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Spectroscopic and electrochemical characterization of the mycofactocin biosynthetic protein, MftC, provides insight into its redox flipping mechanism.

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Abstract

Mycofactocin is a putative redox cofactor and is classified as a ribosomally synthesized and post-translationally modified peptide (RiPP). Some RiPP natural products, including mycofactocin, rely on a radical S-adenosylmethionine (RS, SAM) protein to modify the precursor peptide.

Mycofactocin maturase, MftC, is a unique RS protein that catalyzes the oxidative decarboxylation and C-C bond formation on the precursor peptide MftA. However, the number, chemical nature, and catalytic roles for the MftC [Fe-S] clusters remain unknown. Here, we report that MftC binds a RS [4Fe-4S] cluster and two auxiliary [4Fe-4S] clusters that are required for MftA modification. Furthermore, electron paramagnetic resonance spectra of MftC suggest that SAM and MftA affect the environments of the RS and Aux I cluster whereas the Aux II cluster is unaffected by the substrates. Lastly, reduction potential assignments of individual [4Fe-4S] clusters by protein film voltammetry show that their potentials are within 100 mV of each other.

Graphical Abstract

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Author Contributions

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Supporting Information.

The Supporting Information is available free of charge on the ACS Publications website. EPR analysis methods, Table S1–S4, and Figures S1–S5 (PDF). List of MftC homologues (Excel Spreadsheet).

Keywords

MftC; mycofactocin; peptide biosynthesis; S-adenosylmethionine; enzyme mechanism; RS-SPASM; iron-sulfur protein

INTRODUCTION

Ribosomally synthesized and post-translationally modified peptides (RiPPs) have emerged as a large and structurally diverse class of natural products. The structural diversity of RiPPs is achieved, in part, by extensive post-translational modifications. Many RiPPs depend on radical-S-adenosylmethionine (RS) enzymes to catalyze post-translational modifications such as intramolecular C-C bond formation^{1–3}, intramolecular C-S bond formation^{4–7}, epimerization^{8,9}, decarboxylation^{1,10,11}, methylation^{12,13}, and splicing of the precursor peptide¹⁴. The chemically modified peptides undergo further alteration (e.g. proteolysis) to yield a biologically active natural product, for which functions include, but are not limited to, antimicrobials, quorum sensing molecules, and redox cofactors^{15–21}.

A subset of the RS proteins that modify peptides belong to the SPASM subfamily (subtilisin A, pyrroloquinoline quinone, anaerobic sulfatase maturing enzyme, and mycofactocin) and are annotated as RS-SPASM proteins^{22,23}. RS-SPASM proteins are comprised of a prototypical TIM barrel fold which binds the RS [4Fe-4S] cluster involved in the homolytic cleavage of S-adenosylmethionine (SAM) (Figure 1A and 1B). In addition, they contain an elongated C-terminal SPASM domain which binds up to two additional auxiliary [Fe-S] clusters^{23–27}. The cluster proximal to the RS cluster (annotated as Aux I) is typically a [4Fe-4S] (with the exception of PqqE in which a [2Fe-2S] cluster is reported) and the distal cluster (designated as Aux II) is a [4Fe-4S] cluster^{2,24–28}. It has been proposed that the Aux clusters could serve to bind the peptide substrate (Aux I) or shuttle electrons to and from the RS-cluster (Aux I and Aux II)^{5,23}. Because their precise functions may be different between

RS-SPASM proteins, delineation of their precise roles is important for gaining insight into the distinct chemistries RS-SPASM enzymes catalyze.

MftC is a RS-SPASM enzyme that performs the first modifying step in the biosynthesis of the RiPP, mycofactocin. Mycofactocin is a putative, peptide derived, redox cofactor found primarily in the *Mycobacteria* genera^{29,30}. Although the exact structure and function for mycofactocin remains largely unknown, the molecule is likely synthesized from the precursor peptide, MftA, co-occurring in the gene cluster *mftABCDEF*. Indeed, recent reports have demonstrated that the RiPP recognition element (RRE) MftB, binds MftA and is required for catalysis by MftC^{10,31}. Additionally, it has been shown that MftC carries out the SAM-dependent oxidative decarboxylation of a C-terminal tyrosine followed by a second, SAM-dependent, C-C bond formation between the C β of the penultimate valine and the C α of the former tyrosine, forming a 3-amino-5-[(*p*-hydroxyphenyl) methyl]-4,4-dimethyl-2-pyrrolidinone (AHDP) moiety (MftA*, Figure 1B)^{1,10,11}. Recently, it was shown that, following MftC modification, MftE hydrolyzes MftA* at the valine position, freeing AHDP from the peptide³². Subsequent steps in mycofactocin biosynthesis have not yet been elucidated.

The notion that MftC catalyzes two distinct chemistries in the same active, an oxidative decarboxylation of the C-terminus and a subsequent redox neutral C-C bond formation (Scheme 1), is intriguing. Whereas most reactions within the RS protein family can be classified as either oxidative or redox neutral (for a review see ³³), MftC seems to be an outlier in that it can “redox-flip.” The ability of MftC to redox-flip, or accommodate both oxidative to redox neutral reactions, is certainly unique to the RS-SPASM subfamily, in which all proteins characterized to date have been shown to catalyze net oxidative reactions on the peptide substrate. In order for MftC to catalyze both reactions, we propose that two independent or combinatorial scenarios must play out: 1) the active site undergoes stepwise rearrangement, setting up MftC for both types of redox reactions and/or 2) the potentials of the [Fe-S] clusters within MftC are modulated to accommodate both types of redox reactions. Although efforts are underway to answer the former scenario, herein we address the latter. To do so, the number and state of [Fe-S] clusters were established using site-directed mutagenesis, elemental analysis, and Mössbauer spectroscopy. Electron paramagnetic resonance (EPR) spectroscopy was used to spectroscopically characterize the [Fe-S] centers in MftC. Lastly, direct electrochemistry techniques were used to measure the redox potential for all [Fe-S] clusters. From the combination of these experiments, a general picture has emerged that could explain how MftC is capable of catalyzing a redox-flipping mechanism.

