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Mechanistic and functional versatility of radical SAM enzymes

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Abstract

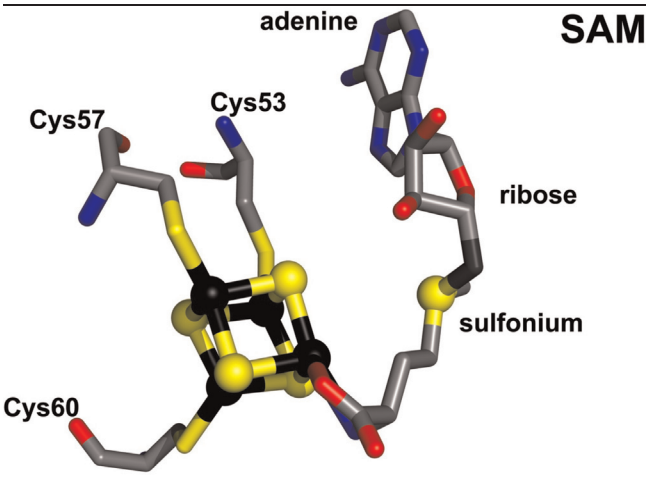
Enzymes of the radical SAM (RS) superfamily catalyze a diverse assortment of reactions that proceed via intermediates containing unpaired electrons. The radical initiator is the common metabolite S-adenosyl-L-methionine (SAM), which is reductively cleaved to generate a 5′-deoxyadenosyl 5′-radical, a universal and obligate intermediate among enzymes within this class. A bioinformatics study that appeared in 2001 indicated that this superfamily contained over 600 members, many catalyzing reactions that were rich in novel chemical transformations. Since that seminal study, the RS superfamily has grown immensely, and new details about the scope of reactions and biochemical pathways in which its members participate have emerged. This review will highlight only a few of the most significant findings from the past 2-3 years, focusing primarily on: RS enzymes involved in complex metallocofactor maturation; characterized RS enzymes that lack the canonical CxxxCxxC motif; RS enzymes containing multiple iron-sulfur clusters; RS enzymes catalyzing reactions with compelling medical implications; and the energetics and mechanism of generating the 5′-deoxyadenosyl radical. A number of significant studies of RS enzymes will unfortunately be omitted, and it is hoped that the reader will access the relevant literature – particularly a number of superb review articles recently written on the subject – to acquire a deeper appreciation of this class of enzymes.

Introduction and context

A pivotal paper published in the year 2001 by Heidi Sofia *et al.* [1] identified a superfamily of metalloenzymes that catalyze a rich assortment of reactions involved in numerous important biological pathways, such as the biosynthesis of a large number of enzyme cofactors, antibiotics and other natural products, the biosynthesis and repair of DNA, and general bacterial metabolism. Although these reactions were diverse, they all shared the property of being initiated via removal of a target hydrogen atom (H•) from their relevant substrate by a 5′-deoxyadenosyl 5′-radical (5′-dA•) generated from a reductive cleavage of S-adenosyl-L-methionine (SAM). The authors coined the title 'radical SAM' for this superfamily of enzymes to distinguish them from the classical SAM-dependent reactions that proceed via polar (e.g., S_N2) mechanisms. The thrust of that paper was the identification of telltale features within the primary structures of these proteins, most notably a CxxxCxxC motif, which has facilitated the rapid discovery of radical SAM (RS) proteins by sequence gazing. Spectroscopic and biochemical studies on canonical members of the RS superfamily showed that each contained a $[4Fe\text{-}4S]^{2+/+}$ cluster in which three of the four irons of the cubane structure are ligated by single cysteinyl residues lying in a CxxxCxxC motif. The fourth iron is chelated to the α-amino and α-carboxylate groups of SAM in a bidentate fashion, which presumably facilitates the electron transfer step and ensuing cleavage reaction (Figure 1) [2,3].

At the time of the study by Sofia *et al.*, the RS superfamily was predicted to contain over 600 members. A recent review article by Frey, Hegeman, and Ruzicka [2] entitled 'The radical SAM superfamily' indicates that there are at

Figure 1. Binding mode of SAM in radical SAM proteins



Binding of SAM (S-adenosyl-L-methionine) to the [4Fe-4S] cluster of biotin synthase. Color scheme: black, Fe; blue, N; yellow, S; red, O; grey, C. Structure prepared using Pymol Molecular Graphics System [74] from Protein Data Bank entry 1R30.

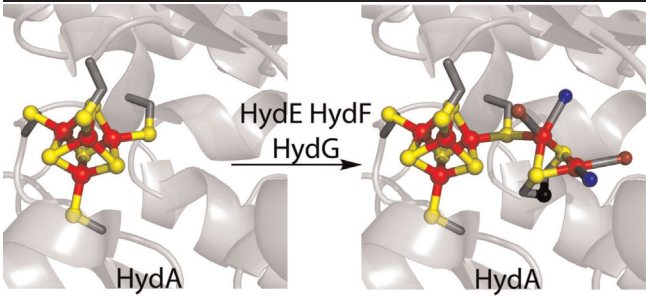
least 2845 proteins in 781 microbial genomes that contain the CxxxCxxC signature sequence; however, as detailed below, this sequence, though overwhelmingly common, is not strictly conserved among all RS proteins, suggesting that this superfamily may be more diverse than previously imagined. Several review articles on RS enzymes have appeared recently, and the reader is encouraged to seek them out to gain a deeper understanding of the most significant issues and a broader appreciation of the reactions involved. The most comprehensive of the reviews is by Frey, Hegeman, and Ruzicka [2], which discusses in broad terms how the field has developed over the past 10 years. The review by Booker entitled 'Anaerobic functionalization of unactivated C-H bonds' discusses the use of the 5′-dA• to catalyze functionalization of small molecules and proteins [4], while the review by Duschene *et al.*, entitled 'Control of radical chemistry in the AdoMet radical enzymes', focuses on the energetics and mechanism of generating the 5′-dA• [5].

