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Self Sacrifice in Radical S-adenosylmethionine Proteins

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Abstract

The radical SAM superfamily of metalloproteins catalyze the reductive cleavage of S-adenosyl-L-methionine to generate a 5'-deoxyadenosyl radical (5'-dA•) intermediate that is obligate for turnover. The 5'-dA• acts as a potent oxidant, initiating turnover by abstracting a hydrogen atom from an appropriate substrate. A special class of these enzymes use this strategy to functionalize unactivated C–H bonds by insertion of sulfur atoms. This review will describe the characterization of three members of this class—biotin synthase, lipoyl synthase, and MiaB protein—each of which has been shown to cannibalize itself during turnover.

The radical SAM superfamily of enzymes

The radical S-adenosylmethionine superfamily of enzymes constitutes a class of metalloproteins that catalyze a reductive cleavage of S-adenosyl-L-methionine (SAM) to L-methionine and a 5'-deoxyadenosyl 5'-radical (5'-dA•) (Figure 1) [1,2]. The 5'-dA• is a key and common intermediate, and functions to remove a hydrogen atom from an appropriate substrate. In several instances the C–H bond that is cleaved is completely unactivated and comparable in energy to C–H bonds cleaved by enzymes that generate high-energy oxidants via the concerted action of a metal-containing cofactor and dioxygen [3]. The closest relative of this radical-generating system is 5'-deoxyadenosyl 5'-cobalamin (coenzyme B₁₂), which reversibly generates a 5'-dA• via a homolytic cleavage of the cofactor's cobalt–5'-carbon bond [2]. Because the sulfur atom in SAM does not contain low-lying *d*-orbitals that are capable of stabilizing an unpaired electron, as does the cobalt atom in coenzyme B₁₂, a 5'-dA• cannot be generated via simple homolysis of the sulfur–5'-carbon bond of SAM. Radical SAM (RS) proteins require the input of an electron that derives from a reduced iron–sulfur (Fe/S) cluster, [4Fe–4S]⁺, which is an obligate cofactor. In almost all RS proteins the Fe/S cluster is ligated by three cysteine thiolate side chains that are arranged in a CX₃CX₂C motif, which has become a signature sequence for this superfamily. This motif, in combination with glycine-rich segments that signify SAM binding, allows RS proteins to be identified by bioinformatics methods. As of the year 2001, the RS superfamily was predicted to contain over six hundred members spanning all three domains of life [4]. With the increasing rate at which DNA sequences of genomes from myriad and diverse organisms are determined, membership is growing rapidly. RS proteins have been shown, or are predicted, to catalyze key steps in general metabolism, DNA biosynthesis and repair, and the biosynthesis of a number of cofactors,

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coenzymes, antibiotics and herbicides [4]. Some are also predicted to participate in a variety of other cellular functions such as host defense against viral invasion and cell-cycle regulation [4-6].

With the exception of lysine 2,3-aminomutase, on which an impressive amount of detailed mechanistic information exists, much of the focus of investigations of RS proteins has been on the characterization of their Fe/S clusters. Elegant spectroscopic studies performed on pyruvate formate-lyase activating enzyme (PFL-AE) and lysine 2,3-aminomutase (LAM) indicate that SAM binds in contact with a unique iron atom of the Fe/S cluster—the one that is not coordinated by a cysteine thiolate side chain—forming a bidentate chelate through its α -amino and α -carboxylate groups [7-10]. These findings have been substantiated with the three-dimensional determination of SAM-bound structures of coproporphyrinogen III oxidase (HemN) [11], biotin synthase (BioB) [12,13], MoaA [13], and LAM [14]. In all instances, the sulfonium atom of SAM is ~ 4 Å away from the nearest atom of the Fe/S cluster, which should allow for facile electron transfer to effect the cleavage reaction. Undoubtedly the coordination of SAM to the cluster somehow influences cleavage, perhaps by modulating the redox potentials of the Fe/S cluster and SAM; however, the detailed mechanism that underlies this process is still obscure.

