Sensitivity of Radical SAM Enzyme MftC to Molecular Oxygen

Vishnu Govindarajan

Follow this and additional works at: https://digitalcommons.du.edu/etd

Part of the Biological and Chemical Physics Commons
SENSITIVITY OF RADICAL SAM ENZYME MFTC TO MOLECULAR OXYGEN

A Thesis

Presented to

the Faculty of the College of Natural Sciences and Mathematics

University of Denver

________________________

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

________________________

by

Vishnu Govindarajan

March 2020

Advisor: John A. Latham
ABSTRACT

MftC is a radical S-adenosyl L-methionine (SAM) enzyme that catalyzes the first step in the production of mycofactocin. It is a ribosomally synthesized post-translationally modified (RiPP) redox cofactor shown to be essential for the survival of bacteria in the Mycobacterium genus in the presence of cholesterol as a carbon source and for the sequestration of ethanol. MftC catalyzes the C–terminal decarboxylation of tyrosine and the subsequent cross-linking of tyrosine to the penultimate valine on the precursor peptide MftA. The product formed out of the reaction is processed into mycofactocin in downstream processes. The bacteria in the Mycobacterium tuberculosis complex are pathogens that primarily affect mammalian lung systems and are subjected to oxygenated environments. It is also known that the bacteria in Mycobacterium species require highly aerobic conditions to survive. Radical SAM proteins require anaerobic conditions to catalyze reactions as their [4Fe-4S]^{2+} clusters are vulnerable to degradation by oxidation. MftC has to function when Mycobacterium species is present in oxygen rich environments such as lung tissue. Discerning the mechanism by which such an enzyme works in oxygen rich environments could provide insights into radical SAM chemistry. In order to investigate the effect of aerobic condition on the MftC reaction, here we seek to measure and demonstrate the change in efficacy of the MftC reaction after varying levels of oxygen exposure. We show that while the rate of the first part of the MftC reaction is slowed on exposure to oxygen, the second part of the reaction – the C–C cross-linking remains relatively unaffected on oxygen exposure.
# TABLE OF CONTENTS

Chapter One: Introduction........................................................................................................... 1  
  Ribosomally synthesized and post-translationally modified peptides (RiPPs)..........1  
  Radical SAM enzymes in RiPP Biosynthesis........................................................................ 14  
  SPASM subfamily of radial SAM enzymes.......................................................................... 20  
  The Mycofactocin biosynthetic pathway and MftC............................................................ 23  

Chapter Two: Materials and Methods.................................................................................... 27

Chapter Three: Results ........................................................................................................ 34
  Deterioration of [4Fe-4S] clusters of MftC........................................................................ 34
  Uncoupled and full reactions of both anaerobic MftC and oxygenated MftC ....... 35

Chapter Four: Discussion and Summary ............................................................................ 41

References..........................................................................................................................46
LIST OF FIGURES

**Figure 1.** Illustrative schematic of a typical RiPP pathway. The precursor peptide is expressed with an N–terminal region and a C–terminal core which are cleaved off and processed into the final product.

**Figure 2.** Lacticin 3147 α, a representative member of the Lanthipeptide family of RiPP molecules. The PTMS (crosslinks) originating from Cys residues are marked in blue whereas the PTMs (segments) originating from Ser/Thr residues are shown in red.

**Figure 3.** Polytheonamide B – a proteusin is shown here with PTMs are color coded. Epimerization modifications are shown in green, methylation in blue, dehydration in red and hydroxylation in purple.

**Figure 4.** Plantazolicin – a member of LAPs. PTMs are color coded. Thiazoles derived from Cys residues are shown in green while the methyloxazoles derived from Ser/Thr residues are shown in purple.

**Figure 5.** A. Microcyclamide and B. Trunkamide are representative examples of Cyanobactins. The PTMs in Microcyclamide are the heterocyclizations of Cysteine residues which are shown in green and the heterocyclization of a Serine residue showin in purple. The PTMs in Trunkamide are the heterocyclization of cysteine shown in green and prenylations shown in blue.

**Figure 6.** Micrococcin, a representative thiopeptide structure. The thiazole and the thiazoline rings which are the PTMs are shown in red.
Figure 7. Bottromycin A2 – an example of Bottromycin structures.

Figure 8. Specialicin – an example of Lasso peptides isolated from Streptomyces specialis.

Figure 9. General PTM of a class IIb microcin shown in blue. A linear trimer of 2,3-dihydroxybenzoyl L-serine is linked via a C-glycosidic linkage to a β-D-glucose. The glucose is anchored to the carboxylate of the C-terminal serine residues of the peptides shown here in black.

Figure 10. Microviridin B from M. aeruginosa. The Post-translationally modified ω-ester and ω-amide bonds are shown in blue and red respectively.

Figure 11. Subtilosin A, one of the defining members of the sactipeptide family. The residues involved post-translationally modified D-configured thioether bonds are shown in blue while the residues involved in L-configured bonds are shown in red.

Figure 12. Structures of A. PQQ (Pyrroloquinoline quinone) B. Pantocin A and C. Putative structure of MFT (Mycofactocin)

Figure 13. Crystallographic structure of anSME, an rSAM enzyme (PDB ID 4K36) shown in ribbon format. The SAM cluster hosting Triose-phosphate isomerase (TIM) barrel is shown in green while the SPASM domain is shown in purple which hosts both the auxiliary [4Fe-4S] clusters.

Figure 14. The conserved Cysteine motif which co-ordinates the SAM cluster in anSME (PDB ID 4K36) shown with the SAM molecule coordinating the iron-sulfur cluster prior to being cleaved.
Figure 15. Reductive cleavage of S-Adenosyl Methionine (SAM) by the rSAM cluster resulting in a 5’-deAdo• radical.

Figure 16. TsrM, a cobalamin binding rSAM enzyme catalyzes the first step in the Biosynthesis of Thiostrepton where the indole ring of L-Tryptophan is methylated at the C2 position on the precursor peptide TsrA. The modification is shown in red.

Figure 17. Reaction catalyzed by AlbA, an rSAM enzyme harboring three [4Fe-4S] clusters which is catalyzes the thioether bond formation between thiol groups of Cys residues and α- carbon atoms on the precursor peptide SboA as the first step in the biosynthesis of subtilosin A.

Figure 18. Reaction catalyzed by YydG, a two [4Fe-4S] cluster containing rSAM enzyme that catalyzes the epimerization of Val36 and Ile44 on the precursor peptide YydF in the RiPP epipeptide biosynthesis pathway.

Figure 19. Reaction catalyzed by NosL, the second rSAM enzyme in the RiPP nosiheptide biosynthetic pathway which catalyzes the transformation of L – tryptophan into 3 – methyl – 2 indolic acid along with byproducts.

Figure 20. Reaction catalyzed by StrB, a two [4Fe-4S] cluster carrying rSAM enzyme which catalyzes the installation of the Lys-to-Trp crosslink as a step in the Streptide biosynthesis pathway.

Figure 21. SPASM domain of anSME (PDB ID 4K36) shown in purple and in ribbon format. The domain holds both the auxiliary clusters – Aux I and Aux II shown as stick representations and being coordinated by conserved cysteine motifs.
Figure 22. The mycofactocin gene cluster from Mycobacterium tuberculosis strain H37Rv.

Figure 23. Basic reaction scheme of the mycofactocin biosynthetic pathway.

Figure 24. Major steps of the MftC reaction where MftA is converted into MftA*.

Figure 25. Attenuation of 410nm absorbance over 24 hours of MftC in oxygenated buffer. Absorption spectrum reading taken at every 30 minutes for 24 hours and normalized absorption readings at 410 nm (mAu) plotted against time in hours.

