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Mechanistic study of the non-oxidative decarboxylation catalyzed by the radical S-adenosyl-L-methionine enzyme BlsE involved in blasticidin S biosynthesis

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Decarboxylation is a fundamentally important reaction in biology and involves highly diverse mechanisms. Here we report mechanistic study of the non-oxidative decarboxylation catalyzed by BlsE, a radical S-adenosyl-L-methionine (SAM) enzyme involved in blasticidin S biosynthesis. By a series of biochemical analysis with isotopically labeled reagents, we show that the BlsE-catalyzed reaction is initiated by the 5′-deoxyadenosyl (dAdo) radical-mediated hydrogen abstraction from a sugar carbon of the substrate cytosylglucuronic acid (CGA), and does not involve a carboxyl radical as has been proposed for 4-hydroxyphenylacetate decarboxylase (HPAD). Our study reveals that BlsE represents a mechanistically new type of radical-based decarboxylase.

Blasticidin S (BLS) is a peptidyl nucleoside antibiotic produced by *Streptomyces griseochromogenes*,[1] which is biosynthesized from the primary precursors cytosine, glucose, arginine, and methionine.[2] The cytidine moiety of blasticidin S binds tightly to the corresponding guanine base at the P site of the ribosome 50S subunit and forms a Watson-Crick base pair,[3] thereby inhibiting peptide bond formation and causing translation termination. It has also been shown that BLS can rapidly inhibit DNA synthesis.[4] As a result, this compound exhibits potent activity against both prokaryotic and eukaryotic cells,[5] and has been widely used in protecting rice plants from infection by the fungus that causes rice blast. Recently, BLS has also been used as a selectable marker in biological research to select transformed cells that carry a resistance gene.[6]

The BLS biosynthetic gene cluster was first identified in 1998 by Zabriskie and coworkers,[7, 8] which expands approximately 20 kilobase pairs (kbs) and encodes a series of proteins, including a unique radical S-adenosyl-L-methionine (SAM) enzyme BlsE. Disruption of *blsE* completely abolished BLS production and resulted in accumulation of cytosylglucuronic acid (CGA) (Fig. 1A) in the culture of the mutant strain.[9] He and coworkers later showed that BlsE catalyzes a radical SAM-dependent decarboxylation reaction that converts CGA to cytosylarabinopyranose (CAP) (Fig. 1A).[10]

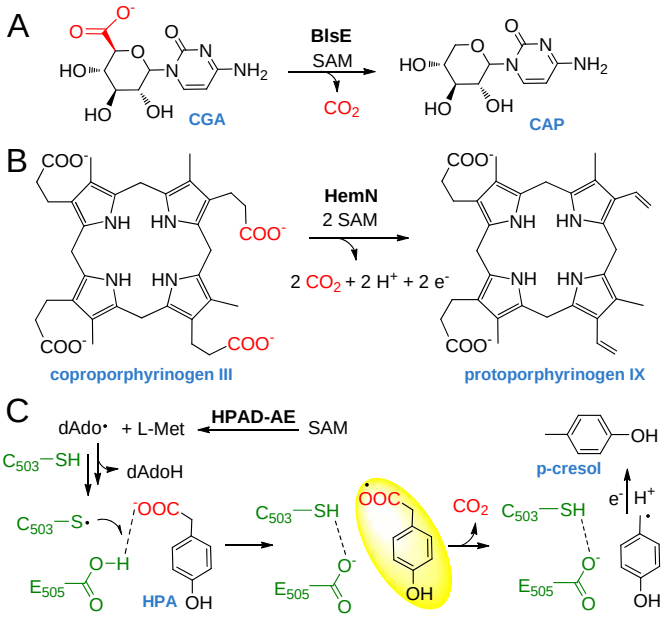

Fig. 1. Radical-SAM dependent decarboxylations. (A) The BlsE-catalyzed non-oxidative decarboxylation. (B) Oxidative decarboxylation catalyzed by HemN. (C) The Kolbe-type decarboxylation reaction catalyzed by 4-hydroxyphenylacetate decarboxylase (HPAD). The dAdo radical produced by the HPAD activating enzyme (HPAD-AE) produces a glycyl radical in HPAD, which, upon substrate binding, generates a thiyl radical that initiates the Kolbe-type reaction. Residue numbers are shown for the HPAD from *Clostridium scatologenes*.