During the last ten years a new class of RS proteins has emerged, in which the participant members catalyze anaerobic oxidations that involve the insertion of sulfur atoms into unactivated C–H bonds. Three proteins within this class have been isolated and characterized: biotin synthase (BS), which catalyzes the insertion of one sulfur atom between two unactivated C–H bonds, resulting in the formation of a thiophane ring; lipoyl synthase (LS), which catalyzes the insertion of two sulfur atoms into two different unactivated C–H bonds; and MiaB protein (MiaB), which catalyzes the insertion of a sulfur atom into an aromatic C–H bond as well as its methylation (Figure 2). The unique aspect of this class of RS proteins is that none of the characterized members have been shown to catalyze more than one turnover. In fact, all are linked by common experimental observations that suggest that each acts as both catalyst—in the sense that they facilitate and accelerate turnover—and reagent—since the inserted sulfur atoms derive from the proteins themselves. This overview will describe the current state of understanding of each of these proteins and the reactions they catalyze, with a particular focus on detailing the experimental observations that support the contention that they play a sacrificial role in their respective reactions.

Biotin synthase

E. coli Biotin synthase (BS) is the best characterized RS protein that catalyzes sulfur insertion. It catalyzes the final step in the biosynthesis of biotin, which is the insertion of a sulfur atom between carbons 6 and 9 of dethiobiotin (DTB), affording a thiophane ring (Figure 2a) [15, 16]. The reaction proceeds with removal of only two hydrogens from DTB; one hydrogen is removed from the *pro-S* position of C-6, and the other is removed from C-9. Since the stereochemical designation at C-6 of biotin is *S*, it follows that sulfur insertion takes place with retention of configuration at this carbon [17,18].

E. coli BS is the product of the *bioB* gene, which encodes a protein of 346 amino acids and molecular mass 38,648 Da. When isolated aerobically, it typically contains approximately one [2Fe–2S] cluster per polypeptide; however, this is not the mature form of the protein. The mature form is generated upon further reconstitution with excess iron and sulfide under anaerobic and reducing conditions, and contains one [2Fe–2S]²⁺ and one [4Fe–4S]²⁺ cluster per polypeptide [15,19]. Catalysis requires, in addition to SAM and DTB, the input of at least one electron, which in *E. coli* is provided by flavodoxin. The three-dimensional structure of *E. coli* BS in complex with SAM and DTB has been solved to 3.4 Å resolution by X-ray

diffraction methods [12]. The protein is homodimeric, as predicted from previous biochemical studies, with each monomer adopting an (α/β)₈ barrel (TIM barrel) fold (Figure 3a). The active site is located deep within the barrel, and contains in addition to SAM and DTB, both the [4Fe–4S] and [2Fe–2S] clusters, which flank the two organic substrates (Figure 3b). As predicted from spectroscopic studies, the α -amino and α -carboxylate groups of SAM coordinate to the unique iron of the [4Fe–4S] cluster, while the remaining three irons are coordinated by Cys53, Cys57, and Cys60; the [2Fe–2S] cluster is coordinated by Cys97, Cys128, Cys188, and Arg260. The six cysteine residues that contribute ligands to the two Fe/S clusters are strictly conserved, and site-directed mutagenesis studies indicate they are essential for turnover [20,21]. Interestingly, Arg260, an unusual ligand for an Fe/S cluster, is also conserved; however, substitution of this residue with Ala, Cys, His, or Met afforded single turnover activities that ranged from 30–120% of that of wild type [22]. Therefore, the rationale for conservation of this residue remains enigmatic, since it appears to play no essential role in catalysis under in vitro reaction conditions.

