How a Subfamily of Radical S-Adenosylmethionine Enzymes Became a Mainstay of Ribosomally Synthesized and Post-translationally Modified Peptide Discovery

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ABSTRACT: Radical S-adenosylmethionine (rSAM) enzymes are a large and diverse superfamily of enzymes, some of which are known to participate in the biosynthesis of ribosomally synthesized and post-translationally modified peptides (RiPPs). Specifically, a subfamily of rSAM proteins with an elongated C-terminus known as a SPASM domain have become a fixation in the discovery of new RiPP natural products. Arguably, a structural study, a bioinformatic study, and a functional study built the foundation of the research for rSAM-SPASM-protein-modified RiPPs. In this Review, we focus on these three studies and how they initiated what has become an increasingly productive field. In addition, we discuss the current state of RiPPs that depends on rSAM-SPASM proteins and provide guidelines to consider in future research. Lastly, we discuss how genome mining tools have become a powerful means to identify and predict new RiPP natural products. Despite the state of our current knowledge, we do not completely understand the relationship of rSAM-SPASM chemistry, substrate recognition, and the structure−function relationship as it pertains to RiPP biosynthesis, and as such, there remain many interesting findings waiting to be discovered in the future.

KEYWORDS: RiPP, SPASM/Twitch domains, radical S-adenosylmethionine enzyme, peptide modification, bioinformatics

INTRODUCTION

Ribosomally synthesized and post-translationally modified peptides (RiPPs) have emerged as a structurally diverse class of secondary metabolites.[1] Interest in RiPPs has arisen due to their immense distribution in bacteria, their potential engineer ability, and their potential as a source of new therapeutics.[3] RiPP production begins with a genetically encoded precursor peptide being synthesized by the ribosome. Following its synthesis, modifying enzymes sequentially process the precursor peptide, ultimately yielding the mature RiPP (Figure 1).[2] Precursor peptides usually contain a N-terminal leader sequence that is recognized and bound by post-translationally modifying enzymes and/or a pathway-associated RiPP recognition element.[3−6] The classification of RiPPs is defined by the types of modifications installed on the peptide, which are carried out by the modifying enzymes.

One family of enzymes that has become increasingly common in RiPP maturation is the radical S-adenosylmethionine (rSAM) superfamily. rSAM enzymes have been shown to catalyze a remarkable range of post-translational modifications on RiPP precursor peptides, oftentimes using unprecedented chemical strategies to modify peptides into complex natural products.[7] rSAM enzymes are effective, because they utilize an [4Fe-4S] cluster to reductively cleave SAM, generating a 5′-deoxyadenosyl radical (Figure 2A). The 5′-deoxyadenosyl radical abstracts a
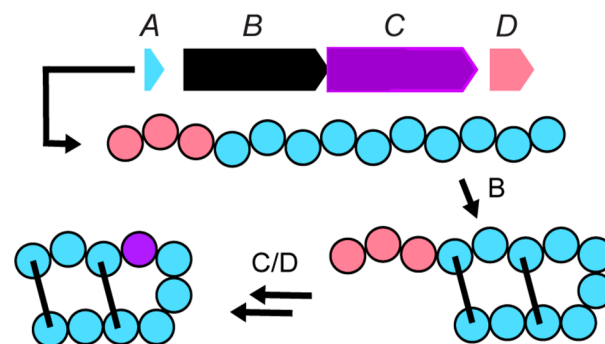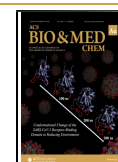

Figure 1. (A) Representation of RiPP biosynthesis where a precursor peptide is modified by biosynthetic genes resulting in a mature natural product.
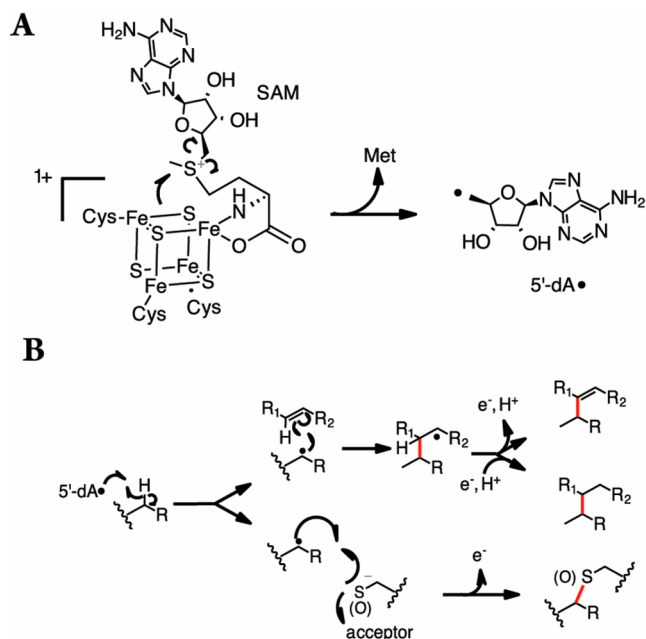
Figure 2. (A) Schematic representation of the homolytic cleavage of SAM resulting in a 5′-deoxyadenosyl radical (5′-dA●). (B) Mechanistic scheme for rSAM-enzyme-catalzyed bond formation.