Major recent advances

Maturation of complex metallocofactors

RS enzymes are involved in the maturation of at least three classes of complex metallocofactors, the iron-molybdenum cofactor (FeMo-co) of nitrogenase, the H-cluster of the [FeFe]-hydrogenase, and the mononuclear cluster of the [Fe]-hydrogenase. The study by Sofia *et al.* [1] suggested that the *nifB* gene product, involved in an unknown step in the biosynthesis of FeMo-co, was a RS protein. A subsequent report by Curatti, Ludden, and Rubio [6] showed that purified and reconstituted NifB was able to support *in vitro* reconstitution of FeMo-co in the presence of SAM; however, little progress has been made in characterizing the reaction or identifying the exact nature of its substrate. By contrast, significant gains have been made in the past 2 years in understanding the biosynthesis of the H-cluster of the [FeFe]-hydrogenase, one of the enzymes responsible for the reversible reduction of protons to H_2 [7]. This cluster consists of a 2Fe subcluster coordinated by cyanide and carbon monoxide ligands, as well as a dithiolate moiety (-SCH_2-X-CH_2S-), which is then bridged to a [4Fe-4S] cluster via a protein cysteinate ligand (Figure 2) [8,9]. The exact identity of the dithiolate moiety has not been confirmed; X has been suggested to be C, N, or O [9]. Genetic and biochemical studies indicate that three accessory proteins are required to synthesize and insert the H-cluster into the hydrogenase protein (HydA) [7]: HydE, HydG, and HydF (Figure 2). HydE and HydG are RS enzymes, while HydF contains GTPase activity [10-13]. The X-ray crystal structure of HydE was recently solved to 1.35 Å, the highest resolution structure of any RS enzyme. The structure revealed a [2Fe-2S] cluster separated from the RS [4Fe-4S] cluster by approximately 20 Å in a spatial arrangement similar to that of the two [4Fe-4S] clusters in MoaA, which is involved in molybdopterin biosynthesis [14]. It is not clear whether this [2Fe-2S] cluster – which may be a degradation product of a second [4Fe-4S] cluster observed spectroscopically in another study [11] – is actually required for maturation, because substitution of its coordinating Cys residues with those containing noncoordinating R-groups did not eliminate hydrogenase activity in an *in vivo* assay. Moreover, the ligands to the second cluster are not conserved among all

Figure 2. Maturation of the H-cluster of the [FeFe]-hydrogenase



The structure on the left represents HydA, the hydrogenase from *Desulfovibrio desulfuricans*, with a [4Fe-4S] cluster bound. In the presence of HydE, HydF, HydG, and appropriate small molecules, the H-cluster is formed on HydA. Color scheme: red, iron; yellow, sulfur; grey, carbon; blue, nitrogen; black, unidentified atom (X). Structure prepared using Pymol Molecular Graphics System [74] from Protein Data Bank entry 1HFE.

HydE proteins. Although the substrate for HydE is unknown, Nicolet *et al.* [15] provided evidence that the protein can bind thiocyanate, which led them to speculate that it might be involved in generating the cyanide ligands to the H-cluster.

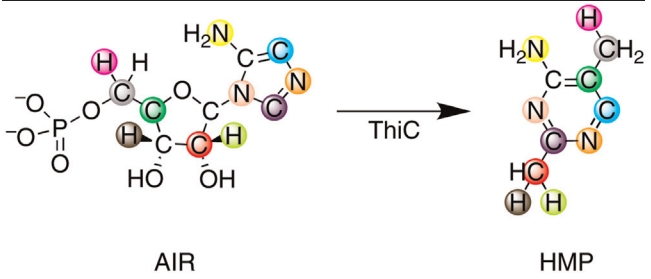
The protein HydG bears 27% sequence identity to the *Escherichia coli* enzyme ThiH, a RS protein that catalyzes a key step in the formation of the thiazole ring of the cofactor thiamine diphosphate. The 5′-dA• produced by ThiH is proposed to abstract the phenolic hydrogen atom from L-tyrosine, initiating a fragmentation reaction that liberates *p*-cresol and dehydroglycine. Dehydroglycine is then condensed with ThiFS thiocarboxylate and 1-deoxyxylulose 5-phosphate to give thiazole-phosphate in a reaction catalyzed by ThiG [16]. The sequence similarity between HydG and ThiH inspired investigation by Pilet *et al.* [17] to ascertain whether the substrate for HydG was also L-tyrosine. HydG did in fact catalyze liberation of *p*-cresol from L-tyrosine, leading the authors to postulate that HydG is the site for the synthesis of a dithiomethylamine ligand (-SCH_2-NH-CH_2S-; X suggested to be N) – derived from the presumed dehydroglycine product – onto a [2Fe-2S] cluster scaffold. There was no mention, however, as to whether dehydroglycine was also observed as a product [17].

A different group investigating the role of HydG in the maturation of the H-cluster of hydrogenase also found that HydG catalyzes the cleavage of L-tyrosine. Not only was *p*-cresol found as a product, there was clear evidence for the formation of cyanide in almost equivalent amounts. The authors proposed that the cyanide produced could derive from a facile oxidative decarboxylation of dehydroglycine, but more interestingly, suggested that both cyanide and carbon monoxide could be produced in a single reaction via a decarbonylation of dehydroglycine, which they stated has chemical precedent [18]. Therefore, it appears that the role of HydG is to use RS chemistry to catalyze formation of the cyanide ligands of the 2Fe subcluster, and perhaps the carbon monoxide ligands as well.