METHODS AND MATERIALS

Bioinformatic analysis of MftC.

The amino acid sequence for *M. ulcerans* Agy 99 MftC (Uniprot: A0PM49) was used in a BLAST search of the Uniprot database. The BLAST search used default settings except that the E-threshold was set to 0.001 and the number of hits was set to 1000. Approximately 760 hits with a sequence identity of >35% were carried forward for the remaining analysis. Proteins with 85% sequence identity (~310) were classified as MftC by default. The

genomes of the organisms that contained homologous proteins with sequence identities between 85–35% were examined to verify that the putative homologues were indeed MftC. The curated genomes of the organisms found on the EnsemblBacteria database or the NCBI Gene database were examined for the precursor peptide MftA. The homologues of the genomes containing an annotated MftA with the conserved sequence –CGVY were validated as MftC²⁹. Those genomes that did not have an annotated MftA were further examined to identify at least two other genes associated with mycofactocin biosynthetic cluster; such as *mftB*, *mftD*, *mftE*, or *mftF*²⁹. If the genome containing the MftC homologue contained two of the aforementioned genes within a 10 kb window, the homologue was validated as MftC. If a curated genome in the two databases was unavailable for the organism that contained the putative homologue, the homologue was classified as annotated and carried forward in the analysis as if it were MftC. Of the remaining ~720 homologues, species duplicates were removed, leaving 625 unique species containing a putative MftC (for a complete list of homologues found here, please see the Excel file supplied in the Supplementary Information).

To identify which cysteines on MftC are strictly conserved, a sequence alignment of all 625 unique MftC amino acid sequences was carried out using the Constraint-based Multiple Alignment Tool (COBALT) provided on the NCBI website (ncbi.nlm.nih.gov/tools/cobalt/cobalt.cgi?CMD=Web). The sequence alignment file was downloaded and used as the input in the server program, Weblogo³⁴, for visualization.

MftC protein structure models were generated by the server program, Phyre2.0³⁵. Using the expert mode program, one-to-one threading, the amino acid sequence for *M. ulcerans* MftC was input along with the Protein Databank identifier for anSME (PDBID: 4K36)²⁷, CteB (PDBID: 5WHY)²⁵, or SuiB (PDBID: 5V1T)²⁴. The threaded MftC structural models were overlaid with their respective parent structure using Chimera's Match Maker tool to visualize the conserved cysteines of MftC and their vicinity to the parent structure's [Fe-S] clusters³⁶.

Generation of RS, Aux I, and Aux II KO variants of MftC.

To create the RS cluster knockout (RS KO), the auxiliary I cluster knockout (Aux I KO), and the auxiliary II cluster knockout (Aux II KO) variants of MftC, alanine replacements of cysteine residues identified in the bioinformatic analysis were used. Site directed mutagenesis and sequence verification were performed by Genscript. For the RS KO variant, the C30A/C37A double mutant was used. For the Aux I KO variant, the single C323A mutant was used. For the Aux II KO variant, the C310A/C341A double mutant was used.

MftC purification.

Purification of MftC, and variants thereof, followed previously reported protocols^{1,10}. In short, a starter culture containing *E. coli* BL21 (DE3) transformed with pPH151 and *mftC*/pET28a, or variants thereof, was grown overnight. The overnight culture was used to inoculate 10 L of TB broth supplemented with phosphate buffer (10 mM) which was grown at 37 °C with shaking until an OD₆₀₀ ~ 1.2 was reached. At this point, 1 mM IPTG, 0.75 g/L of sodium fumarate, and 1X autoinduction metals were added to the cultures and the flasks

were sealed. The induced cultures were incubated at 20 °C overnight with shaking and the cells were harvested by centrifugation. The cell pellet was introduced to the anaerobic chamber (Coy Labs) and was suspended in anaerobic lysis buffer (50 mM Hepes, 200 mM NaCl, 40 mM imidazole, pH 7.5). To the suspension, 1% w/v CHAPS, lysozyme (0.1 mg/g cell paste), and DNase (0.01 mg/g cell paste) was added. The cell suspension was stirred at room temperature for 30 min, transferred to sealable centrifuge tubes, and removed from the chamber. The lysate was clarified by centrifugation (20,000 *g*, 10 min) and reintroduced to the chamber. The his-tagged MftC protein in the supernatant was bound to a 5 mL HisTrap FF Ni-NTA column (GE Healthcare) using an AKTA Start FPLC (GE Healthcare), washed with lysis buffer, and eluted with elution buffer (50 mM Hepes, 200 mM NaCl, 300 mM imidazole, pH 7.5). The MftC containing fractions were combined and buffer exchanged into storage buffer (50 mM Hepes, 200 mM NaCl, 10% glycerol, 10 mM DTT, pH 7.5) using a PD-10 column (GE Healthcare). The protein was then used for reconstitution.

Reconstitution of MftC.

The following procedures were performed in an anaerobic chamber. All reagents were introduced to the chamber in powder form and dissolved in anaerobic water. DTT (10 mM) was added to purified MftC (100–200 μ M), or variants thereof, and stirred at 22 °C for 30 min. To the protein solution, 8 molar equivalents of FeCl₃ and Na₂S were added. After stirring for an additional 30 min at 22 °C, the protein solution was centrifuged to remove particulates. The supernatant was buffer exchanged into storage buffer using a PD-10 column. The protein fractions were collected, pooled, and concentrated using a 30 kDa spin concentrator (Millipore). Concentrated protein was aliquoted, flash frozen, and stored at –80 °C.

Preparation of MftC for Mössbauer spectroscopy.

