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Biochemical Characterization of an Arginine 2,3-aminomutase with Dual Substrate Specificity

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Summary of main observation and conclusion The radical S-adenosylmethionine (SAM) aminomutases represent an important pathway for the biosynthesis of β-amino acids. In this study, we report biochemical characterization of BlsG involved in blasticidin S biosynthesis as a radical SAM arginine 2,3-aminomutase. We showed that BlsG acts on both L-arginine and L-lysine with comparable catalytic efficiencies. Similar dual substrate specificity was also observed for the lysine 2,3-aminomutase from *Escherichia coli* (LAM_{EC}). The catalytic efficiency of LAM_{EC} is similar to BlsG, but is significantly lower than the enzyme from *Clostridium subterminale* (LAM_{CS}), the latter acts only on L-lysine and not on L-arginine. Moreover, we showed that enzymes can be grouped into two major phylogenetic clades, each corresponding to a certain C3 stereochemistry of the β-amino acid product. Our study expands the radical SAM aminomutase members and provides insights into enzyme evolution, supporting a trade-off between substrate promiscuity and catalytic efficiency.

Background and Originality Content

β-amino acids serve as building blocks for a wide range of bioactive natural products, including antibiotics, insecticides, and antifungal, antitumor, and antiviral agents.[1] These amino acids have similar polarity with α-amino acids but offer additional properties, such as resistance to proteolysis. β-amino acids are biosynthesized via various pathways, including decarboxylation of aspartate and aspartate derivatives, Michael-type addition of ammonia/amino groups onto α, β-unsaturated carboxylates, transamination of β-ketocarboxylates, and aminomutase-catalyzed reactions with α-amino acids.[1] Thus far, three types of aminomutases have been identified. The first type is a family of enzymes that use 4-methylideneimidazol-5-one (MIO) as a key cofactor, which is produced autocatalytically from an Xxx-Ser-Gly (Xxx represents an Ala or a Thr) motif at the enzyme active site.[2] The second type is the adenosylcobalamin-dependent glutamate mutase, which produces 3-methylaspartate via a radical-mediated C-C bond fragmentation and a re-addition process.[3,4] The third type of aminomutase is a group of radical S-adenosyl-L-methionine (SAM) enzymes, which are pyridoxal-5'-phosphate (PLP)-dependent and utilize SAM as a catalytic cofactor to catalyze the amino group rearrangement.[5,6]

The radical SAM superfamily enzymes represent the largest known enzyme family consisting of more than 125,000 members found in all three domains of life.[5-8] These enzymes contain a [4Fe-4S] cluster to bind SAM and reductively cleave its carbon-sulfur bond to produce L-methionine and a highly reactive 5´-deoxyadenosyl (dAdo) radical. The dAdo radical then abstracts a hydrogen from the substrate to produce 5´-adenosine (dAdoH) and a substrate-based radical, thereby initiating diverse reactions relevant to the modification of nucleic acids and proteins, and the biosynthesis of cofactors and natural products.[5-8] Recently, a new type of radical SAM chemistry is emerging, in which the dAdo radical is added to an sp^2 carbon to result in an adenosylation

reaction.[9] This type of radical SAM chemistry has been found naturally in menaquinone biosynthesis and bacteriohopanepolyol biosynthesis,[10-13] and can be achieved with unnatural substrates.[14-17] Very recently, it has been shown that the dAdo radical-based adenosylation can even be achieved via homolytic substation reactions.[18]

Lysine 2,3-aminomutase (LAM), which catalyzes the interconversion of L-α-lysine to β-lysine, represents the first biochemically characterized member of the radical SAM superfamily.[19,20] In contrast to the adenosylation reactions and most other radical SAM-dependent reactions, in which SAM is consumed stoichiometrically, LAM uses SAM as a catalytic cofactor and the dAdo radical is regenerated from dAdoH in the end of each reaction cycle, making LAM a highly efficient enzyme in terms of bioenergy and atom economy. The LAM from *Clostridium subterminale* (LAM_{CS}) catalyzes the conversion of L-α-lysine to (*3S*)-β-lysine,[21] which is the first step of lysine catabolism by clostridia (Figure 1A).[22] LAMcs has a turnover number around 2000 min^{-1},[20,23] representing the most efficient enzyme among the radical SAM superfamily. In contrast to LAM_{CS}, the LAM from *Escherichia coli* (LAM_{EC}) produces (*3R*)-β-lysine,[24] which is utilized for post-translational β-lysylation of elongation factor P (Figure 1B).[25,26] Besides LAM, an L-glutamate 2,3-aminomutase from *Clostridium difficile* (EMA_{CD}) was also biochemically characterized, which is homologous to LAM and have a turnover number around 400 min^{-1}.[27] LAM-homologous enzymes have been found in various organisms, and the exact functions of these enzymes remained to be determined.

