Biosynthesis Hot Paper

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A Single Enzyme Transforms a Carboxylic Acid into a Nitrile through an Amide Intermediate

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Abstract: *The biosynthesis of nitriles is known to occur through specialized pathways involving multiple enzymes; however, in bacterial and archeal biosynthesis of 7-deazapurines, a single enzyme, ToyM, catalyzes the conversion of the carboxylic acid containing 7-carboxy-7-deazaguanine (CDG) into its corresponding nitrile, 7-cyano-7-deazaguanine ($preQ_0$). The mechanism of this unusual direct transformation was shown to proceed via the adenylation of CDG, which activates it to form the newly discovered amide intermediate 7-amido-7-deazaguanine (ADG). This is subsequently dehydrated to form the nitrile in a process that consumes a second equivalent of ATP. The authentic amide intermediate is shown to be chemically and kinetically competent. The ability of ToyM to activate two different substrates, an acid and an amide, accounts for this unprecedented one-enzyme catalysis of nitrile synthesis, and the differential rates of these two half reactions suggest that this catalytic ability is derived from an amide synthetase that gained a new function.*

Nitrile-containing products are widespread in nature and are found in secondary metabolites such as cyanoglucosides and alkanenitriles.[1] Although these are increasingly being recognized as important next-generation antibiotics and cytotoxic agents with pharmaceutical potential,[2,3] the biosynthesis of these compounds is very poorly understood. Only one biosynthetic route, which utilizes at least three separate enzymes, has been described in mechanistic detail, and it involves aldoxime formation[4] and a subsequent dehydration[5,6] (Figure 1). The discovery of the 7-deazapurine biosynthetic operon of *Streptomyces rimosus* revealed the first enzyme capable of acting on a carboxylic acid to form a nitrile[7] (Figure 1). This nitrile synthetase, ToyM, was shown to be ATP dependent and to use ammonia as the source of the nitrile nitrogen.[8] Homologues of ToyM are widely distributed since this nitrilation is essential to the biosynthesis of the modified nucleosides archaeosine and the ubiquitous queuosine, as well as many pyrrolopyrimidine nucleoside antibiotics, including toyocamycin and sangivamycin.[9]

ToyM bears evolutionary similarity to glutamine-dependent asparagine synthetases and the lysidine-forming enzyme TilS, both of which employ ATP in the adenylation of substrate molecules, thus poising them for condensation with

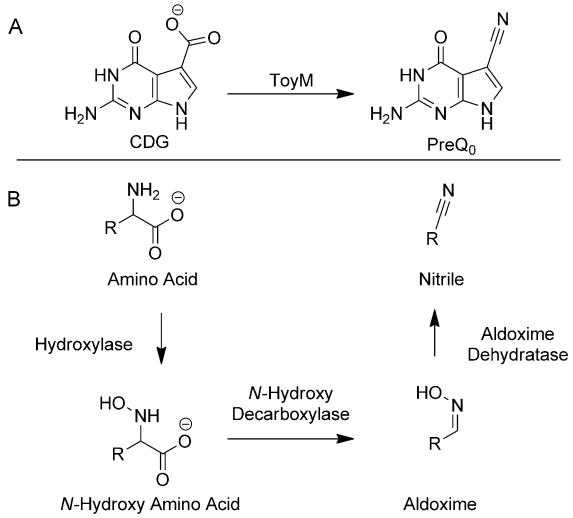

Figure 1. Two biosynthetic pathways to nitrile secondary moieties in nature.

amine nitrogens.[10,11] The crystal structure of ToyM, solved before its enzymatic function was known, showed the presence of a structural zinc divalent cation and revealed that it adopts a PPi loop structure characteristic of ATP-utilizing enzymes.[12] This suggests that the mechanistic strategy employed by ToyM may involve adenylation. The simplest mechanism for ToyM (Figure 2) would be adenylation to activate 7-carboxy-7-deazaguanine (CDG), followed by the addition of ammonia to generate 7-amido-7-deazaguanine (ADG). Activation of ADG by a second equivalent of ATP would precede collapse to form the nitrile product 7-cyano-7-deazaguanine ($preQ_0$). Such a mechanism would be a rare example of a single enzyme activating two functional

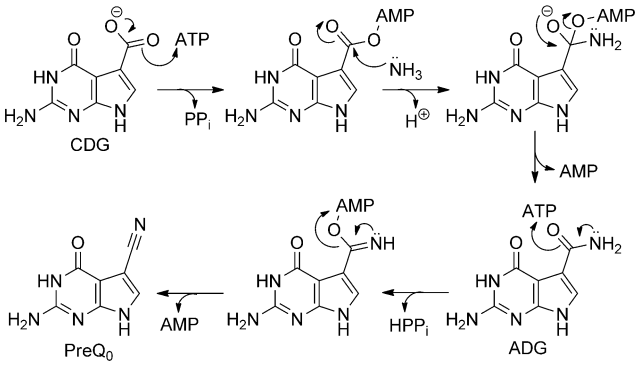

Figure 2. Proposed mechanism of ToyM.