Radical SAM superfamily is the largest known enzyme superfamily that currently consists of more than 16,500 members found in all three domains of life.[11-13] These enzymes utilize a [4Fe–4S] cluster to bind SAM and reductively cleave its carbon-sulfur bond to produce a highly reactive 5′-deoxyadenosyl (dAdo) radical, which initiates a highly diverse variety of reactions.[11-13] A well-known radical SAM decarboxylase is the oxygen-independent coproporphyrinogen III oxidase HemN, which catalyzes protoporphyrinogen IX production by sequential decarboxylation of the two propionate side chains of coproporphyrinogen III (Fig. 1B).[14-17] This decarboxylation reaction is oxidative, in which a carboxyl

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group is released as a carbon dioxide with a simultaneous loss of two electrons. Similar radical-mediated oxidative decarboxylation is also found for other radical-based enzymes, such as the heme-containing enzyme $OleT_{JE}$.[18-21]

Unlike HemN, however, the BlsE-catalyzed decarboxylation is non-oxidative and no electron is released externally (Fig. 1A). The radical-mediated non-oxidative decarboxylations are relatively rare in biochemistry. A well-characterized example is the reaction catalyzed by 4-hydroxyphenylacetate decarboxylase (HPAD), an enzyme involved in the last step of tyrosine fermentation in clostridia, which converts 4-hydroxyphenylacetate (HPA) to 4-methylphenol (p-cresol) (Fig. 1C).[22, 23] In this reaction, the dAdo radical generated by the radical SAM HPAD activating enzyme (HPAD-AE) abstracts a hydrogen from HPAD to produce a stable glycyl radical, which, upon substrate binding, abstracts a hydrogen from the neighbouring Cys to produce a key thiyl radical. This thiyl radical then abstracts the hydrogen from the 4-hydroxyphenylacetate carboxyl group, resulting in a carboxyl radical and a Kolbe-type decarboxylation reaction (Fig. 1C).

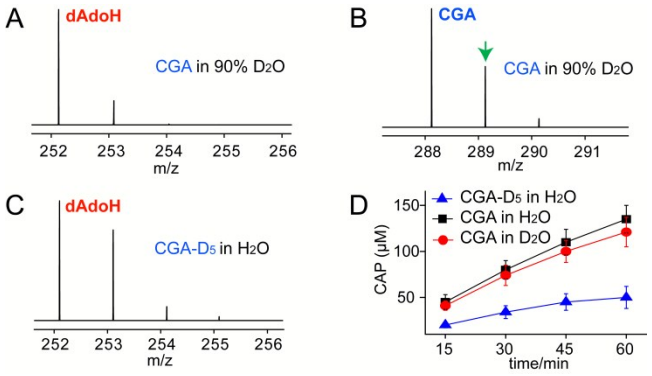
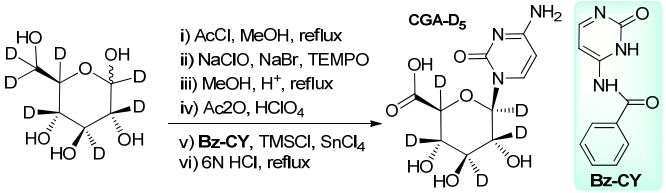