Figure 26. Uncoupled SAM cleavage reactions. 5’deoxyadenosine (5’daDo•) radical formation plotted against time. (A) Fully Anaerobic MftC cleavage of SAM. (B) SAM cleavage by MftC incubated in aerobic buffer for 0.5 hours. (C) SAM cleavage by MftC incubated in aerobic buffer for 6 hours. (D) SAM cleavage by MftC incubated in aerobic buffer for 12 hours.

Figure 27. Coupled anaerobic MftC reaction. (A) exponential decay of MftA fitted to a two-phase disassociation model which shows a fast phase and a slow phase over a period of 60 minutes. (B) The panel shows the disassociation of MftA** over 60 minutes and (C) shows the formation of MftA*.

Figure 28. Coupled peptide modification reaction with MftC exposed to oxygen for 3 hours. (A) Decrease in concentration of MftA w.r.t time fitted on to a two-phase-exponential-decay model. (B) Increase and decrease in concentration of MftA** w.r.t time. (C) Increase in concentration of MftA* w.r.t time fitted to a one-phase-association model.

Figure 29. Coupled peptide modification reaction with MftC exposed to oxygen for 6 hours. (A) Decrease in concentration of MftA w.r.t time fitted on to a two-phase-
exponential-decay model. (B) Increase and decrease in concentration of MftA** w.r.t time.
(C) Increase in concentration of MftA* w.r.t time fitted to a one-phase-association model.

**Figure 30.** Major steps of the MftC reaction where MftA is converted into MftA*. 
CHAPTER ONE: INTRODUCTION

Ribosomally synthesized and post-translationally modified peptides (RiPPs)

Natural products can be defined as molecules or substances derived from natural sources and that which are produced biosynthetically [1]. Natural products have been an intrinsic part of nature almost since the beginning of life and have been a major source of bioactive compounds [2] which play major roles in nearly all life processes [3]. Natural products have also played a fundamental role in changing our way of life [4] and have been a source of lifesaving antibiotics throughout history [5]. Examples include the discovery of aspirin in its earliest forms and penicillin [6] to modern therapeutic agents for congestive heart failure and hypertension [7]. Natural products have also been useful in providing inspiration for the development of new materials and photovoltaic cells [8].

Ribosomally synthesized and post-translationally modified peptides (RiPPs) are a class of natural products which have gained considerable significance in the last decade. They differ from non-ribosomally synthesized natural products which require extensive enzymatic complexes for their biosynthesis [9]. Instead, RiPPs are generally biosynthesized by the addition of post-translational modifications (Figure 1) on a core, ribosomally synthesized peptide [10] which enables them to considerably increase their functional repertoire, often rivaling the non-ribosomally produced natural products in their
structural and chemical diversity [11]. Initially, the ribosomally synthesized and post-translationally modified peptides were classified according to the either their biological activities [12] or the organisms they had originated from [13]. The classification system was redefined and the acronym ‘RiPPs’ decided upon for ‘Ribosomally synthesized Post-translationally modified Peptides’ after a consensus was reached by leading researchers in the field on classifying them based on the type and functionality of Post-translational Modifications (PTM) [10]. A size restriction of 10 kDa was also imposed on the classification system for the precursor peptides which undergo the PTM’s to exclude post-translationally modified proteins [10].

The shift of methods for identifying potential natural products from traditional methods of targeted genera selection [14], exploration of organisms found in new ecologies and unexplored environments [15] and enhanced techniques of sensitivity and separation
to genome mining methods [17] have revolutionized both the quality and quantity of RiPPs pathway discoveries.

Following the development of the classification system [10], RiPPs have been classified into subfamilies based on the structural features and functionalities that their biosynthetic pathways confer on them. A brief review of prominent members of the RiPP family, their functions and origins – including mycofactocin are discussed below to add contextual information before discussing the mycofactocin pathway in detail.

**Lanthipeptides**

The term ‘Lanthipeptide’ is an abbreviation which stands for ‘Lanthionine-containing-peptides’ [10]. The defining PTM of this class results in lanthionine (Lan) and 3- methylanthionine (Melan) residues which are formed by two alanine residues crosslinked through a thioether linkage of their β-carbons and an additional methyl group in the linkage respectively [18]. Lanthipeptides were some of the earliest natural products to be identified to have a ribosomal origin [19]. The final Lan and MeLan residues are introduced as PTMs through a two-step process involving a dehydration step and a Michael-type addition [20][21] with the steps usually also involving the formation of a phosphorylated intermediate [22] between the two steps. The cross link generating enzymes usually require the presence of a leader peptide [23] [24] although exceptions to this norm exist [25]. Lanthipeptides have traditionally known to antibiotics as many them are lantibiotics (lanthipeptides functioning as antibiotics) [26]. Emerging research has
however revealed a wider variety of lanthipeptides involved in non-antibiotic roles [27] [28].

![Figure 2. Lacticin 3147 α, a representative member of the Lanthipeptide family of RiPP molecules. The PTMS (crosslinks) originating from Cys residues are marked in blue whereas the PTMs (segments) originating from Ser/Thr residues are shown in red.](image)

**Proteusins**

Proteusins are cytotoxic polytheonamides first reported in 1994 [31]. They feature many D-amino acids in alternation with L-amino acids and feature an unusual lipophilic N-acyl unit [32]. The cytotoxicity of these polytheonamides has its basis in part due to their ability to form membrane channels [32]. The biosynthesis of a typical proteusin molecule begins with the expression of the precursor peptide molecule (PoyA) which undergoes a unidirectional epimerization of specific L – amino acids in the first step [33]. This is followed by the formation of a unique N-acyl residue by the dehydration of a Thr residue [34] [35] which is again followed by the cleavage of the leader sequence by hydrolysis. The post translational modifications happens on 18 residues in the precursor peptide and all the posttranslational modifications including the formation of the unique N-acyl residue
constitute a total of 48 modifications in total which makes Proteusins some of the most post translationally modified RiPP molecules [34].

![Figure 3. Polytheonamide B – a proteusin is shown here with PTMs are color coded. Epimerization modifications are shown in green, methylation in blue, dehydration in red and hydroxylation in purple.](image)

**Linear azol(in)e-containing peptides (LAPs)**

LAP’s were discovered to have RiPP like properties in the year 2000 [36]. They play a significant part in the pathogenesis of *Streptococcus pyogenes* and other strains of various bacteria [37]. The PTMs that characterize this subfamily are azol(in)e rings on the precursor peptide [38]. The biosynthesis of this class of RiPPs begins with the recognition of the precursor peptide and the formation of the enzyme substrate complex which then proceeds on to an ATP dependent cyclodehydration step where a water molecule is expelled from an amide carbonyl forming an azoline heterocycle [39]. The second step in the biosynthesis proceeds with an FMN-dependent dehydrogenase oxidizing all or some of the azolines to azoles which is generally followed by cleavage of the leader peptide from the core precursor peptide [38] [40].
Cyanobactins

Cyanobactins are cytotoxic peptide products found predominantly in marine organisms like the ascidians and cyanobacteria [41]. The first examples of these RiPPs were isolated from Lissoclinum patella [42] and unlike Proteusins, these RiPPs are small, ranging between 6 – 20 amino acids in length with about 200 different cyanobactins identified so far [43]. The biosynthesis of Cyanobactins begins with the usual expression of the precursor peptide containing the leader or recognition sequence, the core sequence and the C – terminal sequence. The Cys, Ser and Thr residues are modified into oxazoline and thiazoline as a first step in the production of the cyanobactins. The N terminal recognition sequence as well as the C terminal sequence are cleaved off to prepare the core peptide for C-N macrocyclization [44]. Some further processing is found in specific instances where incorporation of isoprenoids can occur [45]. Cyanobactins while being cytotoxic, have been known for their transition metal binding properties [46], they have been found to be selectively toxic to certain human cancer cell lines [47] and are also known to act as a blocking agent towards P-glycoprotein [48].