hydrogen atom from the peptide substrate, leading to the formation of an alkyl radical (Figure 2B). Subsequently, the alkyl radical recombines with an electron-rich functional group, leading to various outcomes. The types of post-translational modifications that have been observed include, but are not limited to, the formation of intramolecular carbon−carbon bonds, carbon−sulfur bonds, carbon−oxygen bonds, and epimerization, among others, on the precursor peptide. As a result of these modifications, rSAM-dependent natural products have been shown to be important biological molecules such as antibiotics,[8,9] essential redox cofactors,[10,11] and quorum sensing molecules,[12,13] among others. Here, we discuss how a subfamily of rSAM enzymes, rSAM-SPASM proteins, has become a focus in the study of RiPP biosynthesis, RiPP bioinformatics, and the coinage of interesting RiPP names.

■ FROM THE FOUNDATION UP

Arguably, the most transformational set of studies in the rSAM-dependent RiPP field has involved a protein structure, a bioinformatic study, and a protein function. To begin with, the rSAM protein structure referred to stemmed from the study of sulfatase maturation in bacteria. For anaerobic sulfatase to be functional, it must first undergo post-translational oxidation of serine or cysteine to form the catalytic residue α-formylglycine (FGly).[14,15] In prokaryotes, the enzyme performing this oxidation was found to be a member of the rSAM superfamily, AtsB.[16] AtsB was shown to oxidize serine through a 5′-deoxyadenosyl-radical-mediated abstraction of the C_β-hydrogen yielding an alkyl radical.[16] Loss of an electron and proton through an unknown mechanism results in the formation of FGly.[17] From these pioneering studies, related enzymes with a similar function have been classified as the anaerobic sulfatase maturating enzymes (AnSMEs) subfamily.[18] Structural characterization of AnSME revealed the protein to contain an elongated C-terminal domain that coordinates two auxiliary [4Fe-4S] clusters, in addition to the rSAM [4Fe-4S] cluster

bound by the TIM barrel domain (Figure 3).[19] This extra C-terminal domain is annotated as a SPASM domain, named after the founding members <u>s</u>ubtilosin A, <u>p</u>yrroloquinoline quinone, <u>a</u>naerobic <u>s</u>ulfatase, and <u>m</u>ycofactocin.[20,21]
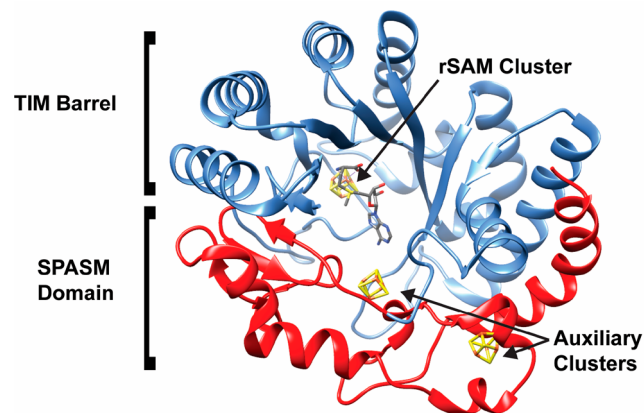

Figure 3. Crystal structure of AnSME (PDBID: 4K37) showing the TIM barrel domain (blue), SPASM domain (red), and other significant features of the prototypical rSAM-SPASM protein.