⁵⁷Fe₂O₃ (Cambridge Isotope Laboratories) was converted to ⁵⁷FeCl₃ by incubating the oxide in 1 M HCl at 80 °C overnight. An overnight starter culture containing *E. coli* B121 (DE3) transformed with pPH151 and *mftC*/pET28a was used to inoculate 8 L of M9 minimal media (Sigma Aldrich) supplemented with 20 amino acids (0.2 g/L) and 1X auto induction metals, excluding iron. The large-scale culture was incubated at 37 °C with shaking until an OD₆₀₀ ~ 1.2 was reached. At this point, 1 mM IPTG, 0.75 g/L sodium fumarate and ⁵⁷Fe (75 μ M) were added to the cultures and the flasks were sealed. The induced cultures were incubated at 20 °C overnight and the cells were harvested by centrifugation. The cell pellet was introduced into the anaerobic chamber, suspended in anaerobic lysis buffer and MftC was purified as described above. Reconstitution of MftC was carried out as described above except that ⁵⁷FeCl₃ was substituted for FeCl₃ and the reconstituted protein was buffer exchanged over a PD-10 column twice. Iron and sulfur quantification were performed for all protein samples prior to being characterized by Mössbauer spectroscopy.

Iron and sulfur quantification.

Protein concentrations were determined using a Bradford assay. Iron and sulfur quantification was carried out following previously described procedures³. In short, to determine the iron concentration, 100 μ L of 20 μ M protein was mixed with 10 μ L 3 M

trichloroacetic acid. The precipitated protein was centrifuged at 15,000 *g* for 10 min. The supernatant was carefully removed and diluted with 330 μL of dH_2O . To the solution, 20 μL sodium ascorbate, 20 μL of 10 mM ferrozine, and 20 μL of saturated sodium acetate was added. The absorbance change due to the ferrozine-iron complex was measured at 562 nm ($\epsilon = 27.9 \text{ mM}^{-1}\text{cm}^{-1}$). To determine the sulfur concentration, 200 μL of 10 μM protein was mixed with 600 μL of 1% (w/v) zinc acetate and 50 μL of 7% (w/v) sodium hydroxide. The solution was incubated at room temperature for 15 min after which 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) was added. The solution was vortexed and incubated at room temperature for 20 min. The absorbance from methylene blue was measured at 670 nm ($\epsilon = 34.5 \text{ mM}^{-1}\text{cm}^{-1}$). For both assays, blanks were generated by substituting protein with dH_2O .

Size exclusion chromatography of MftC variants.

Analytical size exclusion chromatography (SEC) of MftC variants was carried out anaerobically using a Superdex 200 10/300 GL column (GE Healthcare), a flow rate of 0.5 mL/min, and in 50 mM Hepes (pH 7.5) and 200 mM NaCl buffer. A standard curve was prepared using Gel Filtration Standards (Biorad) which contained protein standards for 670 (thyroglobulin), 158 (γ -globulin), 44 (ovalalbumin), 17 (myoglobin), and 1.35 (vitamin B_{12}) kDa. Injections of 100 μL for each MftC variant ($\sim 1 \text{ mM}$) were used for analysis.

MftC activity assays.

MftC variants were assayed for uncoupled cleavage of SAM and complete modification of MftA following previously described procedures¹. For SAM cleavage assays, a reaction containing 2 mM SAM, 10 mM DTT, 2 mM dithionite, and 100 μM of MftC variant in 50 mM HEPES, 200 mM NaCl was incubated in an anaerobic chamber overnight. The reactions were analyzed by HPLC over a Phenomenex Jupiter C4 5 μm 300 $\text{\AA}$ reverse phase column using 0.1% TFA and acetonitrile as solvents. For peptide modification assays, the same reaction was set up with the addition of 100 μM MftA and 100 μM MftB^{32,37}. The reactions were analyzed over the same Phenomenex Jupiter C4 except that the solvents were changed to 5 mM phosphate buffer (pH 8.0) and 5 mM phosphate buffer in 70% acetonitrile (pH 8.0). All HPLC assays were performed on a Shimadzu iProminence HPLC equipped with a diode array.

EPR measurements on MftC.

Continuous wave (CW) EPR spectra were collected between 9.3663 and 9.3721 GHz on a Bruker E580 spectrometer with an SHQE resonator and a Bruker/ColdEdge Stinger cryogenic system set to 20 K. Samples were transferred into 4 mm OD quartz tubes in the inert atmosphere chamber and flash frozen in liquid nitrogen. Tubes were temporarily capped with clamped tygon tubing, removed from the chamber, partially evacuated, and flame sealed. Samples were stored in liquid nitrogen and inserted frozen into the pre-cooled resonator. Spectra were acquired under non-saturating conditions with 4 G modulation amplitude at 100 kHz and 2.3 mW microwave power. This power was selected to be in a range where signal amplitude increases linearly with square root of power. CW EPR signals were averaged for 4 to 9 scans with sweep widths between 1000 and 4000 G. A background signal for a tube containing 1:1 water:glycerol was recorded under identical conditions and

subtracted from the spectra of the FeS cluster samples. The Bruker Xepr software was used for the background subtractions after x-axis offset to correct for differences in the microwave frequencies at which data were acquired. Spectral subtractions were performed using the Bruker Xepr software. Pairs of spectra that were used in subtractions were acquired at frequencies that differed by less than about 3 MHz, which corresponds to shifts in resonance fields of less than about 0.1 mT. These offsets are insignificant relative to linewidths of 3.5 to 5 mT. Signal amplitudes for the subtractions were scaled for differences in sample heights and protein concentrations.