Figure 4. Plantazolicin – a member of LAPs. PTMs are color coded. Thiazoles derived from Cys residues are shown in green while the methyloxazoles derived from Ser/Thr residues are shown in purple.
Thiopeptides are a class of RiPPs whose defining features are the presence of piperidines, dehydropiperidines and pyridines [49]. The biosynthesis of thiopeptides begins with expression of the precursor peptide which undergoes a series of modifications which include the dehydration of Ser and Thr residues to produce Dha and Dhb residues[50] followed by the cyclization of the linear peptide [51]. Thiopeptides are mostly known for their antibacterial properties through impedance of protein synthesis [52]. Certain thiopeptides are also known to exhibit anti-cancer and anti-malarial activity through the induction of apoptosis [53], [54].

Figure 5. A. Microcyclamide and B. Trunkamide are representative examples of Cyanobactins. The PTMs in Microcyclamide are the heterocyclizations of Cysteine residues which are shown in green and the heterocyclization of a Serine residue shown in purple. The PTMs in Trunkamide are the heterocyclization of cysteine shown in green and prenylations shown in blue.

Thiopeptides
**Bottromycins**

Bottromycins are RiPPs that contain C – methylated amino acids, decarboxylated C – terminal thiazole and macrocyclic amidines [55]. The biosynthesis of Bottromycins follows a unique process where the precursor peptide does not have a defined N – terminal recognition sequence but has an extended C – terminal sequence whose function has not been established [56], while the order of the posttranslational modifications is currently unknown, their antimicrobial activity against drug resistant Staphylococci has been well documented [57].
Lasso peptides

Lasso peptides are RiPPs primarily produced by actinobacteria and proteobacteria and are of 16 to 21 amino acid residues in size [60], [61]. The Lasso peptides are named after a characteristic fold present in the final product which confers resistance to proteases and other means of denaturation [62], [63]. The Biosynthesis of lasso peptides occurs through acquisition of the characteristic lasso fold from the precursor peptide and the cleavage of the leader peptide through an ATP dependent process [64], [65]. Lasso peptides are employed as anti-bacterial agents by the producing bacteria to outcompete phylogenetically similar bacteria in colonizing biotopes [66]–[68].
Microcins

Microcins are generally plasmid encoded small, antimicrobial RiPPs usually smaller than 10 kDa in size and are produced by Enterobacteria [58]. Microcins are presently classified into two classes – class I which is composed of microcins below 5kDa in size while class II consists of microcins which are from 5 to 10 kDa in size [59]. Most microcins are produced in a traditional manner that is typical of RiPP biosynthesis where a precursor peptide is expressed with an N – terminal leader domain and a C – terminal
core which undergo post-translational modifications, resulting in the formation of mature microcin peptide [58].

**Figure 9.** General PTM of a class IIb microcin shown in blue. A linear trimer of 2,3-dihydroxybenzoyl L-serine is linked via a C-glycosidic linkage to a β-D-glucose. The glucose is anchored to the carboxylate of the C-terminal serine residues of the peptides shown here in black.

**Microviridins**

Microviridins are RiPPs produced by freshwater cyanobacteria[69], [70] which are primarily produced involved in protease inhibition of elastase[71], subtilisin[72] and trypsin [73]. The Microviridin family consists of N – acetylated peptides that contain Lactams and lactones after undergoing post translational modifications with ω-amide and ω-ester bonds [69], [74].

11
Sactipeptides

Sactipeptides are RiPP molecules which are characterized by the presence of post translational modifications in the form of intramolecular bonds between cysteine sulfurs and the α-carbon of a different residue [75]. The first member of this family – Subtilosin A was discovered in 1985[76] and structurally characterized in 2003 [77]. Subtilosin A
has been reported to have spermicidal and antimicrobial activity over a narrow range of bacteria [78]–[80]. The Biosynthesis of sactipeptides is thought to proceed in a fairly typical manner with the expression of the precursor peptide and the formation of thioether bond formation as the first step as demonstrated in the case of Subtilosin A [81]. The N-terminal leader peptide is then thought to be cleaved off of the C–terminal core followed by a cyclization step as the final step in the production of Subtilosin A [82].

**Figure 11.** Subtilosin A, one of the defining members of the sactipeptide family. The residues involved post-translationally modified D-configured thioether bonds are shown in blue while the residues involved in L-configured bonds are shown in red.

**RiPP redox cofactors and other small RiPP molecules**

An increasing number of small molecules are being discovered which are produced through RiPP biosynthesis, prominent examples of which are pyroloquinone quinone (PQQ), mycofactocin (MFT) and pantocin A [83]–[85]. PQQ is a known redox cofactor used in substrate oxidation which is utilized in the production of cellular ATP via a non-glycolytic pathway in a wide variety of bacterial genera [86] while MFT is a putative redox cofactor shown to be essential for the utilization of cholesterol for metabolism and assimilation of ethanol [87][88]. Pantocin A is a peptide derived antibiotic known to be effective against plant pathogens [85]. All three of the mentioned RiPP products start with
a precursor peptide which is processed further resulting in the final finished RiPP molecule.

The downstream processing of MFT is elaborated in the following segments.

**Figure 12.** Structures of A. PQQ (Pyrroloquinoline quinone) B. Pantocin A and C. Putative structure of MFT (Mycofactocin)
Radical SAM enzymes in RiPP Biosynthesis

Radical S-adenosyl methionine (rSAM) enzymes are characterized by the presence of a conserved Cx₃Cx₂C motif which coordinates a redox active [4Fe-4S]²⁺ cluster and were categorized as a superfamily early in 2001 [89]. They are ubiquitous and are present in all phylogenetic kingdoms of life. The enzymes of this superfamily tend to generally adopt a partial triose-phosphate isomerase fold or (TIM) barrel fold (β/α)₆ whose C or N terminals can have different customized extensions for specific reactions or structures [10]. An example of a rSAM enzyme is shown in Figure 13 which depicts the structure of the Anaerobic sulfatase maturating enzyme (anSME), a homolog to MftC, the radical SAM protein employed by the mycofactocin pathway, determined by X-ray crystallography (PDB ID: 4K36).

![Figure 13. Crystallographic structure of anSME, an rSAM enzyme (PDB ID 4K36) shown in ribbon format. The SAM cluster hosting Triose-phosphate isomerase (TIM) barrel is shown in green while the SPASM domain is shown in purple which hosts both the auxiliary [4Fe-4S] clusters.](image-url)
The characteristic Cx3Cx2C motif binds three out of the four iron atoms in the [4Fe-4S]2+ cluster and the fourth iron is labile and is referred to as the unique iron. The unique iron of the SAM cluster is coordinated by the amino and carboxylate moieties of S-adenosyl methionine (SAM) (Figure 14) and this complex forms the defining part of the whole catalytic machinery of rSAM enzymes [90].

![Figure 14. The conserved Cysteine motif which co-ordinates the SAM cluster in anSME (PDB ID 4K36) shown with the SAM molecule coordinating the](image)

Radical S-adenosyl methionine enzymes facilitate a homolytic reductive cleavage of SAM which generates a methionine and a 5’deoxyadenosine radical (Figure 15) [91]. The highly reactive 5’deoxyadenosine radical in turn abstracts a hydrogen atom from the substrate molecule in a regionally specific and a stereospecific manner [92]. This mechanism is employed by MftC. The substrate radical which is typically formed on
precursor peptide usually undergoes further modifications by downstream enzymes before becoming the final product.