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groups, both the acid as well as the resultant amide intermediate, and presents a new paradigm for the biosynthesis of nitriles from carboxylic acids.

To examine the nitrilation catalyzed by ToyM, the recombinant protein was produced in an *E. coli* expression host. Protein was quantified by amino acid analysis, and metals analysis confirmed the presence of 1.1 ± 0.2 equivalents of Zn^{2+} per monomer. To test the mechanistic proposal in Figure 2, we incubated ToyM with CDG, ATP, and magnesium, and monitored the reaction by LC–MS (Figure S1 in the Supporting Information). We found that magnesium supplementation significantly increased product formation (Figure S2 A in the Supporting Information), and it was routinely included in the assays. The HPLC traces showed that $preQ_0$ was produced as previously shown, as well as an intermediate that increased as CDG was consumed and decreased as $preQ_0$ formed (Figure 3 A). This intermediate exhibited a UV/Vis spectrum highly similar to that of CDG (Figure S3 in the Supporting Information) and a mass consistent with 7-amido-7-deazaguanine (ADG; $[M+H]^+$ *m/z* of 194.0670; theoretical 194.0678). As with $preQ_0$, added magnesium was required to observe maximal quantities of the intermediate (Figure S2 B in the Supporting Information).

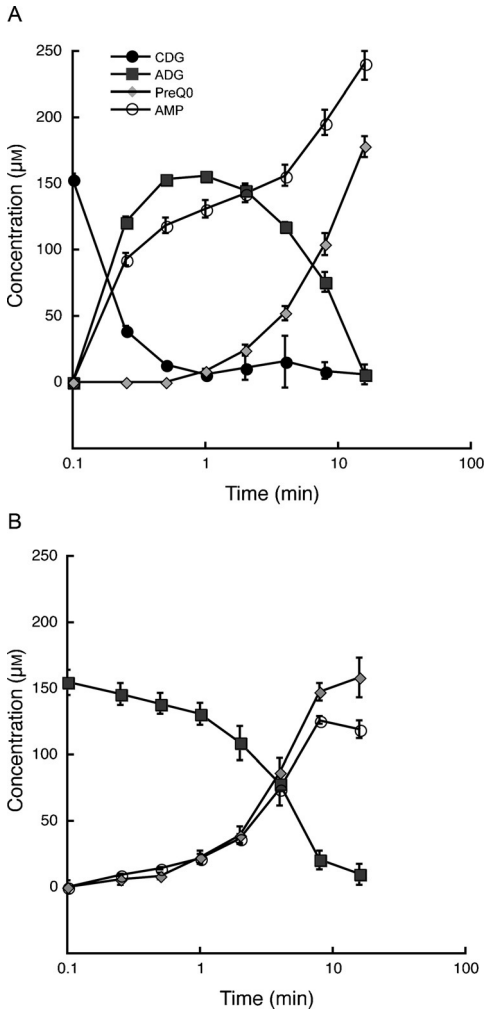

Figure 3. A) Reaction of ToyM with CDG, ammonium chloride, and ATP. B) Reaction of ToyM with ADG and ATP.

The identity of this intermediate was confirmed by comparison with the UV/Vis spectra and mass spectra of authentic ADG, which was synthesized enzymatically from $preQ_0$ by using ToyJKL, a nitrile hydratase from the same pathway as ToyM.[13] To determine whether ADG is a genuine intermediate, it was incubated with ToyM and ATP. In these experiments (Figure 3 B), $preQ_0$ was formed at the expense of ADG with nearly identical $t_{1/2}$ values (ca. 4 min). In addition, AMP formed with a profile that nearly mirrored the formation of $preQ_0$, thus suggesting that adenylation of ADG is the likely mode of activation. Therefore, the formation of $preQ_0$ from CDG appears to occur in two ATP-dependent half reactions, the first of which leads to the formation of ADG, which is converted into $preQ_0$ in the second step.

Direct evidence of the nature of the activated intermediate was obtained by combining ToyM, CDG, and ATP in the absence of ammonia. This reaction was filtered to remove the enzyme and analyzed by ion pairing LC–MS in positive-ion mode. In these experiments, we detected the formation of an intermediate that elutes at ca. 25 min. Examination of the mass spectrum suggested that the peak appears at *m/z* 524.1030, which is consistent with adenylated CDG (theoretical *m/z* 524.1044; Figure 4). The appearance of this new peak is dependent on the presence of ToyM, CDG, and ATP, but it is abolished in the presence of ammonia (Figure 4 A).