For the redox titration curves the samples were prepared similar to previously described procedures³⁸. In short, MftC was introduced into an anaerobic chamber (Coy Labs) and diluted with anaerobic buffer (50 mM Hepes, 100 mM NaCl, and 10% glycerol, pH 7.5). A redox mediator mixture (methyl viologen, benzyl viologen, safranin O, anthraquinone-2-sulfonate, 2-hydroxy-1-4-napthoquinone, and resorufin) was added to MftC for a final concentration of 38 μ M each mediator and 72 μ M of MftC. The protein solution was transferred to a three-neck, water jacketed, glass cell with a stir bar, and cooled to 4 °C. The cell was equipped with a platinum wire working electrode (BASi) and an Ag/AgCl₂ reference electrode (BASi) which were connected to a voltmeter for continuous monitoring of the potential. Potentials were corrected to the standard hydrogen electrode (SHE) by the addition of +0.194 V. While stirring, various concentrations of sodium dithionite was used to titrate the potential to -0.500 V *v.* SHE, near the lowest standard potential of the mediator methyl viologen ($E'^{\circ} = -0.440$ V). At various potentials, aliquots of protein were extracted and prepared for EPR as described above. The observed EPR signal intensities were scaled to take account of differences in sample height and the number of scans averaged. The microwave B₁ and effective modulation amplitude vary with position in the resonator. This variation was calibrated by recording the spectrum of a point sample of BDPA (1:1 α,γ -bisdiphenylene- β -phenylallyl:benzene) as a function of position in the resonator at 9.37 GHz and microwave power of 0.24 μ W. This position-dependence was fit with a polynomial function. The intensity of the [Fe-S] signal was scaled to find the signal intensity that would have been observed if the sample extended over the full length of the cavity for which signal could be observed, using the functional dependence found for the BDPA sample.

Mössbauer spectroscopy.

Mössbauer spectra were recorded at helium temperatures on a spectrometer from WEB Research (Edina, MN), which is equipped with a Janis SVT-400 variable-temperature cryostat. The external magnetic field (0.078 T) was applied parallel to the γ beam. All isomer shifts quoted are relative to the centroid of the spectrum of α -iron metal at room temperature. Simulation of the Mössbauer spectra was carried out by using the WMOSS spectral analysis software (www.wmoos.org, WEB Research, Edina, MN).

Electrochemical measurements of MftC.

Electrochemical experiments were carried out anaerobically in a MBraun Labmaster glovebox using a PGSTAT12 potentiostat. A three-electrode configuration was used in a water-jacketed glass cell. A platinum wire was used as the counter electrode and a standard

calomel electrode was used as the reference electrode. Reported potentials are relative to the standard hydrogen electrode.

Baseline measurements were collected using an edge-plane graphite (EPG) electrode pretreated by mechanical polishing with sandpaper followed by incubating overnight with 14 μ L of 3 mg of multi-walled carbon nanotubes (MWCNT) (Sigma, 10 nm $\pm$ 1 nm x 4.5 nm $\pm$ 0.5 nm x 3- ~6 μ m) dissolved in 1 mL of dimethylformamide (DMF) by sonication for 15 minutes. The electrode was then rinsed and placed into a glass cell containing a mixed buffer solution (10 mM HEPES/CHES/MES/TAPS/CAPS, 200 mM NaCl) and pH 7.5. A 4 μ L aliquot of either wild-type MftC, the RS KO, or the Aux II KO variant was applied directly to the electrode surface and allowed to sit for approximately two minutes at room temperature before being placed into the buffer cell solution.

Cyclic voltammograms were collected at 4 $^{\circ}$ C with a scan rate of 100 mV/s and electrochemical signals were analyzed using QSoas³⁹, where the data is presumed to represent all three iron-sulfur clusters of MftC. Voltammograms were fit to three nernstian components as described where the surface coverage for all components is the same, and n_{app} and the redox potentials are fit as floatable parameters⁴⁰. Square wave measurements were collected at 4 $^{\circ}$ C with a frequency of 10 Hz and an amplitude of 20 mV.

RESULTS

Identification of conserved cysteines in MftC.

A bioinformatic analysis was carried out to identify possible residues involved in [Fe-S] cluster coordination (on the basis of their conservation) on all probable MftC homologues obtained from a BLAST query of the Uniprot database. 625 putative MftC sequences were retrieved and aligned and 11 cysteines were found to be strictly conserved. On the basis of their location sequence-wise and residue spacing, Cys30, 34, and 37 were assigned as the RS ligands (characteristic CX₃CX₂C sequence motif) (Figure S1). In addition to the RS sequence motif, residues Cys251, 258, 269, 310, 313, 319, 323, and 341 were also found to be strictly-conserved among all 625 species (Figure S1). These last eight cysteine residues are expected to reside in the C-terminal SPASM domain and could bind one or two auxiliary [Fe-S] clusters. The conserved cysteine residues were then mapped on a homology model of MftC that was generated based on the structures for anSME (PDBID: 4K36)²⁷, CteB (PDBID: 5WHY)²⁵, or SuiB (PDBID: 5V1T)²⁴. In the representative overlay between the modeled MftC and the parent anSME (sequence identity ~ 25%) shown in Figure SI 2A, the predicted RS motif Cys30, 34, and 37 align accordingly with the corresponding RS binding motif of anSME. Likewise, the modeled MftC residues Cys310, 313, 319, and 341 overlay well with those from anSME involved in binding the Aux II cluster. Of the remaining four modeled MftC Cys residues, only residues 269 and 323 overlay with agreement to the Cys residues of anSME involved in binding the Aux I cluster. Residue Cys251 is modeled in the vicinity of the Aux I cluster of anSME but the modeled residue Cys258 is located in the second shell around the Aux I cluster of anSME. All models generated for MftC provide similar findings (Figure SI 2B, and C, sequence identities ~ 20%). The poor overlay of two of the four Cys residues of Aux I, is most likely due to the low sequence similarity, and neither preclude nor confirm binding of a cluster in the Aux I position in MftC.

Generation of soluble MftC single- and multiple-point cysteine variants.