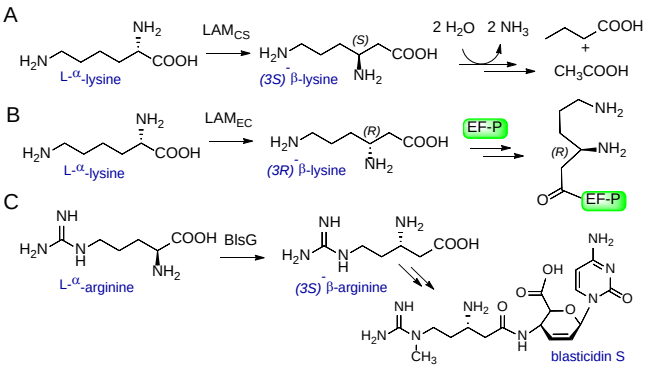

Figure 1. Reactions catalyzed by the radical SAM-dependent aminomutases. (A) The LAM_{CS}-catalyzed reaction is the first step in lysine catabolism by clostridia. (B) (*3R*)-β-lysine produced by LAM_{EC} is utilized for post-translational of elongation factor P (EF-P). (C) (*3S*)-β-arginine produced by BlsG is an intermediate in blasticin S biosynthesis.

Results and Discussion

Blasticidin S, produced by S*treptomyces griseochromogenes*, is a peptidyl nucleoside antibiotic that exhibits potent activity against both prokaryotic and eukaryotic cells.[28] Blasticidin S contains a (*3S*)-β-arginine moiety, which has been shown to be derived from L-α-arginine by isotope labeling experiments (Figure 1C).[29] The biosynthetic gene cluster of Blasticidin S encodes an LAM-homologous enzyme BlsG,[30,31] which is likely responsible for the production of (*3S*)-β-arginine. To investigate the function of this enzyme, the *blsG* gene was amplified from the genomic DNA of *S. griseochromogenes* and expressed in *E. coli* with an N-terminal hexa-histidine tag, and the protein was purified by Ni^{2+} affinity chromatography under strictly anaerobic conditions. After chemical reconstitution of the [4Fe-4S] cluster, the reaction was performed by incubation of the reconstituted protein with SAM, L-α-arginine, and sodium dithionite. The reaction mixture was then treated with phenyl isothiocyanate (PITC) to derivatize the amino acids, and analyzed by liquid chromatography high resolution mass spectrometry (LC-HRMS), which clearly revealed

a new product in the BlsG reaction. Compared with the PITC-derived L-α-arginine, this compound exhibited exactly the same molecular weight but was eluted slightly earlier in LC analysis (Figure 2, trace i), supporting production of β-arginine in the BlsG reaction.

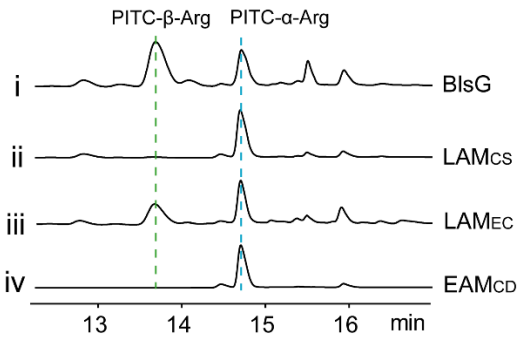

Figure 2. HPLC analysis of the PITC-treated reactions of L-α-arginine with four aminomutases. (i) BlsG, (ii) LAM_{EC}, (iii) LAM_{CS} and (iv) EAM_{CD}.