Radical SAM enzymes lacking the canonical CxxxCxxC motif

ThiC, an enzyme involved in thiamine diphosphate biosynthesis in prokaryotes, was not identified as an RS member by Sofia *et al.* [19]. The penultimate step in the *de novo* thiamine diphosphate biosynthetic pathway involves a condensation of the thiazole and pyrimidine moieties of the cofactor, each synthesized in two independent branches of the pathway, to furnish thiamine monophosphate, which is subsequently phosphorylated to the active cofactor [19]. In contrast to

Figure 3. The reaction catalyzed by ThiC



Color-coding depicts the change in positioning of certain atoms during the rearrangement as determined by labeling experiments. AIR, 5-aminoimidazole ribonucleotide; HMP, 4-amino-5-hydroxymethyl-2-methylpyrimidine. Figure is adapted from reference [21].

ThiH, which participates along with other proteins in the formation of the thiazole moiety, ThiC alone catalyzes formation of the pyrimidine moiety [19]. The reaction is among the most complex in all of mechanistic enzymology, which is the conversion of 5-aminoimidazole ribonucleotide to 4-amino-5-hydroxymethyl-2-methylpyrimidine phosphate (HMP-P). Figure 3 highlights the results of labeling studies, illustrating the complex nature of the reaction [19]. Recently, the enzymes from *Arabidopsis thaliana* [20], *Salmonella enterica* [21], and *Caulobacter crescentus* [22] have been characterized to be iron-sulfur (Fe/S) proteins, the latter two of which were shown to catalyze *in vitro* formation of HMP-P or 4-amino-5-hydroxymethyl-2-methylpyrimidine (HMP) in the presence of substrate, SAM, and dithionite [21,22].

The recent X-ray crystal structure of apo-ThiC from *C. crescentus* with bound HMP-P identified three structural domains: an N-terminal domain, a central domain, and a disordered C-terminal domain. The latter bears a conserved CxxCxxxxC motif, the Cys residues of which could ligate an Fe/S cluster. However, note that the sequence differs from the canonical RS CxxxCxxC motif, and its position in the protein at the C terminus instead of near the N terminus is also distinct [22]. Typical RS enzymes contain the CxxxCxxC motif in the N-terminal half of their primary structures [1]. The structure revealed the protein to be dimeric, and the [4Fe-4S] cluster, shown to be present on the reconstituted enzyme by Mössbauer and electron paramagnetic resonance (EPR) spectroscopy, was modeled into the protein using the structure of biotin synthase as a template. SAM was modeled into the active site pocket to coordinate the unique iron of the [4Fe-4S] cluster in a bidentate fashion, in common with other RS enzymes, which places the 5′-carbon in a suitable position to abstract a hydrogen atom from the ribose ring of the substrate by a generated 5′-dA•.

Evidence for a mechanism involving organic radicals was provided in an EPR study. When SAM was added to the dithionite-reduced enzyme, a new signal centered at g = 2.002 emerged, which had line width and temperature-dependent properties that were consistent with an organic radical. A sample prepared in D_2O allowed determination that the radical was centered on the α-carbon of an amino acid residue other than glycine or alanine. Exposure of the protein bearing the organic radical to oxygen led to rapid destruction of the EPR signal and cleavage of the polypeptide chain between Gly436 and His437 [23]. Whether this organic radical is an intermediate in this reaction remains to be resolved.

Elp3 from *Methanocaldococcus jannaschii* and HmdB from *Methanococcus maripaludis* S2 are two partially characterized enzymes similarly found to lack the canonical CxxxCxxC motif. Elp3 is a component of the Elongator complex, required for transcription elongation. Elongator is composed of six subunits, Elp1 to Elp6. Elp3 is thought to be the catalytic subunit, given that it is one of the subunits that forms the core of the complex, and that it displays histone acetyl transferase (HAT) activity [24]. In addition to its C-terminal HAT domain, Elp3 has a domain potentially related to RS enzymes despite a $CX_4CX_9CX_2C$ motif deviating from the canonical CxxxCxxC RS motif. It was speculated that the RS domain might catalyze demethylation of methylated lysyl residues on histones [25]. The RS domain of Elp3 from *M. jannaschii* (residues 63-371) was subsequently purified and shown to bind SAM and small amounts of iron [26]. The Cys motif in this archaeal Elp3 (CxxxxCxxC) is different from both the canonical motif and that found in eukaryotic proteins.

Small amounts of Elp3 from *Saccharomyces cerevisiae* were recently isolated, allowing the involvement of possible Fe/S clusters in catalysis to be investigated. Substitution of individual Cys residues by Ala residues in the proposed RS domain of Elp3 resulted in phenotypes that were indistinguishable from those observed upon deletion of the entire *ELP3* gene, suggesting that the proposed cluster is important for normal Elongator function. Further studies showed that the Cys→Ala substitutions affected assembly of the Elongator complex, but had little effect on HAT activity or the ability of the complex to bind to RNA polymerase II in chromatin. In addition, no histone demethylase activity was detected, and no evidence for the ability to bind SAM was found. The authors concluded that the Fe/S cluster, if present, serves a structural rather than catalytic role [27].