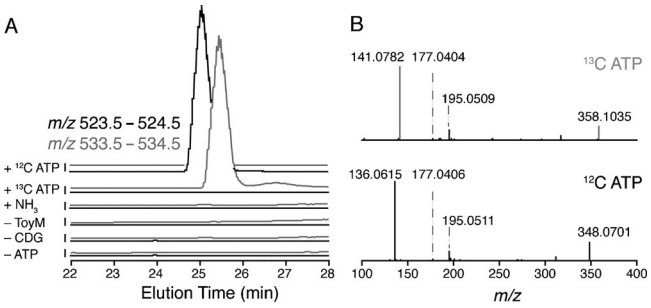

Figure 4. A) Extracted ion traces from ToyM reactions with CDG and ATP in the absence of ammonium ions, showing the adenylated intermediate forms only when all three are present. B) MS/MS analysis of the adenylated intermediate showing fragments of CDG and AMP.

The identity of adenylated CDG was confirmed by using U-$^{13}C_{10}$-labeled ATP. The resulting covalent adduct exhibited the expected mass shift from *m/z* 524.1030 to *m/z* 534.1376 (theoretical *m/z* 534.1379), which is consistent with the incorporation of ten ^{13}C atoms from the labeled ATP. MS/MS analysis of the intermediate further confirmed its identity by revealing fragments consistent with expulsion of $[CDG+H]^+$ (*m/z* 195.0511) and a ketene-like fragment of $[CDG]^+$ (*m/z* 177.0406). We also observed fragments corresponding to [adenosine phosphate $+H]^+$ (*m/z* 348.0701) and [adenine $+H]^+$ (*m/z* 136.0615), both of which shift when ^{13}C-labeled ATP is used. With the labeled substrate, [$^{13}C_{10}$-adenosine phosphate $+H]^+$ is observed at *m/z* 358.1035 and [$^{13}C_5$-adenine $+H]^+$ at *m/z* 141.0782 (Figure 4 B and Figure S4 in

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the Supporting Information). We note that the presence of magnesium abolished the buildup of the adenylated intermediate, presumably as a result of magnesium catalyzing the hydrolysis of the adenylated intermediate in solution. Therefore, these assays were all carried out in the absence of added divalent cation.

To further examine whether the acid and amide substrates are activated in a similar manner, ToyM was incubated with ATP, CDG, and ammonia or ADG for 8.5 h, after which these reaction mixtures were tested by using a coupled enzyme pyrophosphate detection assay.[14] Reactions with both substrates showed the characteristic signal for the presence of pyrophosphate in at least the same stoichiometry as that of the $preQ_0$ product (Figure 5). Taken together with the formation of AMP with both substrates, this result demonstrates conclusively that ToyM is capable of catalyzing the same adenylation reaction on two substrates, CDG and ADG, to eventually form the nitrile $preQ_0$.

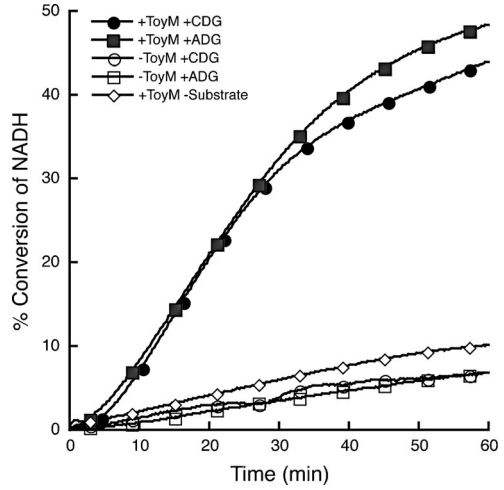

Figure 5. Pyrophosphate-dependent consumption of NADH through a coupled enzyme assay showing that with both substrates (CDG and ADG), ToyM produces pyrophosphate, whereas with either no substrate or no enzyme, only background consumption of NADH is observed.

Enzyme promiscuity has been integral to the formation of the immense biosynthetic repertoire found in nature.[15,16] According to this model, the efficiency of enzymes for their ancestral primary reaction is likely higher than that for the reactions for which they gain catalytic ability over time.[17] ToyM bears tantalizing clues to its evolution in that the first half reaction, the amidation, proceeds more rapidly than dehydration to the nitrile in the second half reaction. It is thus likely that this unprecedented and simplified nitrile synthesis is an elegant example of finding new chemical capability through the promiscuity of an amide synthetase enzyme to achieve what requires at least three enzymes in other known synthetic pathways.

In conclusion, ToyM is an amide synthetase, as well as a nitrile synthetase. Its ability to accept both the acid and amide forms of CDG enables sequential amidation and dehydration to the nitrile. This provides a new paradigm for studying the origin of the many nitrile-containing secondary metabolites, the biosynthetic provenance of which is currently unknown, and it gives molecular detail to the synthesis of an important intermediate in many pathways, the nitrile-containing compound $preQ_0$.

Keywords: bioorganic chemistry · biosynthesis · enzymes · evolution · nitriles

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