We also expressed and purified LAM_{CS}, LAM_{EC}, and EMA_{CD} for parallel assays. LC-HRMS analysis of the reaction mixture of LAM_{CS} suggest that production of β-arginine was not observed (Figure 2, trace ii), and this is consistent with the previous study by Frey et al showing that LAM_{CS} has a very stringent substrate specificity and does not act on L-α-arginine.[32] In contrary, the parallel assay with LAM_{EC} showed that β-arginine was apparently produced in the LAM_{EC} reaction (Figure 2, trace iii), suggesting that LAM_{EC} can act both on L-α-lysine and L-α-Arginine. Production of β-arginine was not observed in the reaction with LAM_{CD} (Figure 2, trace iv).

Table 1. Kinetic parameters of the four radical SAM 2,3-aminomutases investigated in this study. See Figure S1-S6 for the detailed information of the kinetic analysis.

Substrate	enzyme	k_{cat} (min^{-1})	Km (mM)	K_{cat}/Km (mM/min)
L-α-arginine	BlsG	13.8 ± 0.8	2.9 ± 0.3	4.8
	LAM_{EC}	9.6 ± 0.9	4.7 ± 0.8	2.0
L-α-lysine	BlsG	40.9 ± 5.2	5.7 ± 1.3	7.2
	LAM_{EC}	34.9 ± 5.0	5.2 ± 1.0	6.7
	LAMcs	2208.1 ± 122.0	4.8 ± 0.6	460.0
L-α-glutamate	EAM_{CD}	417.2 ± 1.5	3.2 ± 0.1	130.3

To quantitatively analyze the reaction efficiency, kinetic studies were performed for the reaction with BlsG and LAM_{EC}. The results showed that BlsG has a turnover number of 13.8 min^{-1}, which is slightly higher than LAM_{EC} (9.6 min^{-1}), and the Km values of BlsG and LAM_{EC} are 2.9 mM and 4.7 mM, respectively (Table 1). These results demonstrate that both BlsG and LAM_{EC} are L-Arginine 2,3-aminomutases, and the catalytic efficiency of BlsG is about 2-fold higher than that of LAM_{EC}.

The comparable efficiencie of BlsG and LAM_{EC} on L-α-arginine inspired us to further investigate the BlsG reaction with L-α-lysine. To this end, we performed the reaction by incubation of BlsG with L-α-lysine, SAM, and sodium dithionite, and the reaction mixture was analyzed by LC-HRMS after derivatization with PITC. Parallel reactions were also performed on LAM_{CS}, LAM_{EC}, and EAM_{CD}. This analysis showed that production of β-lysine was observed for all the enzymes except for EAM_{CD} (Figure 3). Subsequent kinetic analysis showed that LAM_{CS} has a k_{cat} of 2208.0 min^{-1} and a Km of 4.8 mM, which are very close to those reported previously.[20,23] For LAM_{EC}, the Km is 5.2 mM and the k_{cat} is 34.2 min^{-1}, the latter value is much higher than that reported previously but still within an order of magnitude.[24] Notably, for BlsG the k_{cat} is 40.9 min^{-1}, which is about 3-fold higher than its k_{cat} on L-α-arginine (13.8 min^{-1}). The Km value on L-α-lysine (5.7 mM) is about 2-fold higher than that on L-α-Arginine

(2.9 mM). These results suggest that although BlsG has higher affinity toward L-α-arginine than L-α-lysine, the latter compound actually has higher reactivity in BlsG reaction, raising a possibility that BlsG may have evolved from a LAM progenitor. These observations reveal that unlike LAM_{CS}, which is highly specific and efficient toward L-α-lysine, BlsG and LAM_{EC} have dual activity and can act both on L-α-arginine than L-α-lysine, but only have moderate catalytic efficiencies. We also tested the BlsG activity on L-α-glutamate, but no activity was observed. In the positive control assay with EAM_{CD}, the kinetic study revealed a k_{cat} of 417.2 min^{-1} and a Km of 3.2 mM (Table 1), which are close to that reported previously.[27]

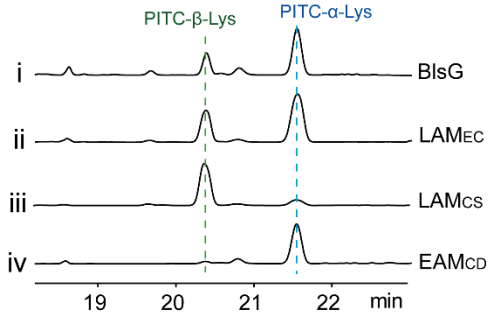

Figure 3. HPLC analysis of the PITC-treated reactions of L-α-lysine with four aminomutases. (i) BlsG, (ii) LAM_{EC}, (iii) LAM_{CS} and (iv) EAM_{CD}.