To gain insight into the roles of the RS, Aux I, and Aux II clusters in catalysis and to further identify Cys residues involved in binding the clusters, Ala variants of MftC were constructed. Using the bioinformatic information described above, Cys residues were systematically replaced with Ala yielding soluble RS KO, Aux I KO, and Aux II KO variants. For the RS KO variant, Cys30 and Cys37 were mutated to generate the C30A/C37A double mutant. The triple mutant C30A/C34A/C37A was insoluble. For the Aux I KO variant, only the single mutants C269A and C323A were soluble. Both the C251A single mutant and the double C269A/C323A mutant resulted in insoluble protein. The C258A variant did not impact protein solubility or MftC activity and was not considered an important mutation. Since the C269A and C323A variants were found to have identical enzymatic activity and since C323A could be produced in larger quantities, the C323A variant was used as in all experiments as the Aux I KO. To make the Aux II KO, Cys310 and Cys341 were mutated to generate the C310A/C341A double mutant. Additional mutations of the Aux II site led to insoluble protein. The quality of MftC variants were checked by SDS-PAGE and size exclusion chromatography (SEC). For all variants of MftC, the proteins were purified to near homogeneity as determined by SDS-PAGE. In addition, the SEC profile of MftC variants showed a major peak at a retention time consistent with ~42 kDa. This suggests that the RS KO, Aux I KO, and Aux II KO had minimal effect on the oligomeric state of MftC.

MftC contains three [4Fe-4S] clusters.

It has been previously reported that MftC contains [Fe-S] clusters however, the exact quantity and configuration (e.g. [4Fe-4S] v. [2Fe-2S]) has not been established^{10,11}. Mössbauer spectroscopy was carried out to gain independent additional insights into the chemical nature and number of [Fe-S] clusters in MftC. The 4.2-K/78-mT Mössbauer spectrum of the as-isolated wild-type MftC is shown in Figure 2 and can be best analyzed considering two quadrupole doublets, which account for 88% of the total iron in the sample. The dark green-shaded doublet has an isomer shift $\delta = 0.26$ mm/s and quadrupole splitting $E_Q = 0.55$ mm/s, parameters that are characteristic of all-ferric [2Fe-2S] clusters in their 2+ state. The light green-shaded doublet has an average isomer shift $\delta = 0.45$ mm/s and an average quadrupole splitting $E_Q = 1.01$ mm/s, parameters that are characteristic of valence-delocalized Fe²⁺-Fe³⁺ cysteine-coordinated pairs in oxidized [4Fe-4S]²⁺ clusters. The mismatches between the summations of the theoretical spectra with the experimental data reveal the presence of a minor and poorly defined subspectrum (~ 10% intensity), which we attribute to nonspecifically bound iron. Considering the number of Fe ions per cluster and those obtained from elemental analysis, the as-isolated MftC sample contains 0.67 [2Fe-2S]²⁺ clusters and 0.61 [4Fe-4S]²⁺ clusters per polypeptide.

Since the as-purified protein already contains an appreciable number of [Fe-S] clusters, the protein employed for Mössbauer analysis to interrogate the number of clusters was chemically reconstituted with 2 equiv. of [Fe-S] centers (see Materials and Methods). The 4.2-K/78-mT Mössbauer spectrum of this sample is shown in Figure 2 (bottom) and can be best analyzed considering a single quadrupole doublet that accounts for 92–94% of the total iron in the sample. The light green-shaded quadrupole doublet has an isomer shift $\delta = 0.45$

mm/s and a quadrupole splitting $E_Q = 1.11$ mm/s, parameters consistent with the presence of oxidized $[4\text{Fe-4S}]^{2+}$ clusters. The remaining small broad absorption in the spectrum accounts for ~6% and corresponds to nonspecifically bound iron. There are no signals detectable for $[2\text{Fe-2S}]^{2+}$ clusters, suggestive that their presence in the as-purified MftC could be an oxidative byproduct. After correction for the number of Fe ions per cluster and elemental analysis, the sample contains 2.7 ± 0.15 $[4\text{Fe-4S}]^{2+}$ clusters per polypeptide, confirming that MftC contains three $[4\text{Fe-4S}]$ clusters.

All three [Fe-S] clusters are required for MftC modification of MftA.

As previously mentioned, the role of for the Aux I and Aux II clusters in MftC catalysis has yet to be assigned. To gain insight into their possible functions or their functional roles in MftC, as well as the role of the RS cluster, activity assays for the RS KO, Aux I KO, and Aux II KO proteins were carried out. Reactions containing the appropriate components (see Methods and Materials) were analyzed by reverse phase HPLC, monitoring the absorbance at 260 nm (SAM cleavage) or 280 nm (peptide modification). As shown in Figure 3A and 3B, MftC catalyzed the cleavage of SAM to form dAdo and it converted MftA to MftA*. Conversely, the RS KO could neither cleave SAM nor modify MftA, consistent with the successful knockout of the RS cluster. Activity assays for Aux I and Aux II KO's also provided insightful results. Both Aux I and Aux II KO's were capable of catalyzing the reductive cleavage of SAM to form dAdo (Figure 3A), suggesting that the RS cluster remained intact and in an active conformation in the mutated proteins. However, when assayed against MftA, both Aux I and Aux II KO's were incapable of converting MftA to MftA* or MftA** (Figure 3B). This suggests that the Aux I and Aux II are required for catalysis, consistent with results shown for QhpD, AnSME, SCIFF, and PqqE^{2,7,27,41}.

EPR analysis of MftC variants with substrate bound provides insight to the function of the [Fe-S] clusters.