![Image of S-Adenosyl Methionine (SAM) with a reaction](image)

**Figure 15.** Reductive cleavage of S-Adenosyl Methionine (SAM) by the rSAM cluster resulting in a 5'-deAdo● radical

There are several RiPP pathways which employ rSAM enzymes as important steps in RiPP maturation and production [93]. Different RiPP pathways employ rSAM enzymes for different ends with varied functionalities [94] and can be loosely grouped into categories based on the type of reactions and Post-translational Modification (PTMs) that they catalyze [95]. Based on all the rSAM enzymes characterized so far, they are broadly grouped into enzymes that catalyze C – methylation[96], thioether bond formation[75], epimerization[97], rearrangement, C – C bond formation[98] and decarboxylation[95].

C – methylation reactions catalyzed by rSAM enzymes proceed through the methylation of nucleophilic (e.g.) and non-nucleophilic (e.g.) positions in the precursor peptides in the process of the production of a RiPP molecule [35], [99]–[101]. Radical S-adenosyl methionine enzymes that catalyze C – methylation reactions have been grouped
subclasses which include class A which utilize active site cysteines for the methylation reactions, class B includes enzymes with an N–terminal cobalamin binding domain, enzymes in Class C employ two SAM molecules to perform methyl transfer and Class D enzymes are characterized by the use of methylenetetrahydrofolate as methyl donors in the methyl transfer reactions [102]. Examples of RiPP pathways that employ C–Methylation reaction catalyzing rSAM enzymes are the Thiostrepton biosynthesis pathway [103][104][105], Polytheonamide biosynthesis [34][106][107], Nosiheptide biosynthesis [108][109][110] and Thiomuracin biosynthesis [111][112][113].

**Figure 16.** TsrM, a cobalamin binding rSAM enzyme catalyzes the first step in the Biosynthesis of Thiostrepton where the indole ring of L-Tryptophan is methylated at the C2 position on the precursor peptide TsrA. The modification is shown in red.

Thioether bond formation reactions in RiPP biosynthesis involve the formation of bonds between the sulfur atom in Cys residues to either α–carbon of an acceptor amino acid [114] or the β–carbon of Ser/Thr residues [115]. Examples of pathways where rSAM enzymes are used for catalyzing thioether bond formation include Subtilosin A biosynthesis[116], Sporulation killing factor biosynthesis[117], Thurincin H biosynthesis [118][119] and SCIFF peptide biosynthesis[120][121].
Epimerization reactions, as the name implies involves the catalyzation of introduction of D-amino acids into RiPP molecules by rSAM molecules. The most prominent examples of rSAM molecules that facilitate these reactions are PoyD from the Proteusin biosynthesis pathway [34][107], YydG from the Epipeptide biosynthesis pathway [122].
Rearrangement reactions are characterized by extensive rearrangements catalyzed by rSAM enzymes upon substrate radicals as the name suggests. One of the most important examples of rSAM enzymes of this category are again Nosipeptides [123].

![Figure 19](image1.png)  
**Figure 19.** Reaction catalyzed by NosL, the second rSAM enzyme in the RiPP nosiheptide biosynthetic pathway which catalyzes the transformation of L-tryptophan into 3-methyl-2 indolic acid along with byproducts.

C–C crosslinking modifications are post-translational modifications where peptide crosslinks are catalyzed by the rSAM enzyme encoded in the RiPP pathway. The most common examples of pathways involving rSAM enzymes that catalyze peptide crosslinks are the Streptide biosynthesis[124][125] and the Pyrroloquinoline quinone (PQQ) biosynthesis pathways[83][126]. The PQQ pathway is also more extensively discussed in the next section.

![Figure 20](image2.png)  
**Figure 20.** Reaction catalyzed by StrB, a two [4Fe-4S] cluster carrying rSAM enzyme which catalyzes the installation of the Lys-to-Trp crosslink as a step in the Streptide biosynthesis pathway.
C – C crosslinking and Decarboxylation reactions are found to be catalyzed together in single pathway in only one RiPP pathway so far – the Mycofactocin pathway [84] which is discussed in detail in the next sections.

**SPASM subfamily of radial SAM enzymes**

The SPASM subfamily of rSAM enzymes was named after the RiPP pathways which produce Subtilosin A, Pyrroloquinoline quinone, Anaerobic sulfatase, and Mycofactocin which involve the rSAM enzymes AlbA, PqqE, anSME and MftC respectively in their pathways [127]. These rSAM enzymes share a C terminal domain which has a CX$_{9-15}$GX$_4$C-gap-CX$_2$CX$_5$CX$_3$C-gap-C (seven cysteine) motif [128] which coordinates two other [4Fe-4S]$^{2+}$ clusters which termed as Auxilliary cluster I (Aux I) and Auxilliary cluster II (Aux II) and they are involved in the modification of the precursor peptide of the pathway [129] [128] [130].

An example of a rSAM enzyme in the SPASM family is anSME from *Clostridium perfringens*. AnSME facilitates the catalyzation of post-translational modification of arylsulfatase active site residues of either cysteine or serine into catalytically important formylglycine moiety [53]. This sulfatase activity is found to be important for both humans and anaerobic bacteria with malfunction or lack of enzymatic activity resulting in disease in humans [131]. The molecular structure of AnSME has been determined through X-ray crystallography up to a resolution of 1.8 Å (Figure15) [132]. The N terminal portion of the protein shows a partial triose-phosphate isomerase fold ($\beta/\alpha$)$_6$ (TIM) barrel fold which
spans from residue 3 to 324 and this domain is termed as the AdoMet domain as it contains the rSAM cluster coordinated by a CX$_3$CXΦC motif (C15, C19 and C22). In addition to the core TIM barrel, there is also a C terminal extension which shows two auxiliary [4Fe-4S]$^{2+}$ clusters coordinated by the seven cysteine motif in which the Aux I cluster is coordinated by C255, C261 and C276 and the Aux II cluster is coordinated by C317, C320 and C326 from the second part of the seven cysteine motif (Figure 10). Hence the iron-sulfur clusters are spread in such a way that the ‘SAM cluster’ is situated near the N terminus, the Aux I cluster is situated near the middle of the protein sequence and the Aux II cluster is situated near the C terminus of the protein. The distances between the SAM cluster and the Aux I cluster is 16.9 Å, the distance between the Aux I cluster and the Aux II cluster being 12.9 Å and the distance between AuxII cluster and the SAM cluster comes to 26. AlbA and SkfB are two rSAM enzymes involved in the biosynthesis of sactipeptides subtilosin A and sporulation killing factor respectively [81] [133]. AlbA belongs to the SPASM subfamily of rSAM enzymes as it contains two auxiliary iron-sulfur

**Figure 21.** SPASM domain of anSME (PDB ID 4K36) shown in purple and in ribbon format. The domain holds both the auxiliary clusters – Aux I and Aux II shown as stick representations and being coordinated by conserved cysteine motifs.
clusters whereas SkfB has only one auxiliary cluster which makes it a member of another subfamily of rSAM enzymes known as the TWITCH whose members contain only one iron-sulfur cluster in their C terminal extensions. AlbA catalyzes the formation of thioether bonds between sulfur atoms of three cysteine residues and the α-carbons of two phenylalanines and one threonine. This reaction forms an essential part of the subtilosin A biosynthesis and maturation [134]. Sactipeptides, as mentioned previously, are produced by soil bacteria such as Bacillus subtilis which have been known to show antibacterial activity against pathogens, gram-negative and gram-positive bacteria [80].

PqqE is an rSAM enzyme in the PQQ biosynthesis pathway, it is a founding member of the SPASM subfamily of rSAM enzymes and is an essential part of the PQQ biosynthesis pathway. PQQ, as mentioned previously, is a redox cofactor involved in an alternate non glycolytic pathway in the production of ATP [86]. It is produced in a wide variety of bacterial genera and is strongly conserved across most of them [135].
The mycofactocin biosynthetic pathway and MftC

Mycofactocin (MFT) is a putative redox cofactor whose biosynthesis is encoded by the mftABCDEF gene cluster (Figure 22). These genes occur in all-or-none fashion in nearly six hundred species of bacteria and especially concentrated in the Mycobacterium genera[136], [137]. Mycofactocin was also recently shown to be important for ethanol assimilation and redox metabolism in Mycobacterium smegmatis [88].