To provide further functional and evolutionary insights into radical SAM-dependent aminomutases, we selected 64 LAM-homologous proteins from bacteria and constructed a phylogenetic tree by using maximum likelihood reference (Figure 4A).[33] This analysis revealed the proteins fall into two major polyphyletic clades that both contain Gram-positive and Gram-negative sequences. BlsG, EAM_{CD}, and LAM_{CS} fall into the same major clade, whereas LAM_{EC} falls into another major clade. This observation is consistent with the fact that LAM_{CS} and BlsG produce *(3S)*-β-amino acids (i.e. *(3S)*-β-lysine by LAM_{CS} and *(3S)*-β-arginine by BlsG), whereas LAM_{EC} produces *(3R)*-β-lysine.

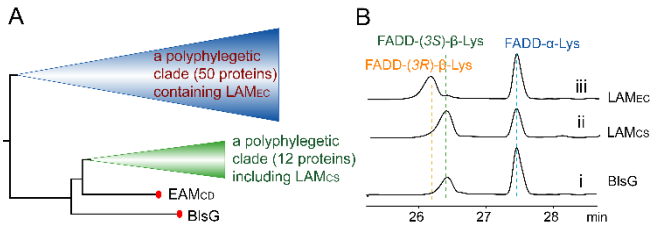

Figure 4. Classification of radical SAM aminomutases. (A) The maximum likelihood phylogeny of the four aminomutases investigated in this study together with other 60 homologous enzymes. See Figure S7 for the full phylogenetic tree. (B) HPLC analysis of the FADD-treated L-α-lysine reactions with (i) BlsG, (ii) LAM_{CS} and (iii) LAM_{EC}.

To confirm that the β-lysine produced by BlsG has a *(3S)*-configuration, we treated the BlsG reaction mixture with N-(5-fluoro-2,4-dinitrophenyl)-L-alaninamide (FDAA) and performed HPLC and LC-HRMS analysis. For comparison, parallel assays were also performed for the reactions from LAM_{CS} and LAM_{EC}. The results showed that the FDAA-derivatized product from the BlsG reaction has exactly the same elution time with that in the LAM_{CS} reaction, and is different from that produced in the LAM_{EC} reaction (Figure 4B). This observation suggests that BlsG produces the corresponding *(3S)*-β-amino acids for both L-α-arginine and L-α-lysine, which is different from LAM_{EC} that produces β-amino acids with an opposite C3 stereochemistry. Although the C3 of β-glutamate is achiral, the stereospecific hydrogen abstraction by the dAdo radical in EAM_{CD} catalysis is likely similar to that of LAM_{CS} and BlsG and is from the 3-pro-*R* position,[24] as EAM_{CD} falls into the same major clade with LAM_{CS} and BlsG (Figure 4A and Figure S7).

Conclusions

In summary, this study characterized BlsG as a radical SAM arginine 2,3-aminomutase and showed it acts both on L-α-lysine and L-α-arginine with similar catalytic efficiencies. Similar dual

substrate specificity and catalytic efficiencies are also observed for LAM_{EC}, although the latter enzyme is evolutionarily distinct and produces β-amino acids with a different stereochemistry. In contrast to these two promiscuous enzymes, LAM_{CS} has a very strict substrate specificity and a significantly higher catalytic efficiency. These observations are consistent with the early hypothesis by Tawfik et al that promiscuity has evolved with the trade-off of catalytic efficiency.[34] It appears that BlsG has evolved from a progenitor of LAM_{CS}, and because the selection pressure toward arginine activity (which is required for the biosynthesis of blasticidin S, a secondary metabolite) has not been strong enough, the promiscuous activity of BlsG retained. Because enzymes homologous to BlsG are widespread in nature, both in prokaryotes in eukaryotes, it is anticipated that many of these homologous enzymes act on different substrates and/or have new activities, which could be a treasure trove for biocatalysis and bioengineering applications.

Supporting Information

The supporting information for this article is available on the WWW under https://doi.org/10.1002/cjoc.2018xxxxx.

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