechanism for the DMAT synthase reaction. It is possible that the mutation simply causes a displacement of the dimethylallyl carbocation in the active site and that an abnormal attack from the C-3 position of Trp occurs. Alternatively, it is possible that an initial attack at C-4 takes place to give the arenium ion intermediate, but in the absence of a base, a Cope rearrangement generates the iminium ion intermediate that cyclizes to give compound **1** (not shown in the figure).

To address the likelihood of these possibilities, we examined the structure of the wild-type DMAT synthase in complex with Trp and dimethylallyl *S*-thiolodiphosphate (DMA-*S*-PP) as this serves as an excellent mimic of the Michaelis complex.[8] The plane of the dimethylallyl group sits directly above the plane of the indole ring such that the distance between C-4 of Trp and C-1 of DMA-*S*-PP is 3.8 Å and the distance between the C-3 of Trp and the C-3 of DMA-*S*-PP is 3.5 Å (see Figure S8). If ionization to form the dimethylallyl carbocation occurs without significant motion in the active site, either mode of attack would be equally likely from a geometrical perspective (Figure 3). Given the well-established preference for the addition of electrophiles to the C-3 position of indoles (even when substituted at C-3),[24−28] an initial C-3 attack onto the tertiary carbon of the dimethylallyl carbocation would be favored from the perspective of intrinsic chemical reactivity. It is therefore difficult to explain how the wild-type enzyme could prevent such an attack from occurring. Taken together with the formation of the "reverse"-prenylated compound **1** by the K174A mutant, we feel it is reasonable to propose an initial "reverse" prenylation at C-3 followed by a Cope rearrangement to give the arenium intermediate in the wild-type DMAT synthase reaction (Figure 2).

Since the interconversion of compound **1** and the iminium intermediate is expected to be reversible in solution, we wished to test whether wild-type DMAT synthase is capable of converting compound **1** into DMAT. Extended incubations were run in either the presence or absence of pyrophosphate; however, no such reaction could be detected. This may be due to the thermodynamic favorability of the ring closure that leaves only vanishingly small amounts of iminium intermediate free in solution, as well as to the inability of the enzyme to catalyze the ring-opening of compound **1**. This finding indicates that ring closure of the iminium intermediate to give compound **1** would have to be prevented during the course of the wild-type reaction. One possible solution is that the enzyme keeps the α-amine in a protonated state during the lifetime of the enzyme-bound intermediate.

A similar finding has recently been made with the enzyme FtmPT1 that catalyzes a "normal" prenylation at the C-2 position of the indole in brevianamide F.[29] An active site mutant of FtmPT1 was also found to produce an unnatural hexahydropyrroloindole alkaloid that is "reverse"-prenylated at C-3. Since this enzyme shares significant structural homology with DMAT synthase and binds its substrates in a nearly identical manner, it is conceivable that a "reverse" prenylation at C-3 is the first step in its mechanism as well.

Enzymes that have been proposed to catalyze pericyclic reactions are somewhat rare and include examples such as chorismate mutase,[30−33] precorrin-8x methyl mutase,[34,35] isochorismate pyruvate lyase,[36,37] and the Diels−Alderases.[38−40] While the jury is still out on DMAT synthase, the observed formation of compound **1**, combined with the structural analysis of the Michaelis complex and the intrinsic reactivity of the indole ring, leads us to consider a Cope rearrangement mechanism as a real possibility for this enzyme. Although pericyclic rearrangements have long been proposed to play roles in the biosynthesis of the indole alkaloids such as ergotamine and echinulin,[2,19,20,28,41−44] mechanistic evidence using purified enzymes has been largely absent until now.

■ ASSOCIATED CONTENT

Supporting Information. Full experimental details outlining synthesis, PIX experiments, and kinetic studies. UV spectra of DMAT and compound **1**. NMR spectra of synthetic and enzymatic samples of compound **1**. This material is available free of charge via the Internet at http://pubs.acs.org.

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