Although AnSME is not an RiPP maturase, its structure is homologous to RiPP-modifying rSAM-SPASM enzymes and thus has been pertinent to the RiPP field. Interestingly, AnSME has structural homology with another C-terminal extension called a Twitch domain. The Twitch domain, found in BtrN and MoaA, is a truncated SPASM domain that binds a single auxiliary Fe-S cluster.[22,23] The scope of this Review is focused on rSAM-SPASM enzymes and the rSAM-Twitch enzymes SkfB and TqqB. We include SkfB and TqqB because of their similarities in chemistry to rSAM-SPASM enzymes. Since publication of the AnSME structure, only three additional rSAM-SPASM proteins, out of ∼60 000 annotated in the Interpro database (IPR023885), have had their structures solved (PqqE, SuiB, and CteB). Notably, the conventional thought is that the SPASM domain binds two auxiliary [4Fe-4S] clusters; however, SPASM domains have also been reported to bind a [2Fe-2S] cluster in place of one auxiliary [4Fe-4S] cluster.[24] Regardless of the composition, a common role for auxiliary iron-sulfur clusters in RiPP modifications has not yet been definitively determined. However, it has been proposed that auxiliary clusters participate in substrate binding and/or electron shuttling.[25,26] For instance, MftC employs auxiliary clusters to catalyze two distinct reactions, oxidative and redox-neutral, on the precursor peptide.[26] Specifically, it is thought that close midpoint potential values of auxiliary clusters allow for the reversible electron transfer between each cluster.[27] A recent study of SuiB suggests a similar role.[28] By trapping the reaction intermediates and characterizing them by electromagnetic resonance spectroscopy, Balo et. al provided evidence that the auxiliary cluster I (Aux I) is the oxidant of Lys-Trp·.[28] In addition, it was suggested that substrate binding regulates the redox potential of Aux I and rSAM.[28] Considering the range of modifications that rSAM-SPASM proteins are capable of, the function(s) of the auxiliary clusters are likely to be system-dependent.

While the structure of AnSME was being elucidated, Haft and Basu reported on the association of rSAM-SPASM proteins to biosynthetic pathways that encoded for a peptide.[21] At the time,

Table 1. Current List of Known rSAM-SPASM Proteins and Their Respective Products

	Protein	Product	PDBID	Linkage	Refs	Example Structures
C–C Bond	PqqE	Pyrroloquinoline Quinone	6C8V	C_γ-C3	24,56	
	StrB/SuiB	Streptide	5V1Q, 5V1T, 5V1S	C_β-C8	51,57	
	MftC	Mycofactocin		C_α-C_β	10,32,33	
	RrrB	Ryptides		C_δ-C3	55	
	WgkB	Benzindole		C_α-C5 C_δ-C6	54	
	XyeB	Cyclophane		C_β-C6 C_β-C4	35,53	
	GrrB	Cyclophane		C_β-C4 C_β-C7	35,53	
	FxsB	Cyclophane		C_β-C4	35,53	
C–S Bond	AlbA	Subtilosin A		C_α-S	25,39	
	SCIFF Maturase (CteB, Tte1186)	Thermocellin	5WHY, 5WGG	C_γ-S	34,47	
	SkfB (Twitch)	Sporulation Killing Factor	6EFN	C_α-S	41,58	
	ThnB	Thuricin H		C_α-S	46	
	QhpD	Mature QhpC		C_β-S C_γ-S	48	
	RumC	Ruminococcin C		C_α-S	44	
	QmpB	Sactipeptide		C_α-S	59	
	NxxcB	Ranthipeptide		C_β-S	49	
	GggB	Streptosactin		C_α-S	45	
	PapB	Freyrasin		C_β-S	50,60	
C–O Bond	TqqB (Twitch)	Rotapeptide		C_α-O	12	
Other	PlpD	Unknown			61	

it was established that the redox cofactor, pyrroloquinoline quinone (PQQ), was synthesized from the precursor peptide PqqA.[29] In addition, it was known that the *pqq* biosynthetic gene cluster encoded for a rSAM-SPASM protein, PqqE, which was later shown to install a C−C cross-link between a conserved Tyr and Glu on the peptide PqqA.[30,24] The association of a peptide and a rSAM-SPASM protein, along with other bioinformatic evidence, led Haft to discover the mycofactocin (MFT) biosynthetic pathway.[31] Like PQQ, the redox cofactor MFT is synthesized from a precursor peptide and hinges on a critical transformation catalyzed by the rSAM-SPASM enzyme MftC.[32,33] Expanding on the association with peptides, Haft and Basu leveraged rSAM-SPASM proteins as molecular markers to discover new multicomponent (>2 modifying enzymes) RiPP biosynthetic pathways.[21] Their approach used partial phylogenetic profiling and hidden Markov models (HMMs) to identify rSAM-SPASM proteins that were genetically clustered with putative precursor peptides.[21] From this study, Haft and Basu identified five previously unknown RiPP natural product families that rely on rSAM-SPASM enzymes, some of which have been experimentally validated.[34,35] In addition, Haft and Basu built a collection of 68 protein subfamilies based on HMMs that are nonoverlapping and functionally distinct.[21] The abundance of rSAM-SPASM proteins and their association with peptides led Haft and Basu to postulate that rSAM-SPASM proteins are commonplace in RiPP biosynthesis, a notion that is coming to fruition.