A more recent study has demonstrated at least partial involvement of the RS domain of mammalian Elp3 in active demethylation of 5-methyl cytosines of the paternal DNA strand at the zygotic stage of fertilization and development [28]. This event is believed to be vital in the reprogramming of germ cells to allow their transition to somatic cells. To show this, Okada *et al.* [28] developed molecular probes to allow determination of the methylation state of DNA in zygotes via time-lapse imaging, which they used in conjunction with RNA interference to allow cellular levels of candidate demethylases to be knocked down. Single interfering RNA (siRNA) molecules targeting Elp1, Elp3, and Elp4 all affected the zygotic paternal methylation status. Interestingly, introduction of mRNA encoding substitutions of the Cys residues within the proposed RS domain of Elp3 affected the paternal methylation status, whereas substitutions in the HAT domain of Elp3 did not. They suggested that demethylation might be mediated through a reaction that requires an intact RS domain.

The *hmdB* gene from *M. maripaludis* S2 was recently found to be adjacent on the chromosome to the *hmdA* gene. HmdA, found in hydrogenotrophic methanogens, catalyzes the reversible reduction of methenyl-tetrahydromethanopterin (H_4MPT^+) to methylene-H_4MPT and H^+, and contains an octahedrally-coordinated nonheme iron atom bearing two CO ligands, a protein cysteinyl ligand, an unknown ligand, and a guanylyl pyridinol cofactor ligand [29]. The primary structure of HmdB contains a CxxxxxCxxC motif and is phylogenetically related to ThiH, HydE, and HydG (see above). The purified protein was shown by UV-visible and EPR spectroscopy to contain a [4Fe-4S] cluster. In addition, it was capable of catalyzing cleavage of SAM to 5′-dA in the presence of dithionite, suggesting its inclusion in the RS superfamily [29]. It was suggested that HmdB might participate in the synthesis of the iron-carbonyl linkage in the Hmd cofactor.

Radical SAM enzymes with multiple iron-sulfur clusters

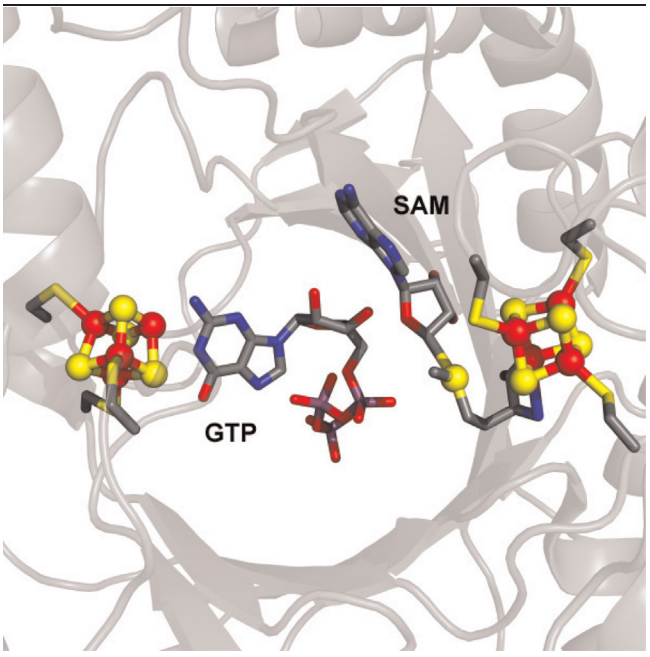
The discovery that biotin synthase from *E. coli* contains two distinct Fe/S clusters per polypeptide, a [4Fe-4S] cluster and a [2Fe-2S] cluster, ushered in a new chapter in RS enzymology, which highlighted the versatility of these enzymes as catalysts [30,31]. With the exception of MoaA, all early RS members containing multiple Fe/S clusters catalyzed the insertion of sulfur deriving from the second cluster into unactivated C-H bonds: a [2Fe-2S] cluster on biotin synthase, and [4Fe-4S] clusters on lipoyl synthase and MiaB [4,32-34]. In addition to sulfur insertion into the hypermodified tRNA nucleoside N^6-(isopentenyl)adenosine-37, MiaB transfers the methyl group from another molecule of SAM onto the inserted sulfur atom. This reaction takes place on the hypermodified tRNA nucleoside N^6-(isopentenyl)adenosine-37,

and involves a net methylthiolation at C2 of the adenine ring. Therefore, it appears that a single polypeptide catalyzes both radical and polar SAM-dependent reactions [35].

Recently, Anton *et al.* [36] showed that the *yliG* gene in *E. coli*, the product of which was designated RimO, catalyzes a similar methylthiolation reaction on a universally conserved Asp residue (Asp88 in *E. coli*) of the S12 subunit of certain bacterial ribosomes. RimO proteins from *E. coli* [37] and *Thermotoga maritima* [38] were subsequently purified by two different groups, and in both instances shown to bind two [4Fe-4S] clusters per polypeptide and to catalyze methylthiolation of a peptide substrate containing an Asp residue in the appropriate sequence context. RimO, like MiaB, contains six conserved Cys residues, all of which reside in the N-terminal region of the protein, suggesting that this second cluster is also ligated by only three Cys residues. In analogy with the previously mentioned RS enzymes that catalyze sulfur insertion, it is believed that the second cluster provides an activated form of sulfide to be inserted into the substrate.