To determine if the local environments around the RS, Aux I, or Aux II clusters are affected by substrate binding, we turned to EPR spectroscopy. EPR spectra were acquired for the wildtype MftC, RS KO, Aux I KO, and Aux II KO proteins with and without SAM, MftA, and/or MftB. Substrate binding events are expected to cause changes in the EPR spectra of the $[4\text{Fe-4S}]^+$ clusters⁴²⁻⁴⁶ however, since MftC contains three EPR active $[4\text{Fe-4S}]$ clusters, any changes in the EPR spectrum would represent the ensemble of changes for all three clusters. To evaluate the changes of a single $[4\text{Fe-4S}]$ cluster, a first approximation of the spectrum for an individual cluster was made by subtracting the spectrum of the knockout from the spectrum of wildtype MftC. If knocking out one cluster does not perturb the EPR spectra of the remaining clusters, the difference spectrum should be a close approximation of the $[4\text{Fe-4S}]^+$ site that was knocked out. It would then be possible to simulate the difference spectrum with parameters that are plausible for this class of clusters. Alternatively, if the absence of one cluster impacts the environment of the remaining two clusters, or if ligand binding to a cluster impacts the environment of the remaining clusters differently, this would also be apparent. In this case the difference spectra would be an approximation of the $[4\text{Fe-4S}]^+$ knockout plus changes of the other $[4\text{Fe-4S}]^+$ clusters and may not resemble the spectrum of an individual cluster. In addition to providing a visual representation of individual clusters, the difference spectra of RS, Aux I and Aux II clusters were simulated to

estimate the g values and g strain for the three $[4\text{Fe-4S}]^+$ clusters (for simulation methods and results see Supplementary Information and Tables S2 and S3). To validate the extracted g values and g strain from the difference spectra, the parameters were used to fit the spectra of wildtype MftC protein. It should be noted that although a previous analysis of EPR spectra of two $[2\text{Fe-2S}]$ ferredoxins at three microwave frequencies demonstrated that the principal axes of the g and g -strain matrices are not co-linear⁴⁷, the resolution of the overlapping spectra for the three $[4\text{Fe-4S}]$ clusters for MftC did not warrant the inclusion of additional angular parameters in the simulations of the spectra. As shown in Figure 4, the estimated g values and g strain from individual clusters provide a fit that closely resembles the experimental data of wildtype MftC, suggesting that they are a reasonable approximation.

When this strategy was applied to wildtype MftC, RS KO, Aux I KO, and Aux II KO the resulting difference spectra for the RS, Aux I and Aux II clusters in the absence of substrate (Figure 5A, B, and C, respectively) resemble those for $[4\text{Fe-4S}]^+$ clusters with similar g_x , g_y , and g_z values (Table S2). The same strategy was applied to spectra of samples to which SAM had been added. Again, the spectra for the Aux I and Aux II centers in the presence of SAM (Figure 5E and 5F) resemble spectra expected for $[4\text{Fe-4S}]^+$ clusters. However, the addition of SAM caused a decrease in the estimated value of g_x for the Aux I cluster with no apparent change to the g_x strain (Table S2 and S3). The change in the difference spectrum for the Aux II cluster is due primarily to a decrease in g_x strain (Table S3) which makes the high-field extrema more pronounced. In contrast, the difference spectrum for the RS cluster when SAM is added (Figure 5D) does not resemble that for a $[4\text{Fe-4S}]$ cluster. This indicates that, in the absence of the RS cluster, SAM could be affecting the environments of the Aux I and/or Aux II clusters differently than the wildtype protein. The parameters for the RS cluster in the presence of SAM were obtained by simulating the spectra of the Aux I and Aux II knockouts and a downward shift in the estimated g_z value (Table S2) was observed. This observation is typical for a RS cluster bound to SAM^{7,41,46,48,49}.

When SAM, MftA, and MftB are added to the system, the difference spectra for the Aux I cluster undergoes a significant downward shift in the estimated g_x values but the estimated g_y and g_z values remain relatively unchanged (Figure 5H, Table S2). These trends to lower values are similar to what has been reported for other RS proteins when substrate is added (for a recent tabulation of RS protein g values see ref⁵⁰). Under the same conditions, the difference spectra for the Aux II cluster shows a decrease g strain (Figure 5I) while the estimated g values remain fixed (Table S2). Again, the difference spectrum for the RS cluster (Figure 5G) does not resemble that for a $[4\text{Fe-4S}]$ cluster. Therefore, the parameters of the RS cluster in the presence of SAM, MftA, and MftB were obtained by simulation of the spectra for the Aux I and Aux II, under the same conditions, and the g values for the RS cluster (Table S2) were found to decrease. From this analysis, we observed that the RS and Aux I clusters are more sensitive to the addition of substrates than the Aux II cluster.

As alluded to earlier, the addition of SAM, MftA, and/or MftB to MftC lowered the estimated g_x , g_y , and/or g_z strain values of the RS, Aux I, and Aux II clusters. Since g strain values are indicative of variations in the environments of the paramagnetic center, this result could indicate that conformational sampling by MftC is limited upon the addition of

substrates. From the trends discussed here, it is reasonable to conclude that the addition of substrates to MftC affect the g values of the RS and Aux I clusters and the g strain of all three clusters. Furthermore, these changes are likely the result of either direct substrate-cluster interaction or global or local changes to the protein conformation. Future work monitoring protein dynamics will be required to validate the restrictions in protein conformation.

Protein film electrochemistry measured the midpoint potentials for all clusters in MftC.

The ability for MftC to catalyze both oxidative and redox neutral reactions suggests that the Aux clusters could participate in the reaction through an electron transfer mechanism. Although activity assays for the Aux KO's suggest that they play a role in catalysis, and EPR data suggests that the environment of the Aux I cluster is altered by substrate binding, the exact nature of that role (structural vs. electron transfer) is not clear. To provide further insight about the possible role(s) for the Aux clusters, protein film electrochemistry (PFE) was used to directly measure the midpoint potentials of the [Fe-S] clusters in MftC (Figure 6). Wild-type MftC was non-covalently adsorbed onto a plan-edge graphite electrode using multi-walled carbon nanotubes as a co-adsorbent. A single and broad feature in the cyclic voltammogram for wild-type MftC was observed between -425 mV and -600 mV (Figure 6A).