A third major influence in the rSAM-dependent field was the study of AlbA and its involvement in synthesis of subtilosin A.[36−38] Subtilosin A is a bacteriocin that is comprised of a circular peptide containing three intramolecular thioether bridges.[36−38] Installation of the thioether bonds in subtilosin A is carried out by the rSAM-SPASM enzyme AlbA. Similar to AnSME, AlbA was found to bind three [4Fe-4S] clusters and catalyzes the 5′-deoxyadenosyl-radical-mediated hydrogen abstraction from the precursor peptide, in this case SboA.[39] Unlike AnSME, AlbA was shown to abstract hydrogen from a C_α, forming the initial alkyl radical.[25] Subsequent radical recombination with the thiol/thiolate of cysteine and loss of an electron and proton forms the intramolecular thioether bond. RiPPs that contain C_α-S bonds are classified as sactipeptides[40] (sulfur-to-alpha carbon thioether cross-linked peptides) and include sporulation killing factor (SKF),[41] the thuricin family,[42,43] ruminococcin C,[44] six-cysteines-in-forty-five (SCIFF),[34] and streptosactin.[45] The pivotal study of AlbA, together with the bioinformatic study discussed above, effectively launched the growing field of rSAM-dependent RiPPs.

EXPANDING THE REALM OF RSAM-SPASM-DERIVED RIPPS

Following the publication of AlbA, the Marahiel group continued to be prolific in the discovery of sactipeptide biosynthesis.[39] They reported on SkfB, demonstrating that it catalyzed the formation of a thioether bond between a C_α-S bond on the precursor peptide to SKF[41] and on ThnB, showing that it installed a C_α-S bond on the precursor peptide of thuricin H.[46] Soon after, the functions of Tte1186,[34] CteB,[47] and RumC,[44] among others, were discovered. While reports of C_α-S thioether bridges were predominant in the early stages of rSAM-

dependent RiPP discovery, it should be noted that C_β-S and C_γ-S bonds, or ranthipeptides (radical non-α-thioether peptides), have since become increasingly common. The first rSAM-SPASM enzyme to catalyze non-C_α-S thioether bonds was QhpD.[48] QhpD installs both C_β-S and C_γ-S thioether bonds during the maturation of the γ-subunit (QhpC) of quinohemoprotein amine dehydrogenase.[48] The family of rSAM-SPASM enzymes involved in ranthipeptide biosynthesis has since expanded to include NxxcB[49] and PapB.[50] While sacti/ranthipeptides predominated early on, a second class of rSAM-dependent RiPPs has emerged.

Carbon−carbon bonds are not trivial to make; yet, rSAM-SPASM proteins have made them commonplace. The first reported *in vitro* C−C bond formation by a rSAM-SPASM in RiPP biosynthesis involved streptide.[51,52] Streptide is a quorum sensing molecule that is synthesized from the precursor peptide, StrA, by the *str* gene cluster.[52] Within the cluster, the rSAM-SPASM protein, StrB, was shown to install a C_β-C7 bond between Lys and Trp, yielding a cyclophane.[51] Soon after, a PqqE was reported to catalyze the formation of a C_γ-C3 bond between Glu and Tyr on the precursor peptide PqqA, a critical step in the formation of PQQ.[24] More recently, XyeB, GrrB, FxsB, WgkB, and RrrB have all been reported to catalyze the formation of cyclophanes on their respective precursor peptide.[35,53−55] The linkages from nonaromatic carbons can occur from C_α, C_β, C_γ, or C_δ and typically are attached to the C3 of Tyr or, less discriminately, to C4−C8 of Trp. From these studies, it is becoming more apparent that rSAM-SPASM proteins have been recruited to install cyclophanes in RiPP biosynthesis. An outlier in the formation of C−C bonds is MftC. As mentioned earlier, MftC is involved in the biosynthesis of the redox cofactor MFT.