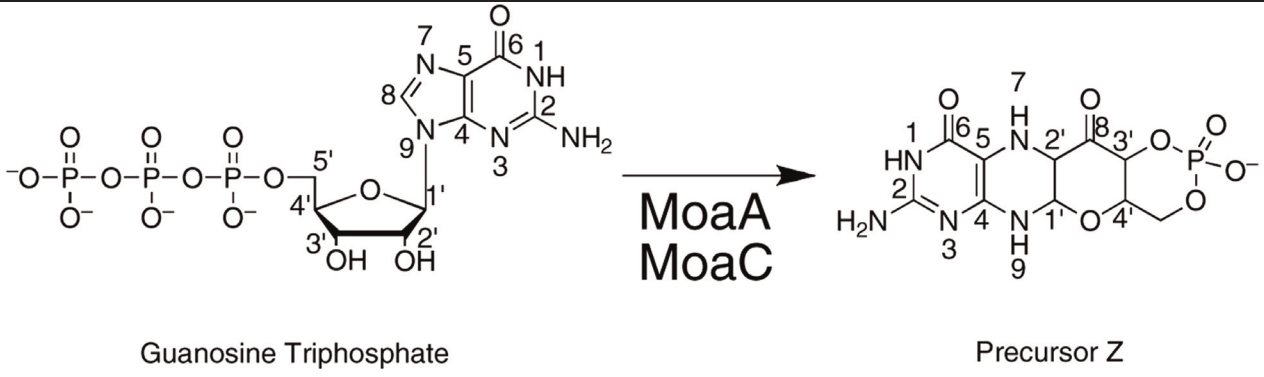
The RS enzyme MoaA has been characterized structurally and shown to bind an additional [4Fe-4S] cluster via three Cys residues located in the C-terminal region of its primary structure [39]. Unlike the enzymes discussed above, its net reaction does not involve sulfur insertion, but is a cryptic rearrangement of GTP to yield precursor Z, an intermediate in the biosynthesis of the cofactor molybdopterin (Figure 4). The MoaA reaction was shown to be dependent on the second cluster and to require the accessory protein MoaC, which participates in some undefined role [14]. The X-ray crystal structure of MoaA containing both clusters and in complex with both SAM and GTP provided valuable insight into the architecture of the active site (Figure 5) [40]. The C-terminal cluster appeared to interact with either the N1 or N3 nitrogen atoms of GTP; however, the poorly defined electron density of the substrate did not allow an exact determination of its binding mode. Recently, electron nuclear double resonance (ENDOR) spectroscopy was used to show that the mode of binding involved coordination of the N1 nitrogen atom to the unique iron atom of the cluster at a distance of 1.94 Å.

Figure 5. The active site of MoaA



Structure of MoaA with both GTP and SAM (S-adenosyl-L-methionine) bound. Color scheme: black, Fe; blue, N; yellow, S; red, O; grey, C. Structure prepared using Pymol Molecular Graphics System [74] from Protein Data Bank entry 2FB3.

Figure 4. The reaction catalyzed by MoaA/MoaC



Numbers highlight changes in positioning of atoms during the rearrangement.

The authors suggested that this interaction should favor guanine binding to the unique iron atom as the enol rather than keto tautomer, which they stated may have mechanistic implications [41]. Currently, little is known about the detailed mechanism of catalysis by MoaA/MoaC. This C-terminal Fe/S cluster has been shown to be redox-active, and it has been speculated that it could play a role in electron transfer [40].

Another RS enzyme purported to be in the MoaA family is PqqE, which is one of six proteins required for the biogenesis of pyrroloquinoline quinone (PQQ). Unlike the other quino-cofactors, which are generated via posttranslational modifications of the core catalytic proteins, PQQ is synthesized as a small-molecule cofactor that subsequently associates with the relevant catalyst via noncovalent interactions [42]. The biogenesis of PQQ is quite complex, involving the crosslinking of the side chains of glutamyl and tyrosyl residues from a core peptide composed of 23 amino acids, which serves as the skeleton of the cofactor [43]. It has been suggested that PqqE might catalyze this crosslinking, generally believed to be the first step in the pathway. Similar to MoaA, PqqE displays two highly conserved cysteine-containing motifs at the N and C termini of the protein, CX_3CX_2C and $CX_2CX_{27}C$, respectively. A recent study by Wecksler *et al.* [44] provided spectroscopic and analytical evidence for the presence of two Fe/S clusters on the enzyme from *Klebsiella pneumoniae*, and showed that the protein can catalyze cleavage of SAM to yield 5′-dA and methionine. However, no evidence for *in vitro* formation of PQQ was forthcoming despite a determined effort to provide it. As described for MoaA, no distinct mechanistic role for this second cluster has been assigned.

The radical SAM dehydrogenases are an emerging subclass of RS enzymes, catalyzing the simple two-electron oxidation of an alcohol or thiol group to the corresponding aldehyde or ketone. Three of these enzymes, spanning two distinct reaction types, have been characterized *in vitro*. The first, BtrN, catalyzes a key step in the biosynthesis of the aminoglycoside antibiotic butirosin B, which is the oxidation of the C3 alcohol of 2-deoxy-*scyllo*-inosamine (DOIA) to amino-2-deoxy-*scyllo*-inosose (amino-DOI) [45]. The second two, anSMEcpe and AtsB, are anaerobic sulfatase modifying enzymes from *Clostridium perfringens* and *K. pneumoniae*, which catalyze the oxidation of a Cys or Ser residue on a cognate protein to generate a formylglycyl cofactor [46,47]. The reactions proceed via abstraction of a hydrogen atom from the carbon to be oxidized by the 5′-dA•, followed by the uptake of an electron by an undetermined acceptor [45,47,48]. Detailed analytical and spectroscopic analysis of AtsB showed that it contained three [4Fe-4S] clusters per polypeptide. It was postulated that one of the clusters binds in contact with the substrate to facilitate loss of an electron from the substrate-radical intermediate via an inner-sphere mechanism [47]. Although the stoichiometry of Fe/S clusters on anSMEcpe has not been determined, the protein shares 48% sequence similarity with AtsB, including 11 conserved Cys residues. By contrast, BtrN was characterized to contain only one [4Fe-4S] cluster, suggesting that the presence of multiple Fe/S clusters is not a prerequisite for RS dehydrogenation [49]. Recently, Mössbauer spectroscopy was used in concert with analytical determinations of iron content to re-evaluate the stoichiometry of Fe/S clusters rigorously, showing that indeed the protein contains two [4Fe-4S] clusters [50].