Fig. 2. Mechanistic investigation of the BlsE-catalyzed non-oxidative decarboxylation of CGA. (A) Mass spectrum of dAdoH produced in BlsE reaction with CGA in 90% D_2O. We noted that a very small proportion of dAdoH was deuterated, which is likely a result of hydrogen abstraction from the newly formed deuterated CGA (see Fig. 2B). (B) Deuterium incorporation into CGA when the reaction was performed in D_2O. For the HR-MS/MS spectrum of the deuterated CGA (highlighted by a green arrow), see Fig. S1, ESI†. (C) Mass spectrum of dAdoH produced in BlsE reaction with CGA-D_5 in H_2O. (D) Time course analysis of CAP production in BlsE reactions. The assays were performed by incubating 250 μM CGA or CGA-D_5 with ~50 μM reconstituted BlsE, 1 mM SAM, 2mM sodium dithionite in 40 mM Tris-HCl buffer (pH 8.0 in H_2O or pH/pD 8.0 in 90% D_2O). Reactions were maintained at room temperature (~25°C) before quenching at different time points by addition of formic acid to a final concentration of 5% (v/v), and the product CAP was quantified by HPLC. Because the reactions were not performed in parallel (reaction in D_2O involves an additional buffer exchange step), whether there is a small solvent KIE in the reaction remains to be determined.

Because radical SAM-dependent reactions are in most cases initiated by a dAdo radical-mediated hydrogen abstraction process, it appears that the BlsE-catalyzed non-oxidative decarboxylation might also be a Koble-type, involving a carboxyl radical resulting from the dAdo radical-mediated hydrogen abstraction from the carboxyl group. Because the carboxyl-associated hydrogen is readily exchangeable with the solvent, we reasoned that, if the BlsE-catalyzed reaction is indeed a Kolbe-type, deuterium incorporation into dAdoH should be observed when the reaction is performed in D_2O. Such a strategy has been extensively used in mechanistic investigation of radical SAM enzymes, such as HydG, CofH, and NosL.[24-28] To this end, we synthesized CGA and performed the reaction with reconstituted BlsE, SAM, and dithionite in 90% D_2O. Liquid chromatography with high-resolution mass spectrometry (LC-HR-MS) analysis of the reaction mixture showed that deuterium incorporation into dAdoH was not apparent (Fig. 2A), suggesting that unlike HAPD, the BlsE-catalyzed reaction is not a Kolbe-type. Furthermore, we observed apparent deuterium incorporation into the substrate CGA (Fig. 2B), which likely resulted from quenching of a carbon-centered substrate radical by a solvent-derived hydrogen equivalent. Careful HR-MS/MS analysis of the deuterated CGA (m/z = 289.1) shows that the deuterium atom is on the sugar moiety (Fig. S1, ESI†). It is noteworthy that similar observations of substrate deuteration have been made in many cases, including the radical SAM dehydratase AprD4,[29] and NosL and DesII with unnatural substrates.[30-33] Together, this analysis suggests that the dAdo radical-mediated hydrogen abstraction in BlsE catalysis does not occur on the carboxyl group, but on a site on which the hydrogen is not exchangeable with solvent.

To further interrogate the hydrogen abstraction site in BlsE catalysis, we synthesized CGA-D_5, which contains five deuterium atoms on the sugar moiety (Scheme 1). LC-HR-MS analysis of the BlsE reaction with CGA-D_5 showed that deuterium was apparently incorporated into dAdoH (Fig. 2C), suggesting that the dAdo radical-mediated hydrogen abstraction occurs on a sugar carbon of CGA. We then carried out a detailed time course analysis of the assays with either CGA or CGA-D_5 as a substrate. This analysis showed that the reaction with CGA is apparently faster than that with CGA-D_5, showing a kinetic isotope effect (KIE) of ~2.3 (Fig. 2D). On the contrary, the enzyme activity did not obviously decrease when the reaction was performed in 90% D_2O (Fig. 2D). These analyses strongly support that the BlsE-catalyzed reaction is not a Kolbe-type decarboxylation but is initiated by the dAdo radical-mediated hydrogen abstraction from a sugar carbon of CGA.