![Diagram of the mycofactocin gene cluster from Mycobacterium tuberculosis strain H37Rv.](image)

MftA is a ribosomally synthesized precursor peptide that contains a highly conserved C-terminal IDGXCGVY sequence. MftB is a small peptide chaperone with predicted homology to the RiPP recognition element (RRE) PqqD in the PQQ biosynthesis pathway [138]. MftC is a rSAM protein belonging to the SPASM subfamily which is also one of its founding members. The enzyme catalyzes the oxidative decarboxylation and carbon – carbon bond formation in the precursor peptide MftA [138], which comprises the first step in the biosynthesis of mycofactocin. MftE is a peptidase [139] that cleaves the leader peptide off the precursor peptide and MftD is an FMN dependent oxidative deaminase [140] which installs the redox center on mycofactocin.
MftC, as mentioned earlier, is a rSAM enzyme catalyzing the SAM dependent oxidative decarboxylation of the C-terminal tyrosine on the precursor peptide MftA. This reaction is followed by another SAM dependent reaction which results in a C – C bond formation between Cβ of the penultimate valine and Cα of the C terminal tyrosine forming a 3-amino-5-[(p-hydroxyphenyl) methyl]-4,4-dimethyl-2-pyrrolidinone moiety or AHDP, hereafter referred to as MftA*.

MftC has been confirmed to have three [4Fe-4S]²⁺ clusters as other members in the SPASM subfamily [141] and it has been shown that while the auxiliary clusters are not needed for SAM turnover, they are required for the coupled reaction for the modification of the precursor peptide. It should also be noted that while all other members of rSAM
family of proteins catalyze net oxidative reactions on the peptide substrate, MftC is unique in that it accommodates both oxidative and redox neutral reactions while modifying the precursor peptide MftA [141]. A brief schema of the reaction mechanism is shown below:

![Figure 24. Major steps of the MftC reaction where MftA is converted into MftA*](image)

Typically, [4Fe-4S]^{2+} clusters are oxygen sensitive and can quickly degrade in the presence of oxygen in the solvent or when exposed to air[142]. Many rSAM proteins are usually found to have [3Fe-4S]^{+} clusters in as-isolated state or when exposed to air [143]. Although reductants like dithiothreitol help with scavenging iron out of the solvent to reform the [4Fe-4S]^{2+} cluster, it is still difficult to initiate reactions in an oxygenated environment.

*M. tuberculosis* infects a highly oxygen rich environment[144][145] and under hypoxic conditions enters stationary phase growth[146]. The effect of oxygen on [4Fe-4S] clusters calls into question the tolerance that MftC has towards oxygen. Investigating the effect of oxygen on rate of degradation of clusters in MftC and the subsequent effect on the rate of the peptide modification has potential to shed light on its in vivo mechanism of
action in the *M.tuberculosis* bacterium when it infects a highly oxygen rich environment as the tissue of the alveolar air sacs [147]. Investigating aerobic degradation of the iron sulfur clusters in MftC can also give further insights into the function of the SPASM family of rSAM proteins and lay foundations for predicting new SPASM like rSAM proteins discovered in the future.
CHAPTER TWO: MATERIALS AND METHODS

Expression and purification of MftC:

The MftC gene (Uniprot: A0PM49) was co-transformed in the BL21 Star(DE3) cell line with suf operon plasmid pH151. An overnight culture from the transformed cells was used to inoculate 4 L of TB (Terrific Broth) medium. The cells were grown at 215 RPM and 37°C till an OD<sub>600</sub> measurement of 0.7 to 0.8. The culture was then cooled to a temperature of ~21°C and protein production was induced by adding 1 mM IPTG (Isopropyl β-d-1-thiogalactopyranoside), 50µM of FeCl₃ (iron (III) chloride) and 0.75g/L Na<sub>2</sub>C₄H₂O₄ (sodium fumarate). The cells were then incubated overnight at 21°C for 16 – 18 hours. Cells were harvested by centrifugation at 6500 g for 10 min and the cell pellets were flash frozen and stored at −80°C for further use.

The frozen cell pellets were thawed inside an anaerobic chamber and suspended in 5 times the cell pellet weight of lysis buffer (50 mM HEPES, 300 mM NaCl and 40 mM Imidazole). Lysozyme (0.5% w/v), CHAPS (0.75% w/v) and DNAse (Bovine Pancreas, 0.05mg/g of thawed cells) were added and the suspension was stirred for 25 min at room temperature inside the anaerobic chamber. The cell suspension was sealed in centrifuge tubes, removed from the chamber, centrifuged at 13000 g for 10 min, and transferred back into the anaerobic chamber where an ÄKTA Start FPLC system was used to load the
supernatant onto two Ni\(^{2+}\)His-trap columns (GE Life Sciences). The columns were washed with 5 column volumes of lysis buffer (50 mM HEPES, 300 mM NaCl and 40 mM Imidazole) to wash out nonspecifically bound protein matter and other cell debris. The columns were then washed with elution buffer (50 mM HEPES, 300 mM NaCl and 300 mM Imidazole), the UV absorbance of the eluate was monitored, and appropriate fractions were collected, pooled and buffer exchanged into storage buffer (50 mM HEPES, 300 mM KCl, 10 mM DTT and 10% v/v Glycerol).

**Reconstitution of MftC:**

Reconstitution of the purified MftC fractions were carried out entirely inside the anaerobic chamber immediately after purification. All reagents used in the reconstitution procedure were taken into the chamber in powder form and dissolved in deoxygenated DI water. DTT (10 mM) was added to the purified protein and the solution was stirred at 4°C for 10 min followed by the addition of 12-fold protein concentration of FeCl\(_3\) and stirred again for 10 min at 4°C. This followed by an addition of 12-fold protein concentration of Na\(_2\)S and the solution was incubated at 4°C while being stirred gently for 45 min. The solution was then centrifuged at 14000 g for 5 min to separate aggregated protein and the supernatant containing active protein was buffer exchanged into storage buffer (50 mM HEPES, 300 mM KCl, 10 mM DTT and 10% v/v glycerol) using PD-10 columns (GE Life Sciences). The protein solution was then concentrated using pall 30 kDa spin columns, divided into 50µl aliquots, flash frozen and stored at −80°C.

29
Determining the concentration of MftC:

The final concentration of MftC in solution was determined by recording absorbance at 280 nm, using a molar extinction Coefficient of 39420 M$^{-1}$·cm$^{-1}$ (assuming cysteines are reduced). The molar extinction coefficient for MftC was computed using the ExPASy ProtParam web tool [1]. All the absorbance measurements were carried out in a Shimadzu UV – 1800 UV-visible spectrophotometer.

Iron sulfur quantification of MftC:

The quantification of iron and sulfur content of reconstituted protein was conducted according to the procedure previously reported [66]. After determining the concentration of the protein through Bradford assay, 100 µl of 20 µM protein was mixed with 10 µl of 3M TCA and the protein precipitate was centrifuged at 15,000g for 10 mins and the supernatant is removed and diluted with 330 µl of diH$_2$O. A volume of 20 µl of 10mM sodium ascorbate, 20 µL of 10 mM ferrozine, and 20 µl of saturated sodium acetate (room temperature) were added to the solution and the absorbance change due to the ferrozine–iron complex was measured at 562 nm (ε = 27.9 mM$^{-1}$ cm$^{-1}$).