Radical SAM enzymes with compelling medical implications

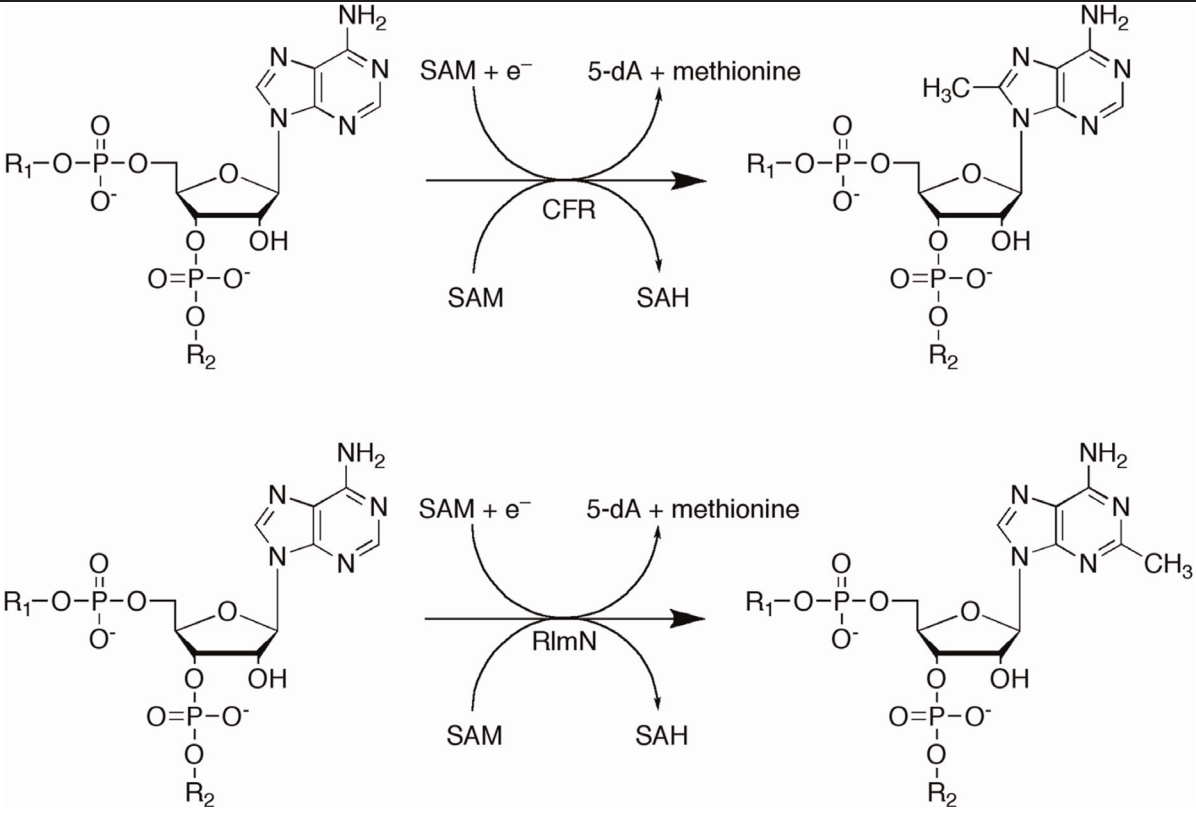
Very recent findings portend that a number of exciting RS-dependent transformations that have compelling medical implications are on the horizon, including reactions involving bacterial defense against antibiotics and host defense against invading viruses. Viperin (virus inhibitory protein, endoplasmic reticulum-associated, interferon-inducible), a protein induced upon interferon stimulation, is involved in the antiviral defense against DNA viruses such as cytomegalovirus, RNA viruses such as hepatitis C and influenza, and retroviruses such as human immunodeficiency virus [51,52]. The protein is highly conserved across species, sharing significant sequence homology with similar proteins from trout and mouse, as well as a protein from rat, best5, which is expressed during osteoblast differentiation and bone formation [1,51]. Viperin is composed of three distinct domains, a variable N-terminal domain, a radical SAM domain, and a C-terminal domain, the last two of which are highly conserved. Mutations in the gene encoding Viperin that give rise to Cys→Ala substitutions at the protein level resulted in loss of antiviral effects against hepatitis C virus, demonstrating the importance of RS chemistry in antiviral activity [53]. At present, the exact mechanism of action of Viperin is unknown, as is its direct target. It has been suggested that Viperin-dependent inhibition of influenza A virus involves perturbing its release from the plasma membrane during its budding cycle by affecting the formation of lipid rafts. This activity is believed to derive from an inhibition of farnesyl diphosphate synthase via an unknown mechanism. Also unknown is the exact pathway downstream of farnesyl diphosphate synthase inhibition that gives rise to viral inhibition [54]. Recently, it was shown that Viperin localizes to intracellular lipid-storage organelles called lipid droplets via an N-terminal amphipathic α-helix, which may mediate its effect against

hepatitis C virus; however, again, the exact mechanism of inhibition is unknown [55].