This suggests that the three [4Fe-4S] clusters have similar reduction potentials that appear as a single envelope when summed up. The envelope signal was fitted using QSoas software and three, one electron transfers with reduction potentials of ~ -550 , -500 , and -460 mV vs SHE were extracted (Table 1)³⁹. The three reduction potentials represent three redox active species and are expected to represent each of the three [4Fe-4S] clusters found on MftC. These findings were corroborated, in part, through EPR redox titration experiments which showed a single transition beginning at ~ -430 mV (Figure SI 4). The fitting shown in Figure 6 allows n (the number of electrons in the redox couple) to be a floatable parameter by using the popular equations describing adsorbed redox species, given in Ref⁵¹. Here n_{app} is close to 1 in all cases; rigorously holding n to 1.0 results in slightly poorer fits (Figure S5C), but shifts of potential of only ± 3 mV.

To resolve which reduction potential belonged to which cluster, the same analysis was conducted on the RS KO and Aux II KO. In experiments containing the RS KO, fitted data suggests that two redox active species, with reduction potentials of -507 and -502 mV vs SHE, are present (Figure 6B, Table 1). Notably, the highest potential of ~ -460 mV was no longer apparent, suggesting that this potential belongs to the RS cluster. It remained unclear which of the two remaining signals belong to the Aux I or Aux II cluster. To clarify this, the cyclic voltammogram for the Aux II KO variant was also acquired and fitted. The fitted data from this experiment indicated that two redox active species, with reduction potentials of -520 and -454 mV vs. SHE, were present (Figure 6C, Table 1). The reappearance of the ~ -460 mV species further suggests that the midpoint potential for the RS cluster is indeed ~ -460 mV. Additionally, the remaining signal of -520 mV could be assigned to the Aux I cluster. It is likely that the -520 mV species found in the Aux II KO dataset corresponds to the -550 mV species found in the wild-type MftC dataset. Therefore, the resting state

reduction potentials measured for MftC could be assigned as the following: ~ -460 mV – RS cluster, -550 mV – Aux I cluster, and -500 mV – Aux II cluster. These potentials are similar to those found for the only other RS-SPASM protein measured to date, SCIFF maturase. SCIFF maturase catalyzes the formation of intramolecular thioether bonds in its precursor peptide, and also contains three [4Fe-4S] clusters.⁴¹ Similar to MftC, the reduction potentials measured for the three [4Fe-4S] clusters on SCIFF maturase were -490 mV, -540 mV, and -585 mV vs. SHE⁵².

DISCUSSION

An apparent common feature of the RS-SPASM protein family is their ability to bind two auxiliary [Fe-S] clusters however, their role in catalysis remains enigmatic. The recent deposition of several RS-SPASM crystal structures (e.g. anSMe, PqqE, CteB, and SuiB) has provided insight into the potential functions of the auxiliary clusters however, the rules by which to assign function has not yet been made clear. For example, in the pyrroloquinoline quinone biosynthetic enzyme PqqE, a RS-SPASM protein responsible for forming a C-C bond between Glu-Tyr in the peptide PqqA, it was previously thought that the Aux I cluster would contain an open coordination site that could be used for binding PqqA²⁶. However, the recently solved partial crystal structure indicates that the Aux I cluster is fully ligated by Cys residues, suggesting that the Aux I cluster does not participate in substrate binding but serves another unknown catalytic function. Moreover, the catalytic role, if any, of the Aux II cluster in PqqE remains unknown. Alternatively, it has been proposed that the Aux I cluster in CteB, a thioether bond forming RS-SPASM protein, does bind the peptide substrate²⁵. Consistent with this proposal, a recently solved crystal structure of CteB with the partial peptide substrate (CteA) bound in the active site indicates that the sulfur atom of a CteA Cys residue is coordinated to the unique Fe atom from the Aux I cluster. This coordination could be used to position the peptide and activate the Cys for thioether bond formation²⁵. Although the role for the Aux I cluster in CteB is rather straight forward, the role of the Aux II cluster remains unknown. It is interesting to note that both PqqE and CteB catalyze oxidative chemistry, yet crystallographic evidence suggests that their Aux I cluster serve divergent roles in catalysis.

Here, we provide additional information for the roles of the Aux I and Aux II clusters in the RS-SPASM family. Similar to QhpD, PqqE, SCIFF, and AlbA^{5,7,26,41}, the Aux I and Aux II clusters in MftC are required for modification of MftA but not for SAM cleavage. This may imply that the Aux I and II clusters participate directly in the decarboxylation and crosslinking reactions on MftA by binding the peptide, electron transfer during the course of the reaction, or a combination of both. Indeed, our bioinformatic and mutational analysis provided evidence that the Aux I cluster may be coordinated by at least three Cys residues (251, 269, and 323), potentially leaving an open coordination site. An open coordination site to the Aux I cluster would be indicative of a substrate binding role similar to that of CteB²⁵. However, since Cys258 is conserved, albeit with little catalytic impact observed for the mutant, and since structural information for MftC remains wanting, this proposal is limited.

Perhaps one of the most intriguing features of MftC catalysis is its ability to catalyze both oxidative and redox neutral chemical reactions (Scheme 1). The ability to redox flip is

unique to the RS-SPASM family and suggests that MftC has means to modulate the electrochemical environment within its active site for each catalytic step. The resting state reduction potential for the Aux I cluster is similar to what was measured for the same cluster in SCIFF maturase⁵². This similarity suggests that the midpoint potential of the Aux I cluster in MftC may be modulated through substrate interaction or active site rearrangement to accommodate both oxidative and redox neutral chemistries. Indeed, unlike SCIFF, MftC catalyzes two distinct chemistries on two electrostatically different compounds. In the first step, MftC catalyzes the decarboxylation of the MftA peptide resulting in the α/β unsaturated bond on MftA^{**}. Since the C-terminus of MftA is presumably deprotonated, the loss of the carboxylate would also result in the loss a negative charge in the active site, which could influence the reduction potential of the Aux I cluster. However, further electrochemical investigations will be required to confirm this hypothesis.