Perhaps the most intriguing aspect of catalysis by BS relates to the immediate source of sulfur in the reaction and the mechanism employed for installing it in its proper location. The culmination of an extensive and arduous hunt for the sulfur donor led to the implication of the protein itself as the source [23,24]. When an apo form of *E. coli* BS was reconstituted with iron and [³⁴S]-sulfide and then incubated under turnover conditions, the biotin isolated from the reaction mixture contained 62% and 45% of the ³⁴S isotope after 2 and 4 h of reaction, respectively. The observed dilution in isotopic content was presumed to have arisen from exchange of [³²S]-sulfide into the cluster from decomposition of DTT present in the reaction [24]. A parallel study using partially purified BS from *E. coli* grown in minimal media supplemented with [³⁵S]-cysteine found a similar observation; the protein produced [³⁵S]-labeled biotin in an in vitro reaction [23]. These observations, and the fact that BS has not been observed to catalyze formation of more than one mol of biotin per mol of monomer in an in vitro reaction, are consistent with the contention that the protein acts as the sulfur donor in the reaction [15,16].

The Jarrett laboratory put forth a working model that specifically implicated the [2Fe–2S] cluster on BS as the immediate source of the sulfur atom in biotin. They were able to achieve almost one full turnover in the absence of excess iron, sulfide, and dithiothreitol (DTT) only with BS containing both the [4Fe–4S] and [2Fe–2S] clusters, and observed UV-visible spectral changes concomitant with biotin formation that were consistent with destruction of the [2Fe–2S] cluster [25]. In a similar study, in which the states of the clusters were monitored by Mössbauer and EPR spectroscopies, destruction of the [2Fe–2S] cluster was observed to occur at a rate that was an order of magnitude greater than that for biotin formation. It was concluded that if the [2Fe–2S] cluster is the immediate sulfur donor, then sulfur insertion cannot be rate-limiting. It was also suggested that the [2Fe–2S] cluster may not, itself, act as the immediate sulfur donor, but might degrade into another species—such as a protein-bound polysulfide or persulfide—that acts in that capacity [26]. Recently, reconstitution of BioB with selenide was shown to generate a [2Fe–2Se] cluster that was amenable to characterization by resonance Raman spectroscopy. Under turnover conditions in the presence of excess selenide, selenobiotin was generated by this form of the protein, while a mixture of biotin and selenobiotin was generated in the presence of excess sulfide. Analysis of the enzyme by resonance Raman spectroscopy before completion of turnover showed that under conditions of excess sulfide a significant amount of sulfide exchanged into the [2Fe–2Se] cluster. The authors concluded that their observations were consistent with the role of the [2Fe–2S] cluster as the immediate sulfur donor [27].

A mechanism that is consistent with most experimental observations is shown in Figure 4 [25]. A 5'-dA• generated from reductive cleavage of SAM abstracts a hydrogen atom from C-9

of DTB, affording 5'-dA, L-methionine, and an organic radical at C-9 of the substrate. The substrate radical attacks a bridging μ -sulfido atom of the nearby $[2\text{Fe}-2\text{S}]^{2+}$ cluster with concomitant inner-sphere electron transfer to one of the iron atoms, resulting in a mixed-valent $\text{Fe}^{\text{II}}-\text{Fe}^{\text{III}}$ Fe/S species. In the second half-reaction the 5'-dA $\bullet$ abstracts the 6-*pro-S* hydrogen atom the Fe^{III} -coordinated 9-mercaptodethiobiotin intermediate. Formation of the thiophane ring occurs by attack of the C-6 radical on the sulfur atom of the intermediate with concomitant reduction of the coordinated Fe^{III} species to Fe^{II} . Consistent with this mechanism, BS has been shown to catalyze formation of 2.8 ± 0.3 equiv of 5'-dA per equiv of biotin; the excess above the 2 equiv predicted by the mechanism is attributed to abortive cleavage of SAM [28]. In addition, when BS reactions were incubated with $[9\text{-}^2\text{H}_3]\text{-DTB}$, $[6\text{-}^2\text{H}_2, 9\text{-}^2\text{H}_3]\text{-DTB}$, and $[6\text{-}^2\text{H}_5, 9\text{-}^2\text{H}_1]\text{-DTB}$, deuterium was found in 5'-dA, suggesting direct hydrogen atom abstraction by a 5'-dA $\bullet$. The near absence of deuterium in 5'-dA when $[6\text{-}^2\text{H}_R, 9\text{-}^2\text{H}_1]\text{-DTB}$ was used is consistent with removal of the *pro-S* hydrogen during turnover [29]. Recent results from Marquet and coworkers indicate that 9-mercaptodethiobiotin (DTBSH) is converted into biotin in an amount and at a rate that is similar to that obtained with DTB as the substrate, although the estimated K_M value for DTBSH was 20-fold greater than that for DTB. It was concluded that the rate-determining step must follow formation of a common intermediate from both DTB and 9-DTBSH, which is in agreement with kinetic analyses from the Huynh and Johnson laboratories [26]. The X-ray structure of BS is also consistent with the above mechanism. It shows that C-9 of dethiobiotin is ~ 3.9 Å away from C-5' of SAM and 4.6 Å away from the nearest bridging sulfido atom of the $[2\text{Fe}-2\text{S}]$ cluster (Figure 3b) [12]