Two reports describing the first *in vitro* characterization of Viperin have recently appeared [56,57]. In the report by Shaveta *et al.* [57], expression analysis of 12 fragments of the Viperin gene showed that a Viperin construct lacking the first 44 amino acids (i.e., 45-361) was a predominantly soluble protein that could be purified under native conditions by immobilized metal affinity chromatography. Analysis of the reconstituted protein by UV-visible spectroscopy supported the presence of an Fe/S species. In the report by Duschene and Broderick [56], a Viperin construct spanning residues 43-360 was generated. The purified protein had low amounts of iron, but was reconstituted to contain 3.7 irons per polypeptide. Both UV-visible and EPR spectroscopy analysis of the protein supported the presence of [4Fe-4S] clusters. In addition, the protein was capable of catalyzing reduction of SAM to 5′-dA and methionine.

Cfr is another recently characterized RS protein with clear medical implications. It confers resistance to five classes of antibiotics (phenicols, lincosamides, oxazolidinones, pleuromutilins, and streptogramin A) – all of which bind to the peptidyl transferase center of bacterial ribosomes – as well as the 16-membered macrolides josamycin and spiramycin. Its mode of action involves methylation of C8 of adenosine 2503 of 23S ribosomal RNA (rRNA), which sits in the center of the peptidyl transferase site [58]. This methylation has a negligible effect on peptidyl transferase activity, but sterically impedes the binding of antibiotics that target the site. A similar protein, RlmN, targets C2 of the exact nucleotide (Figure 6). It is endogenous to a wide number of bacteria and other organisms, functioning in the fine-tuning of translation. By contrast, the *cfr* gene is acquired, and appears to be an evolutionary spin-off of the *rlmN* gene, arising from gene duplication and horizontal transfer [59]. Recently, a structural model of Cfr was generated using the MoaA structure as a template for its central RS domain. The model included the expected [4Fe-4S] cluster ligated by the RS motif as well as two molecules of bound SAM: one as the precursor to the 5′-dA• and one as the donor of the methyl group [59]. The RNA substrate was not modeled into the structure. A particularly significant

Figure 6. The reactions catalyzed by Cfr and RlmN



SAH, S-adenosyl-homocysteine; SAM, S-adenosyl-L-methionine.

aspect of the work was a study of amino acid substitutions at proposed sites for binding the [4Fe-4S] cluster and the two SAM molecules, as well as in the N-terminal and C-terminal domains of the protein. Among other observations, five Cys residues appeared critical for Cfr activity. These included residues 112, 116, and 119, found in the RS signature sequence; Cys338, found at the C-terminus of the protein; and Cys105, found in the binding pocket for the second SAM molecule. Interestingly, although the Cys105→Ala substitution did not support methylation, it appears that some type of reaction took place that caused a stop in primer extension assays similar to the observed effect of C8 methylation [59].

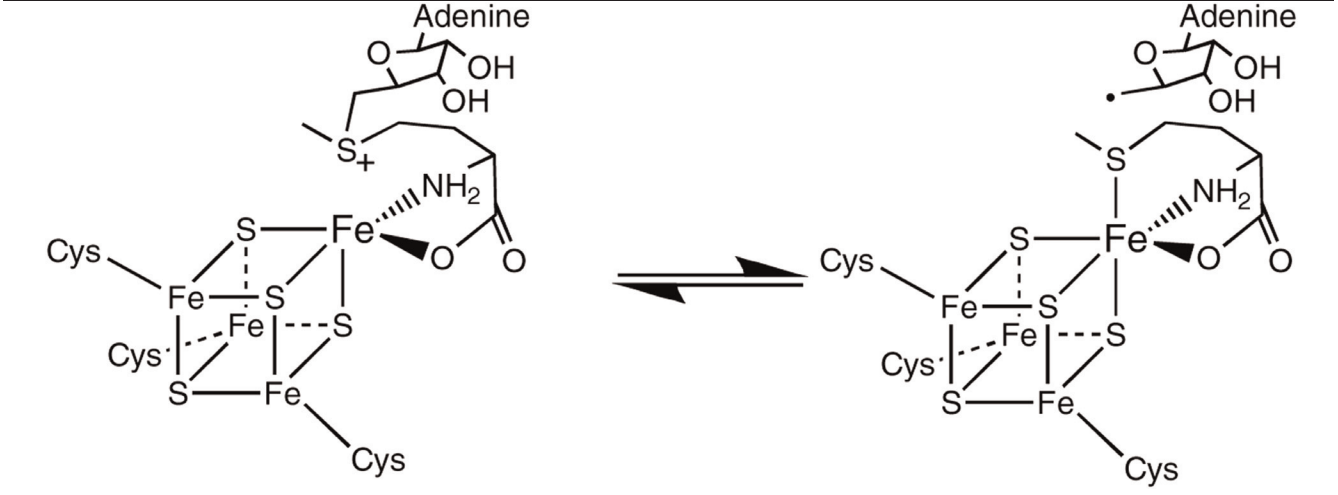
More recently, Yan *et al.* expressed the genes for *E. coli* RlmN and *Staphylococcus aureus* Cfr in *E. coli*, and isolated the corresponding hexahistidine-tagged proteins [60]. They found that the reconstituted protein contained 3.98 and 6.79 irons, respectively, which they concluded was sufficient to support formation of a [4Fe-4S] cluster. *In vitro* activity determinations on the proteins were conducted with a series of potential substrates, including rRNA substrates of different sizes. It was found that neither the intact 70S ribosome nor the isolated 50S or 30S subunits served as substrates for either Cfr or RlmN. Only protein-free rRNA containing adenosine at position 2503 was capable of being methylated, which suggests that these proteins catalyze their reactions before the ribosome is assembled. Further studies provided evidence for the formation of both 5′-dA and S-adenosyl-homocysteine similar to that observed for MiaB and RimO. Moreover, when the reaction was conducted in the presence of S-adenosyl-L-[*methyl*-^{3}H]methionine, radioactivity was found to be transferred to the rRNA substrate, indicating that SAM is the source of the appended methyl group [60].