Although a potential role for the Aux I cluster in MftC catalysis has been provided, the role of the Aux II cluster remains unknown. Our EPR analysis of MftC with SAM, MftA, and MftB bound suggests that the environment around the Aux II cluster is unaffected when MftA and MftB are bound. Indeed, in all RS-SPASM structures reported to date, the Aux II cluster is fully coordinated by Cys or Asp residues and resides in the SPASM domain away from the active site^{24–27}. While it is clear that Aux I and Aux II behave as two separate one-electron redox centers, their close potentials suggest that there should not be a steep barrier for the reversible electron transfer between the two clusters. Taken together with evidence that the Aux II cluster is required for catalysis, this suggests that the Aux II cluster may participate in electron shuttling to and from the active site.

In summary, MftC is a unique RS-SPASM protein capable of catalyzing oxidative and redox neutral reactions in the same active site. Here, we provided spectroscopic, biochemical, and electrochemical evidence about the [4Fe-4S] clusters which provided insight into the unique catalytic capabilities of MftC. Future experiments will be aimed at understanding the influence of the substrates on MftC protein motions and the midpoint potentials of the [4Fe-4S] clusters.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

AHDP	3-amino-5-[(p-hydroxyphenyl) methyl]-4,4-dimethyl-2-pyrrolidinone
DTT	dithiothreitol
DMPD	N,N-dimethyl- <i>p</i> -phenylenediamine

DTH	dithionite
EPR	electron paramagnetic resonance spectroscopy
KO	knockout
PFE	protein film electrochemistry
RS	radical S-adenosylmethionine
SAM	S-adenosylmethionine
SEC	size exclusion chromatography
SCIFF	six cysteines in forty five
SHE	standard hydrogen electrode
SPASM	subtilisin, pyrroloquinoline quinone, anaerobic sulfatase maturing enzyme, and mycofactocin
SW	sweep width
TCA	trichloroacetic acid

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Figure 1.

A) A representative crystal structure for the RS-SPASM protein family (PDBID: 4K36) showing components of the protein including the TIM barrel fold, RS domain (blue), the SPASM domain (red), [4Fe-4S] clusters (yellow/orange), and SAM (purple). **B)** A condensed reaction scheme for the formation of a 5'-deoxyadenosine radical, generated on the radical-SAM cluster (RS cluster) and the known biosynthetic modifications catalyzed by MftC and MftE.

Figure 2.

4.2-K Mössbauer spectra of the as-purified and chemically reconstituted wild-type *Mu* MftC. (Top) The spectrum of the as-isolated MftC (black) consists of a quadrupole doublet corresponding to the presence of [2Fe-2S] clusters (dark green highlight) and [4Fe-4S] clusters (light green highlight). The overall simulation is overlaid on the experimental spectrum as a black solid line. (Bottom) The spectrum of the chemically reconstituted with two equiv. of [Fe-S] clusters MftC (black) consists of a single quadrupole doublet corresponding to the presence of [4Fe-4S] clusters. The spectra were recorded in the presence of a small external magnetic field (0.078 T), applied parallel to the orientation of the γ beam.

Figure 3.

A) Activity assays consisting of 2 mM SAM, 10 mM DTT, 2 mM DTH, and 100 μ M of the MftC variant or **B)** 2 mM SAM, 10 mM DTT, 2 mM DTH, 100 μ M MftA, 100 μ M MftB and 100 μ M of the MftC variant suggest that Aux I and Aux II clusters are not required for SAM turnover and that all clusters are required for MftA modification.

Figure 4.

The extracted g values and g strain for the three $[4\text{Fe-4S}]$ clusters from the difference spectra were used to simulate (red, cyan, magenta) the intact MftC protein (black, blue, and purple) in the absence and presence of SAM, MftA, and MftB. Spectra were obtained at 20 K.

Figure 5.

EPR spectra, measured at 20 K, for individual [4Fe-4S] cluster knockouts were subtracted from MftC, yielding difference spectra (A-C). The experimental difference spectra (black) represent the EPR spectra for the individual cluster knocked out and can be reasonably simulated (red). The EPR difference spectra in the presence of SAM (D-F) suggest that the removal of the RS cluster affects the environments or the remaining clusters when SAM is bound. Conversely, removal of the Aux I or Aux II cluster has little impact on the environments of the remaining clusters when SAM is bound. The experimental difference spectra (blue) for the RS cluster could not be simulated whereas the simulations (cyan) for the Aux I and Aux II cluster are plausible representations of [4Fe-4S]⁺ clusters. The EPR difference spectra in the presence of SAM, MftA, and MftB (G-I) have similar implications to when SAM is bound alone. Likewise, the removal of the RS cluster impacts the remaining [4Fe-4S] clusters when SAM, MftA, and MftB is bound whereas the inverse is not observed. The experimental difference spectra are shown in violet and the simulated data is shown in magenta; the RS cluster could not be simulated.

Figure 6.

Voltammetry of MftC and variants measured at pH 7.5 and 4 °C. (A) Cyclic voltammogram measured with a scan rate of 100 mV/s for wild-type MftC (solid line), fitting for three, one electron transfers (dotted line), and EPG baseline (dashed line). Square wave voltammograms measured with a frequency of 10 Hz and an amplitude of 20 mV for (B) wild-type MftC (solid line), (C) RS KO, and (D) Aux II KO with EPG baselines (dashed line).

Scheme 1.

MftC catalyzes the oxidative decarboxylation of MftA followed by the C-C bond formation between Val29 and Tyr30. The oxidative reaction is highlighted by the blue box and the redox neutral reaction is highlighted by the red box. It is currently unknown if the Aux I or Aux II clusters participate in the reaction via electron shuttling.

Table 1.

Reduction potentials for the RS, Aux I, and Aux II [4Fe-4S]^{2+/1+} clusters in and the midpoint potential for the sweep width (SW).

Variant	$E_{m,1}$ (mV vs. SHE)	$E_{m,2}$ (mV vs. SHE)	$E_{m,3}$ (mV vs. SHE)	SW E_{mid} (mV vs. SHE)
Wild-type MftC	−549	−499	−458	−489
RS KO	−507	−502	--	−484
Aux II KO	−520	--	−454	−475