The sulfur concentration was measured through mixing 200 µl of 10 µM was added to 600 µL of 1% (w/v) zinc acetate and 50 µL of 7% (w/v) sodium hydroxide. The solution was allowed to rest at room temperature and 150 µL of 0.1% (w/v) N, N-dimethyl-p-phenylenediamine (in 5 M HCl) and 150 µL of 10 mM FeCl$_3$ (in 1 M HCl) were added. The solution was vortexed and incubated at room temperature for 20 min. The absorbance
from methylene blue was measured at 670 nm. Blanks were made for both measurements using diH₂O instead of protein.

**Expression and Purification of GST-His MftB:**

A previously constructed pGEX6p-1 plasmid construct containing the *mftB* gene (Uniprot: A0PM48) out of which the first 20 amino acids were truncated to reflect the updated Uniprot annotation was fused with GST (Glutathione-S-Transferase) with an C-terminal His tag was transformed into T7 Express lysY/Iq competent cell line and was allowed to grow overnight on agar plates with appropriate antibiotic selection. An overnight starter culture from the plate grown at 37°C was used to inoculate 4L of LB medium and incubated at 37°C and 215 RPM till the culture reached an OD₆₀₀ measurement of 0.6 to 0.8. The culture was cooled to 21°C and induced by adding 1mM IPTG (Isopropyl β- D -1-thiogalactopyranoside) and incubated overnight for a period of 16 to 18 hr at 215 RPM at 21°C. The cells were then centrifuged at 6500 g for 10 min to spin down the cells in the growth medium. The cells were then aliquoted, flash frozen and stored in the −80°C.

The purification of *mftB* was conducted inside the anaerobic chamber. The cells were thawed inside the anaerobic chamber and resuspended in 5-fold cell pellet volume of lysis buffer (50 mM HEPES, 300 mM NaCl, 40 mM Imidazole). Lysozyme (0.5% w/v) and DNAse (Bovine Pancreas, 0.05 mg/g of thawed cells) were added and the solution was stirred at room temperature for 20 min. The cells suspension was then sonicated and centrifuged at 13000g for 10 mins and loaded onto two 5 ml Ni²⁺ NTA His columns (GE
Life Sciences). The columns were then washed with 5 column volumes of lysis buffer (50 mM HEPES, 300 mM NaCl, 40 mM Imidazole) and then with the elution buffer (50 mM HEPES, 300 mM NaCl and 300 mM Imidazole). The UV absorbance of the eluant was monitored and appropriate fractions were collected and pooled. The eluted protein samples were buffer exchanged into a storage buffer (50 mM HEPES, 250 mM KCl, 1 mM TCEP and 10% v/v Glycerol) using PD-10 columns (GE Life Sciences). The final protein solution was concentrated using pall 30 kDa columns and aliquoted into 50 µL aliquots, flash frozen and stored at −80°C.

**Iron-sulfur Cluster degradation assay:**

An initial absorption spectra reading was taken from 220 nm to 500 nm for 50 µM of purified and reconstituted MftC in anaerobic storage buffer without a reference spectrum reading. Then an aerobic storage buffer without any reductant was prepared (50 mM HEPES, 300 mM KCl and 10% v/v Glycerol). Approximately 10 µL of 2.5 mM MftC was diluted into 490 µL of aforementioned oxygenated buffer mixed thoroughly and brought up to a final concentration of 50 µM in a quartz cuvette which was then sealed in an airtight manner. Absorption spectra readings from 220 nm to 500 nm were taken every 30 minutes for 24 hours. This experiment was performed in triplicate and change in 410 nm absorbance was noted and recorded at the end of the experiment.
Time course reactions for uncoupled (without co-substrate MftA and MftB) SAM cleavage reaction with wildtype MftC (anaerobic MftC):

All reactions were conducted in the anaerobic chamber. All the reagents were prepared as described above. The reactants were mixed in the following order: anaerobic reaction buffer (50 mM HEPES, 300 mM KCl and 10% v/v glycerol), 10 mM DTT, 1.1 mM SAM, 100 µM MftC and 4 mM DTH. The time course reactions were conducted over 24 h at room temperature. The formation of 5’deoxyadenosine was measured at designated time intervals.

Time course reactions for uncoupled oxygenated SAM cleavage reaction with oxygenated MftC:

All reactions were conducted in the anaerobic chamber. Purified and reconstituted MftC was buffer exchanged into an oxygenated buffer (50 mM HEPES, 300 mM KCl and 10% v/v glycerol) and incubated at 4°C for 0.5, 6, and 12 h in separate aliquots. Uncoupled SAM cleavage reactions were set up with each of the different oxygenated samples after buffer exchanging them back in anaerobic buffer (50 mM HEPES, 300 mM KCl and 10% v/v glycerol) containing reductant (DTT) to stop further decay of the iron sulfur clusters[148]. The reactions were set up in the same manner as described in the previous experiment and the same time course experiment was performed for all of the oxygenated varieties in triplicate and formation of 5’deoxyadenosine was monitored using reverse phase HPLC.
**MftA modification reaction with anaerobic MftC:**

A truncated version of MftA (12-30) was bought in synthesized form from GenScript Biotech Corporation and was used in further experiments. All reactions were carried out in the anaerobic chamber and the reactants were added as follows: the reaction buffer (50 mM HEPES, 300 mM KCl and 10% v/v glycerol), 10mM DTT, 200 µM MftA, 205 µM MftB, 800 µM SAM, 350 µM MftC and 4 mM DTH. A time course reaction was set up and aliquots were taken at specific intervals to facilitate measurement of initial rates of formation of MftA**, MftA*, and the decrease of MftA. These were measured through reverse phase HPLC.

**MftA modification reactions with oxygenated species of MftC:**

All experiments were set up inside the chamber as above with the same synthesized version of (12-30) MftA. MftC used in this reaction was buffer exchanged into oxygenated buffer (50 mM HEPES, 300 mM KCl and 10% v/v glycerol) and allowed to incubate 3, 6 and 12 h at 4℃ before being buffer exchanged in anaerobic buffer (50 mM HEPES, 300 mM KCl and 10% v/v glycerol) with DTT as stated in the previous experiments to prevent further oxygen decay. The reactants were added as follows: the reaction buffer (50 mM HEPES, 300 mM KCl and 10% v/v glycerol), 10mM DTT, 200 µM MftA, 205 µM MftB, 800 µM SAM, 350 µM MftC and 4mM DTH. Time course reactions were conducted with each of the oxygenated species and initial rates of formation of MftA**, MftA* and the rate of decreasing MftA was measured through reverse phase HPLC.
CHAPTER THREE: RESULTS

Deterioration of [4Fe-4S] clusters of MftC

It was theorized that since MftC has three iron sulfur clusters (one SAM and two auxiliary clusters) oxidative degradation of MftC would proceed in distinct stages with degradation of one cluster at a time. To test this, the decline in absorbance at 410 nm was measured over 24 h. Specifically, [4Fe-4S] clusters show absorbance specifically at 410 nm and the attenuation of the absorbance shoulder at 410 was taken as a sign of cluster degradation. It was seen that rather than a step wise degradation or distinct stages of the 410 nm absorbance attenuation, the absorbance signal decreased in a steady manner over 24 hours (Figure 25).

![Figure 25](image.png)

Figure 25. Attenuation of 410nm absorbance over 24 hours of MftC in oxygenated buffer. Absorption spectrum reading taken at every 30 minutes for 24 hours and normalized absorption readings at 410 nm (mAu) plotted against time in hours.
Since distinct phases of degradation in the 410 nm absorbance were not observed, it could be that there is no specific order for the degradation of the three clusters and the overall attenuation of the 410 nm absorbance signal is the result of aggregate of populations of MftC undergoing degradation of different clusters simultaneously. The rate of degradation was found to be $0.2 \pm 1\times10^{-2} \text{ min}^{-1}$ after fitting the data on to a one-phase-decay model. It was expected that the rSAM cluster and the Aux I clusters undergo degradation faster than Aux II cluster as it might be nestled in the middle of the protein sequence and therefore shielded better from molecular oxygen in the solution compared to the other two clusters. Even if multiple phases of degradation are considered, the distinct phases cannot be gleaned from the present data as the data reflects the average degradation of different populations and phases of degradations.