There has been a report that BS containing only the $[4\text{Fe}-4\text{S}]$ cluster binds pyridoxal 5'-phosphate, and catalyzes desulfurization of cysteine in addition to biotin synthesis [30]. The cysteine desulfurase activity was proposed to proceed analogously to the prototypical cysteine desulfurases, NifS and IscS, wherein sulfur from cysteine is liberated in the form of a protein cysteine persulfide. Cysteines 97 and 128, two of the ligands to the $[2\text{Fe}-2\text{S}]$ cluster in the structure of BS, were shown to be critical for cysteine desulfurase activity, and were suggested to be candidates for the residue on which the persulfide is formed. This finding was appealing, because a reaction in which sulfur is liberated from cysteine via a PLP-dependent mechanism has the potential to be catalytic. Nevertheless, product formation did not exceed more than 1 mol of biotin per mol of BS monomer in the presence of excess cysteine. Further studies showed that 5'-dA, one of the products of SAM cleavage, potentially inhibits the BS reaction—an observation that was also made in *in vivo* studies [31]—and that an additional turnover could be achieved upon removal of this compound by gel-filtration [32]. Moreover, in contrast to studies from the Marquet laboratory, their data indicated that only one equiv of SAM is cleaved per equiv of biotin formed [32]. It was suggested that the cysteine persulfide might function as a direct intermediate in sulfur insertion, or that it might be an intermediate in the formation of the $[2\text{Fe}-2\text{S}]$ cluster.

To date, there is no clear explanation for the strikingly different observations obtained on BS. Almost all PLP-dependent enzymes bind the cofactor covalently in an internal aldimine linkage with a specific protein lysine residue; however, there have been no reports that describe the trapping of this species in BS by reduction with sodium borohydride or another suitable reagent, and identifying the relevant lysine amino acid by peptide mapping. There have also been no reports of the effect of amino acid substitutions at the lysine amino acid that has been described as highly conserved [30]. In fact, not only did the X-ray structure of BS fail to suggest a clear binding site for PLP, but all lysine amino acids were observed to be at the surface of the protein far from the cysteine residues that were speculated to be involved in persulfide formation [12]. Last, deliberate efforts by two other laboratories to repeat the cysteine desulfurase activity of BS were unsuccessful [19,33].