Currently, it is the only rSAM-SPASM protein involved in C−C bond formation that does not yield a cyclophane. Instead, two independent studies demonstrated that MftC catalyzes the oxidative decarboxylation of the C-terminus of MftA, resulting in the α/β unsaturated tyramine (MftA**).[32,33] However, mechanistic studies later demonstrated that MftC catalyzes a two-step reaction. Following the decarboxylation, MftC installs a lactam moiety by catalyzing the C−C bond formation between the C_β of the penultimate Val and the C_α of the former Tyr.[10] While MftC is the outlier in the family of C−C-bond-forming rSAM-SPASM enzymes, it promises that potentially unusual and difficult post-translational modifications are to come. Lastly, rSAM-SPASM proteins have been shown to install C−O bonds on RiPP precursor peptides. Recently, the Seyedsayamdost group reported on the formation of an aliphatic ether in a streptococcal quorum sensing molecule encoded by the *tqq* biosynthetic gene cluster.[12] TqqB was shown to catalyze the formation of a C−O bond between a Thr-derived alcohol and the C_α of an adjacent Gln on the precursor peptide TqqA.[12]

Now that a foundation has been established for rSAM-SPASM enzymes and their involvement in RiPP biosynthesis, broad guidelines can be followed for investigating new rSAM-SPASM enzymes and pathways:

(1) In the case of multicomponent RiPP biosynthetic gene clusters (>2 modifying enzymes), the rSAM-SPASM protein generally acts on the peptide first, followed by a peptidase. Rationale for this comes from both the PQQ and MFT biosynthetic pathways, where difficult chemical transformations occur on the peptide. Once the skeleton or topology is set on the peptide, it can be freed and modified by other enzymes.

(2) Peptide-derived alkyl radical generation occurs on sp^3 carbons. While this guideline could change in the future, all reports of rSAM-SPASM enzymes to date begin with alkyl radical formation on sp^3 carbons.

(3) In general, rSAM-SPASM proteins catalyze oxidative reactions, and alkyl radicals combine with electron-rich (e.g., S or O or sp^2 carbon) moieties to form bonds. The only exception thus far is MftC, which catalyzes a decarboxylation reaction and a redox-neutral C−C bond formation.

THE RSAM-SPASM STORY CONTINUES TO EVOLVE

We now know that rSAM-SPASM enzymes are widely used peptide-modifying enzymes, with expanding functionality. The development of new bioinformatic tools has led genome-based discovery of rSAM-dependent RiPPs and has been successfully employed in the discovery of sactipeptides,[47] ranthipeptides,[60] and ryptides,[55] to name a few. Different strategies have been implemented for RiPP mining, such as targeting conserved tailoring enzymes[62−64] or targeting the precursor peptide in their search.[65] For example, BAGEL performs a rule-based strategy, where it implements six-frame translation, which is used to search for the motifs and core peptides and classifies them into the RiPPs class.[66,67] The core peptide is detected by homology to the already known core peptides or the expected properties of the given class.[66] In contrast, RODEO implements profile HMM-based local genomic analysis and precursor peptide/structure prediction by implementing heuristic scoring, motif analysis, and machine learning to detect RiPPs.[65] Notably, the sactipeptide RiPP biosynthetic gene cluster was mapped via the rSAM-SPASM enzymes, which catalyze the sactionine bond by employing the recent improved version of RODEO 2.0.[60] A search was performed to identify candidate proteins using rSAM-SPASM sequences of known sactipeptides (AlbA, SkfB, ThnB, ThnC) and two SCIFFs (CteB, Tte1186).[60] Heuristics with support vector machine classification were employed in the putative precursor peptide search.[60] Then, a sequence similarity network (SSN) using the Enzyme Function Initiative Enzyme Similarity Tool (EFI-EST) is employed, to group the similar enzymes and distinguish them from novel RiPPs, for further precursor peptide analysis.[68] By doing so, Hudson et al. were able to characterize the sactipeptide huazacin and identify hundreds of new sactipeptide BGCs. Interestingly, the rSAM-SPASM protein responsible for the biosynthesis of huazacin is related to QhpD.[60] This association led them to determine that the SCIFF family members freyrasin and thermocellin contain S-C_β and S-C_γ linkages, respectively. Subsequently, ranthipeptides have been designated as a new class of RiPPs, since they are structurally distinct from sactipeptides.[60] Taken together, the work performed by Hudson et al. indicates that the precursor peptide or peptide-related tailoring enzymes could be used as markers in the mining of novel RiPPs.[60,67]

CONCLUSION

Despite the growing interest in rSAM-dependent RiPP natural products, little is known about the full extent of modifications that rSAM-SPASM proteins catalyze. Currently, the field has been relegated to discovering sacti/ranthipeptides, cyclophanes, and ether bonds. Do rSAM-SPASM enzymes install other modifications, and what are the "rules" that they follow? How do

rSAM-SPASM enzymes recognize their substrate? Is there a common mechanism for the electrons being evacuated from the active site? What is it about rSAM-SPASM structures that dictate whether a thioether bond is formed rather than a cyclophane or an ether bond? To fully leverage the promise of RiPP mining toolsets, these questions will need to be answered.

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Author Contributions

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Notes

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