**Uncoupled and full reactions of both anaerobic MftC and oxygenated MftC.**

Biosynthesis of mycofactocin was shown to be crucial for the persistence of infection by *M. tuberculosis* while present in the alveolar tissue. In addition, the pathway was shown to be crucial for cholesterol utilization as an energy source which is a crucial energy source while enveloped by macrophages [149]. The alveolar tissue is a very oxygen rich (concentration) environment however the first step in the mycofactocin biosynthesis is the post-translational modification of the precursor peptide MftA in the mycofactocin pathway by the rSAM enzyme MftC.
It was expected that even though MftC was an rSAM enzyme, which are typically oxygen sensitive, the enzyme would have to be at least partially tolerant of oxygen to function in an oxygen rich environment such as the alveolar tissue, which might introduce oxygen rich species into the cell cytosol even if the cytosol in general is reducing in nature [150]. Hence the rate of reactions catalyzed by MftC in various states of oxidative cluster degradation were tested. The purified, reconstituted protein was buffer exchanged into oxygenated buffer incubated for specific intervals of time before being buffer exchanged back into anaerobic buffer to initiate uncoupled (without MftA and MftB) and coupled (with all the co-substrates) full reactions.

![Figure 26. Uncoupled SAM cleavage reactions. 5’deoxyadenosine (5’dAdo•) radical formation plotted against time. (A) Fully Anaerobic MftC cleavage of SAM. (B) SAM cleavage by MftC incubated in aerobic buffer for 0.5 hours. (C) SAM cleavage by MftC incubated in aerobic buffer for 6 hours. (D) SAM cleavage by MftC incubated in aerobic buffer for 12 hours.](image)

Uncoupled SAM cleavage reactions were performed in triplicate inside the anaerobic chamber with anaerobic MftC, MftC incubated in oxygenated buffer for 0.5, 6, and 12 h. The time course experiments showed a highly unusual result where the reaction
rate was sped up with exposure to oxygen. The pseudo-first-order rate constant for the SAM cleavage reaction with fully anaerobic MftC (Figure 26 A) was $4.32 \pm 1.08 \text{ min}^{-1}$, whereas the pseudo-first-order rate constants for 0.5, 6, and 12 h aerobic buffer incubated MftC SAM cleavage reactions (Figure 26 B-D) were $30.72 \pm 4.98 \text{ min}^{-1}$, $13.2 \pm 0.9 \text{ min}^{-1}$ and $14.94 \pm 2.28 \text{ min}^{-1}$ respectively. A number of explanations can be postulated for this result and the most prominent among them are structural changes due to degradation of the Aux I and the Aux II clusters which might cause conditions mimicking a coupled reaction.

![Figure 26](image)

**Figure 26.** Coupled anaerobic MftC reaction. (A) exponential decay of MftA fitted to a two-phase disassociation model which shows a fast phase and a slow phase over a period of 60 minutes. (B) The panel shows the disassociation of MftA** over 60 minutes and (C) shows the formation of MftA*.

In the anaerobic coupled reaction (Figure 27) the conversion of MftA has a fast and a slow process. The fast decay step corresponds with the sharp increase in MftA** accumulation in the beginning of the reaction. The slow steps of the decay of MftA** and the final accumulation of MftA* occur in the same timeframe indicating that the C–C bond formation – the latter, seemingly slower step might be the rate limiting step of the whole peptide modification reaction. The first five data points were taken within a minute of initiation of reaction and the fast decay step happens within the first ten seconds of reaction.
initiation. As a result, even with a two-phase-decay model used for fitting the data which allows the inclusion of a fast step and a subsequent slow step, there were not enough initial data points to assign a very accurate upper limit for the confidence interval for the fast step and as a result, the rate constant for the fast step produced by the fitting $k_{\text{fast}} = 13.33$ min$^{-1}$ has to be taken with relative caution.

The fit was however able to obtain a relatively accurate rate constant for the slow step of the decay and it was calculated to $K_{\text{slow}} = 0.105 \pm 6 \times 10^{-2}$ min$^{-1}$. The rate of formation of MftA** was within the ten seconds of the reaction initiation and hence only the rate of decay of the MftA** was calculated by fitting the data to a one-phase-decay model. The rate constant of the MftA** was calculated to be $0.068 \pm 0.013$ min$^{-1}$. The data for the rate of formation of MftA* was fitted on to a two-phase-association model and the rate of formation of MftA*, $k = 0.074 \pm 0.02$ min$^{-1}$ was found to be comparable to the rate of MftA** decay. This is consistent with the proposed mechanism of reaction where the precursor peptide gets converted into MftA** after the oxidative decarboxylation step and then into MftA* where a C – C bond is conferred on the C-terminal of the peptide.

![Figure 28](image)

**Figure 28.** Coupled peptide modification reaction with MftC exposed to oxygen for 3 hours. (A) Decrease in concentration of MftA w.r.t time fitted on to a two-phase-exponential-decay model. (B) Increase and decrease in concentration of MftA** w.r.t time. (C) Increase in concentration of MftA* w.r.t time fitted to a one-phase-association model.
The coupled reaction was seen to have lowered reaction rates when MftC was exposed to oxygen. Figure 28 shows reactions involving MftC that had been incubated in oxygenated buffer for three hours before being used in a coupled reaction. Panel A in the figure shows the decrease in concentration of MftA with time and again a two-phase-decay model was used to fit the data through which an accurate rate for the slow step was calculated - $K_{\text{slow}} = 0.122 \pm 7 \times 10^{-2}$ min$^{-1}$. The rate of formation and decay of MftA** could not be properly plotted as the absorbance data became inconsistent for MftA** when oxygenated MftC was used in the reactions, however the rate constant for the formation of MftA* could calculated with the data being fitted on to a one-phase-association model. The rate constant for the formation of MftA* was found to be $k = 0.063 \pm 0.015$ min$^{-1}$. It was noted that the rate constant for the formation of MftA* in this experiment with the oxygenated buffer was comparable to the rate constant of MftA* formation in the previous reaction with completely anaerobic MftC.

**Figure 29.** Coupled peptide modification reaction with MftC exposed to oxygen for 6 hours. (A) Decrease in concentration of MftA w.r.t time fitted on to a two-phase-exponential-decay model. (B) Increase and decrease in concentration of MftA** w.r.t time. (C) Increase in concentration of MftA* w.r.t time fitted to a one-phase-association model.
The results of the coupled peptide modifying reaction with MftC that was oxidized longer is consistent with the predicted trend of slowing down of reactions with increasing oxidative degeneration of MftC. The above figure (Figure 29) shows the results of a coupled reaction set up with MftC that had been incubated in oxygenated buffer for 6 hours. The rate of decrease in the concentration of MftA when fitted to two-phase-decay model was found to be $0.088 \pm 0.02 \text{ min}^{-1}$ which is slower than the rate of decay in the experiment with MftC incubated in oxygenated buffer for 3 hours (Figure 28). The rate of formation and decay of MftA** could not be calculated for the same reasons as mentioned in the previous experiment. The rate of MftA* formation however was calculated when the data was fitted to a one-phase-association model and it was found to be $0.061 \pm 0.02 \text{ min}^{-1}$.

It is to be noted that the rate of formation of MftA* for all three reactions with anaerobic MftC and with MftC incubated in oxygenated buffer for 3 hours and 6 hours was very comparable and nearly the same.