Lipoyl Synthase

Significant advances in the understanding of lipoic acid biosynthesis were made upon establishment of the nature of the true substrate for lipoyl synthase (LS) [34-36]. LS catalyzes the final step in the biosynthesis of the lipoyl cofactor, which is the insertion of two sulfur atoms into C–H bonds at carbons 6 and 8 of an *n*-octanoyl chain appended to a specific lysine residue of a lipoyl carrying protein (LCP) [34-37]. Three LCPs are known in *E. coli*: the E₂ subunits of the pyruvate and α -ketoglutarate dehydrogenase complexes, and the H protein of the glycine cleavage system [37]. *E. coli* LS is the product of the *lipA* gene, and is a protein of 321 amino acids and molecular mass 36,072 Da. Metabolic feeding studies similar to those conducted in the study of the BS reaction indicate that the only hydrogens removed from substrate during formation of the lipoyl cofactor—one at C-6 and one at C-8—are those that are replaced by sulfur atoms [18]. In contrast to the BS reaction, the *pro-R* hydrogen is removed at C-6 of the *n*-octanoyl chain, and sulfur insertion takes place with inversion rather than retention of configuration (Figure 2b) [18]. *E. coli* LS contains eight cysteine residues, six of which are conserved in two distinct motifs. One motif, $^{94}\text{CX}_3^{98}\text{CX}_2^{101}\text{C}$, contains the cysteines that contribute thiolate ligands to the [4Fe–4S] cluster that is common to RS enzymes, while the second motif, $^{68}\text{CX}_4^{73}\text{CX}_5^{79}\text{C}$, is found only in lipoyl synthases. Site-directed mutagenesis used in combination with quantitative analyses for iron and sulfide, and UV-visible, EPR, and Mössbauer spectroscopies indicate that the cysteines in the second motif contribute thiolate ligands to an additional [4Fe–4S] cluster [38]. The identity of the fourth ligand to this second cluster is not known; however, substitution of any of the cysteines in the motif with alanine or aspartate results in complete loss of activity [38].

The exact role of the second [4Fe–4S] cluster in LS has not been established. In analogy to BS, it has been suggested that it functions as the direct sulfur donor; however, there are significantly fewer experimental observations that support this proposition in this system [38,39]. One of the major limitations is the difficulty in distinguishing between the two clusters, since they have similar configurations and Mössbauer parameters [38]. Labeling experiments indicate, however, that the protein serves as the direct source of sulfur atoms in lipoic acid, and that both sulfur atoms derive from the same LS polypeptide [39]. When LS was isolated from recombinant *E. coli* grown in minimal media containing [^{34}S]-sulfide as the sole sulfur source, and then added to a reaction in which sulfur atoms in all other sulfur-containing molecules were present at natural abundance (94.93% ^{32}S), the lipoic acid produced contained ^{34}S at the same abundance as the [^{34}S]-sulfide used in the growth media. When an approximately equimolar ratio of [^{34}S]-labeled and [^{32}S]-labeled LS was incubated with other reaction components under turnover conditions, the overwhelming majority of lipoic acid synthesized contained either two ^{34}S atoms or two ^{32}S atoms; very little, if any, contained both ^{34}S and ^{32}S in the same molecule [39]. These experimental results also indicate that monothiolated species are not freely dissociable intermediates, which would result in a 1:2:1 labeling pattern of [^{34}S – ^{34}S]- [^{34}S – ^{32}S]- and [^{32}S – ^{32}S]-labeled lipoic acid.

A working hypothesis for turnover by LS is shown in Figure 5 [18,34,35,39,40]. A 5'-dA• generated from the reductive cleavage of SAM abstracts the *pro-R* hydrogen atom from C-6 of a target *n*-octanoyllysine side chain, affording one equiv of 5'-dA and a C-6 substrate alkyl radical. The substrate radical attacks one of the bridging μ -sulfido atoms of the [4Fe–4S] cluster in the $^{68}\text{CX}_4^{73}\text{CX}_5^{79}\text{C}$ motif with inversion of configuration and concomitant reduction of one of the Fe(III) atoms to Fe(II). In the second half-reaction, another 5'-dA• abstracts a hydrogen atom from C-8, generating a C-8 alkyl radical, which adds to another of the bridging μ -sulfido atoms of the [4Fe–4S] cluster with concomitant reduction of another of the Fe(III) atoms to Fe(II). The addition of two protons facilitates dissociation of the product, which is the reduced form of the lipoyl cofactor. Consistent with this working model, quantification of 5'-dA and the lipoyl cofactor as a function of time showed that each was formed with similar rate