Advances in understanding the reductive cleavage of S-adenosylmethionine

The fundamental chemical transformation common to all radical SAM enzymes is the reductive cleavage of SAM to generate the 5′-dA•, which in solution is thermodynamically unfavorable. Midpoint potentials for the irreversible one-electron reduction of a trialkylsulfonium ion are on the order of -1.8 V, while those for radical SAM proteins tend to be much higher [61]. A study by Wang and Frey [61] investigated the energetics of SAM cleavage by lysine 2,3-aminomutase, which uses RS chemistry to catalyze an interconversion of α- and β-lysine when bound in an aldimine linkage to a required pyridoxal 5′-phosphate cofactor. They found that in the resting state of the enzyme (i.e., with SAM and pyridoxal 5′-phosphate bound) the [4Fe-4S] clusters exhibited a midpoint potential of -430 mV, and that the binding of lysine lowered the midpoint potential by ∼150 mV. Similarly, the midpoint potential for the reductive cleavage of SAM in the enzyme/SAM/lysine complex was estimated to be –990 mV from values obtained using the analog *S*-3′,4′-anhydroadenosyl-L-methionine. Therefore, the enzyme active site environment raises the redox potential of SAM by ∼ 810 mV while lowering the redox potential of the Fe/S cluster upon substrate binding, which corresponds to a decrease in the overall barrier for the reductive cleavage of SAM from 32 kcal/mol in solution to 9 kcal/mol. Additional energy for the process is believed to derive from ligation of the sulfur atom of the generated methionine to the unique iron of the cluster, which generates a hexacoordinate species and facilitates inner-sphere electron transfer (Figure 7) [61,62].

A recent study by Nicolet *et al.* [63] provided additional support for the mechanism of reductive

Figure 7. Model for the reductive cleavage of SAM to generate a 5′-deoxyadenosyl radical



cleavage of SAM proposed by Frey and coworkers, and argued that the mechanism should be common to all RS enzymes containing the canonical CxxxCxxC motif. This conclusion stems from their solving of the X-ray structures of HydE (see above) with SAM bound and with both 5′-dA and methionine bound at 1.62 and 1.25 Å, respectively, and then using these structures in concert with computational methods to calculate the most likely reaction trajectory. Interestingly, their calculated barrier for SAM cleavage of 54.0 kJ/mol (12.9 kcal/mol) agrees well with the experimental estimate made by Wang and Frey (9 kcal/mol) [61]. Moreover, they remarked that in all RS structures solved in complex with SAM, SAM was bound in essentially the same fashion in each case [63].

Future directions

In the near future, much of the focus on RS enzymes will undoubtedly involve characterizing new and novel enzymatic reactions. As described above, the RS superfamily may be significantly larger than previously imagined, and it is no longer safe to rely on the presence of a CxxxCxxC motif in the N-terminal half of a protein sequence as an indicator of membership in this family. Many new discoveries will emanate from studies to identify gene clusters for the biosynthesis of a variety of natural products, such as clorobiocin [64], moenomycin A [65], pactamycin [66], gentamicin [67], nosiheptide [68], unusual lipids [69], and deazapurine-containing secondary metabolites [70], which are just a few of the more recent ones to be discovered. Indeed, RS enzymes involved in the biosynthesis of the antibiotic butirosin B and the antibiotic precursor D-desosamine have already been well characterized [45,49,71].

One particular class of RS methyltransferases, which are distinct from Cfr and RlmN, deserves special attention in the future. This class was highlighted in the study by Sofia *et al.*, and its participant enzymes are annotated as being in the P-methylase family. This name derives from one of the founding members of this subclass of RS enzymes, which catalyzes the methylation of a phosphinate phosphorus atom in the biosynthesis of the herbicide bialaphos [72]. Interestingly, these enzymes are annotated as cobalamin binding proteins, and a number of genetic and *in vivo* biochemical studies support that assignment; however, there have been no reports of the isolation and *in vitro* characterization of one of these proteins [4]. A hypothetical mechanism for these RS methyltransferase reactions was advanced by van der Donk [73], in which he proposed that the added methyl group is transferred from methylcobalamin to the substrate radical generated via hydrogen atom abstraction by the 5′-dA• in a radical process.

Two additional areas of future interest are the elucidation of the mechanisms for re-installing the sacrificed Fe/S clusters in RS enzymes that catalyze sulfur insertion, and the development of more robust bioinformatics methods for identifying RS proteins that do not contain the canonical signature sequence in the N-terminal half or their primary structures. The emergence of the RS superfamily of enzymes has brought renewed vigor to mechanistic enzymology. Many of the known transformations are simply astounding, and the future bodes well for discovering new ones that will remind us of the wonders of nature.

Abbreviations

5′-dA•, 5′-deoxyadenosyl 5′-radical; EPR, electron paramagnetic resonance; FeMo-co, iron-molybdenum cofactor; Fe/S, iron-sulfur; HAT, histone acetyl transferase; HMP-P, 4-amino-5-hydroxymethyl-2-methylpyrimidine phosphate; PQQ, pyrryloquinone; rRNA, ribosomal RNA; RS, radical SAM; SAM, S-adenosyl-L-methionine; Viperin, virus inhibitory protein, endoplasmic reticulum-associated, interferon-inducible.

Competing interests

The authors declare that they have no competing interests.

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