Scheme 1. Chemical synthesis of CGA-D_5 from perdeuterated D-glucose.

The results presented above reveal that BlsE reaction is a new type of enzymatic non-oxidative decarboxylation.

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Although the hydrogen atom abstracted by the dAdo radical has not yet been definitely established, we proposed that it is very likely from the carboxyl β positions (i.e. the C4′ of CGA) (Fig. 3A). Such a hydrogen abstraction process results in a β carbon-centered radical intermediate **1**, whose Cα-C bond is cleaved heterolytically with the assistance of a proton transfer process. The resulting decarboxylated radical **2** is then reduced by a hydrogen equivalent to afford the non-oxidative decarboxylated product CAP (Fig. 3A). Because when running the assay in 90% D_2O, a significant proportion of CGA was di-deuterated (Fig. S2, ESI†), the hydrogen equivalent that reduces the decarboxylated radical **2** should derive from the solvent (Fig. 3A) and not from dAdoH, because otherwise, mono-deuterated CAP is expected.

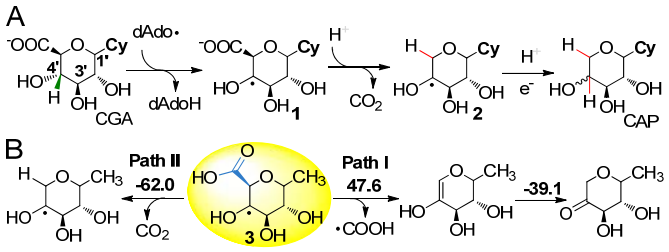

Fig. 3. BlsE catalysis involves a carbon-centered radical resulted from the dAdo radical-mediated hydrogen abstraction. (A) Proposed mechanism for BlsE catalysis. The solvent-exchangeable hydrogen atoms are shown in red, which are incorporated into the final product CAP. (B) DFT calculation of the reactivity of **3**, which serves a chemical model of the key radical intermediate **1** and is highlighted by a yellow ellipse. This analysis shows that when coupled with a proton transfer, heterolytic fission of the Cα-C bond is significantly lower in Gibbs free energy. The Gibbs free energy change (ΔG) for each step is shown in green font above the arrow. Both geometry optimization and energies calculation were conducted at the B3LYP/6-311+G(2d,p)/SMD(water) level of theory.

It should be noted that in most radical-mediated decarboxylation reactions (e.g. the reactions catalyzed by HemN and $OleT_{JE}$), the Cα-C bond of the β carbon-centered radical is cleaved homolytically to release a formyl radical. An intriguing question for BlsE catalysis is how the enzyme chemistry is tuned to heterolytically, not homolytically, cleave the Cα-C bond of **1**. We proposed that when coupled with a suitable proton transfer process, the heterolytic cleavage pathway may be energetically favorable over the homolytic cleavage pathway. To test this hypothesis, we performed a density functional theory (DFT) calculation on **3**, a chemical model compound of **1**, and both homolytic Cα-C bond fission (Path I) and heterolytic fission that is coupled with an internal proton transfer (Path II) are calculated (Fig. 3B). This analysis showed that indeed, Path II is 109.6 $kJmol^{-1}$ lower in Gibbs free energy than Path I; even when the subsequent Keto-enol tautomerization process is considered, Path II is still 70.5 $kJmol^{-1}$ lower in energy than Path I, suggesting the heterolytic cleavage pathway is thermodynamically favored (Fig. 3B). Thus it is apparent that the microenvironment of enzyme active site plays a key role in fine-tuning the reactivity of the radical intermediates to achieve distinct catalytic outcomes, as has been recently reported in the catalysis of several other radical SAM enzymes.[27, 28, 34-39] Further detailed mechanistic investigation on the protein/substrate interaction and thermodynamics that govern BlsE catalysis is currently in progress.

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