This points to possibility that the rate of C – C bond formation reaction instituted by the MftC on MftA** to modify it into MftA* is not affected by the oxidation of MftC whereas the conversion of MftA into MftA** is affected by oxidation. An inverse relationship is seen with the decrease in the rate of decay of MftA with increasing oxidative degradation of MftC.
CHAPTER FOUR: DISCUSSION AND SUMMARY

MftC is a rSAM enzyme in the mycofactocin biosynthesis pathway and is responsible for post-translationally modifying the precursor peptide MftA as the first step in the Mycofactocin biosynthesis[138]. MftC also has been confirmed to have three [4Fe-4S] clusters out of which the rSAM cluster is shown to be essential for the reductive, homolytic cleavage of SAM and the additional two clusters Aux I and Aux II clusters are shown to be involved in peptide modification while not being essential for SAM cleavage[136]. MftC also belongs in the SPASM subfamily of proteins of which it is a founding member[151]. MftC catalyzes the formation of a C – C bond between Cα carbon of the N terminal tyrosine and Cβ of the penultimate valine in the C terminus of the precursor peptide. MftC utilizes two equivalents of SAM to complete the oxidative decarboxylation of the tyrosine followed by the C – C bond formation both of which are SAM dependent. MftC by the nature of its oxygen sensitivity is thought to only catalyze reactions in an anaerobic environment as other types rSAM enzymes[152].
The [4Fe-4S] clusters show absorbance at 410 nm and incubation of purified and reconstituted MftC in oxygenated buffer has shown to result in attenuation of 410 nm absorbance which is indicative of degradation of the [4Fe-4S]$^{2+}$ clusters. There were no distinct phases or segments and the deterioration of the signal was relatively uniform and the rate of signal decay was found to be $0.20 \pm 0.06 \times 10^{-2} \text{ min}^{-1}$.

The effect of oxidation of MftC was tested on rate of uncoupled SAM cleavage reactions and the rate of 5’dA radical formation was counterintuitively found to be enhanced after exposure to oxygen. The rate of 5’dA production with completely anaerobic conditions was $4.32 \pm 1.08 \text{ min}^{-1}$ whereas the 5’dA production rate was increased several orders of magnitude to $30.72 \pm 4.98 \text{ min}^{-1}$, $13.2 \pm 0.9 \text{ min}^{-1}$ and $14.94 \pm 2.28 \text{ min}^{-1}$ for the 0.5 hour, 6 hours and 12 hours oxygen exposed MftC respectively. The rate seems to have decreased after prolonged oxidation as seen in the case of the 6- and 12-hour oxygen incubated samples after the initial increase (0.5 hr of oxygen incubation). Since the enzyme still continues to function even after 12 hours of oxygen exposure, it is highly likely that

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure30.png}
\caption{Major steps of the MftC reaction where MftA is converted into MftA*}
\end{figure}
the r-SAM cluster or the Adomet cluster is solvent protected and does not have extensive contact with the solvent for it to be degraded even after extensive time periods of oxygen exposure. There are many factors which influence the reaction rate and initiation of the SAM cleavage reaction itself, the presence of substrate is shown to increase SAM cleavage rate in general in rSAM enzymes[143]. If the attenuation of the 410 nm signal is assumed to be from the auxiliary clusters, then the degradation of the auxiliary clusters might cause structural rearrangement or change in the structure of the protein back bone which might emulate the same condition as the binding of the substrate, thus increasing the rate of SAM cleavage which can only be confirmed through further structural and biochemical characterization of MftC in oxidized state.

Another reason for the observed increase in rate might be due to changes in polarity of the active site environment [153]. The degradation of the auxiliary clusters could lead to structural changes in the protein which if decrease the polarity of the Adomet cluster active site, the rate of SAM cleavage reactions can significantly go up. An alteration in the redox potentials at the site of the Adomet cluster [154] due changed secondary structure elements and can also lead to increased reaction rate.

The reaction rate of coupled reaction seems to be affected in more intuitive manner following oxidation of MftC. The rate of decay of MftA is decreased with the increase in oxidation of MftC whereas the rate of formation of MftA* remains relatively the same (0.074 ± 0.02 min⁻¹ for anaerobic MftC, 0.063 ± 0.015 min⁻¹ for MftC incubated in oxygen for 3 hours and 0.061 ± 0.02 min⁻¹ for MftC that was incubated in oxygenated buffer for 6 hours) in all the peptide modification experiments. This shows that the second part of the
MftC reaction, the C – C bond formation between the C terminal tyrosine and the penultimate valine might not be oxygen sensitive even though SAM is consumed for the reaction to happen. This can also alternatively indicate that the step is not dependent on the auxiliary clusters even if it is SAM dependent [136].

*M. tuberculosis* invades the human body primarily though inhaled droplets that deposit in the alveolar tissue [155]. Alveoli are highly oxygenated environs within the body [156] and is populated by macrophages which ingest most of the *M. tuberculosis* cells and form the primary line of defense against the pathogen [157]. *M. tuberculosis* has to sustain metabolic functions before forming granulomas which can produce partial anoxic conditions at the core of the granuloma [158]. This leads again to the question of how the pathogenic bacterium manages to produce mycofactocin which is required for metabolic activity while inside the macrophage [87] since one of the steps involves MftC which only works optimally in an anaerobic environment [159]. The results indicate a certain level of oxygen tolerance exhibited by MftC which could explain how the *M. tuberculosis* species successfully invades and thrives in a very oxygen rich environment while requiring an anaerobic step to produce the redox cofactor which in turn is required for metabolic activity. It is also plausible that this oxygen tolerance could be enhanced *in vivo* as the protein is in its native environment.

Further biochemical and structural characterization of MftC is required to fully investigate the role of auxiliary clusters and their oxidative degradation in the peptide modification reaction and to understand oxygen tolerance during SAM cleavage reactions.
One of the ways to isolate the effect of oxygen degradation on the SAM cleavage reaction is to express and purify a truncated version of MftC which has only the partial TIM barrel part of the enzyme which hosts the SAM cluster. Experiments with UV absorbance measurements on the continuous oxygen degradation and SAM cleavage reactions with the partial enzyme would isolate the oxygenation effect to just the SAM cluster. This can open up possibilities of investigation on why there was an observed increase in the rate of SAM cleavage on slight oxidation of the protein. Another possible avenue of investigation would be deletion of the conserved residues hosting the auxiliary clusters and performing oxidative degeneration experiments on mutationally altered versions of MftC which do not host the Auxiliary clusters to test the effect on the SAM cleavage reaction.

Hydrogen–deuterium exchange experiments can be performed on MftC to determine areas of the protein that are more solvent accessible than other regions. This can potentially point out the clusters that are more solvent accessible. This can lead to more information on how cluster degradation proceeds on dissolved oxygen exposure through the solvent.

Exploring these avenues might shed light into another dimension of virulence exhibited by *M. tuberculosis* and also can lead to much better understanding of rSAM enzymes, especially SPASM like enzymes and their mechanism of action in general.
REFERENCES


[14] R. H. Baltz, “MbtH homology codes to identify gifted microbes for genome


[34] M. F. Freeman et al., “Metagenome mining reveals polytheonamides as posttranslationally modified ribosomal peptides,” *Science (80-.)*, 2012.


[72] K. Miyamoto et al., “Cloning and nucleotide sequence of the gene encoding a


[117] L. Flühe, O. Burghaus, … B. W.-J. of the, and undefined 2013, “Two clusters containing radical SAM enzyme SkfB catalyze thioether bond formation during the maturation of the sporulation killing factor,” *ACS Publ.*


[125] M. Ibrahim *et al.*, “Control of the transcription of a short gene encoding a cyclic


[137] D. A. Stahl and J. W. Urbance, “The division between fast- and slow-growing species corresponds to natural relationships among the mycobacteria,” J.