constants, and that the ratio of 5'-dA to the lipoyl cofactor varied between 2.38 at low extents of turnover and 2.75 at high extents of turnover. When a substrate containing a perdeuterated (octanoyl- d_{15}) octanoyl chain was used in the reaction, 5'-dA containing one deuterium atom (5'-dA- d_1) and 5'-dA containing no deuterium atoms (5'-dA- d_0) were both observed, indicating the occurrence of abortive cleavage of SAM, as is observed in the BS reaction. Moreover, the maximum amount of 5'-dA- d_1 observed was approximately one-half of that observed with unlabeled substrate, and no formation of the lipoyl cofactor was detected [34]. These results imply that there is a significant isotope effect associated with abstraction of a deuterium atom from either C-6 or C-8. The subsequent use of substrates containing deuterium label specifically at C-6 or C-8 established that the large isotope effect is associated with deuterium removal from C-8 [40], which also had been observed in early metabolic feeding studies using tritiated precursors [18]. Incidentally, an isotope effect of this magnitude was not observed in either in vitro or in vivo experiments on BS, despite the identical nature of the hydrogen-abstrating species and similar bond dissociation energies of the C-H bonds cleaved [18,29]. Recently, the Roach laboratory provided evidence that sulfur insertion takes place at C-6 before C-8 in LS from *Sulfolobus sulfataricus*. They incubated LS under turnover conditions, and then separated the reaction components by high-performance liquid chromatography (HPLC) before completion of turnover. NMR and chromatographic methods were then used to show that in addition to octanoylated and lipoylated components, the mixture contained a 6-mercaptooctanoylated species. By contrast, an 8-mercaptooctanoylated species was not observed [40]. This finding appears somewhat contradictory to what was observed in early metabolic feeding studies, wherein 8-mercaptooctanoic acid was a much better precursor to lipoic acid than 6-mercaptooctanoic acid [18,41]. A suitable kinetic analysis of the reaction has not yet been conducted to ascertain whether the 6-mercaptooctanoylated species is a true intermediate or whether it is generated via mechanisms that are off the normal catalytic pathway.

MiaB

MiaB catalyzes the final step in the biosynthesis of the hypermodified tRNA nucleoside 2-methylthio- N^6 -(isopentenyl)adenosine (ms^2i^6A) (Figure 2c). This modification, or its hydroxylated derivative, is found throughout eubacterial and eukaryotic tRNAs that read codons that begin with U—except for the serine codons UCC and UCU—and typically is found at position 37, or just adjacent to the first base of the anticodon on the 3' side [42]. The first committed step in the biosynthesis of ms^2i^6A is catalyzed by MiaA, and involves the transfer of a dimethylallyl group from dimethylallyl diphosphate (DMAPP) to the exocyclic amine (N^6) of the appropriate adenosine nucleoside, affording N^6 -(isopentenyl)adenosine-37 (i^6A). The second step, catalyzed by MiaB, involves methylthiolation at position 2 of the modified adenine ring (Figure 2c) [42,43]. Although this position is remote from the dimethylallyl group, both in vivo and in vitro evidence indicate that the group must be present for MiaB to catalyze its reaction [42,43].

The MiaB reaction involves sulfur insertion into an unactivated C-H bond that is orthogonal to an aromatic system. The homolytic bond dissociation energy (HBDE) of this bond is expected to be approximately 98 kcal mol⁻¹ [44], similar to the HBDEs of the relevant C-H bonds in biotin and lipoic acid biosynthesis [45]. In the late 1960s it was reported that the occurrence or extent of the ms^2i^6A modification in *E. coli* is dependent on the presence of iron in the growth medium [46,47]. Three decades later, the subsequent identification and sequence determination of the *miaB* genes from both *Salmonella typhimurium* and *E. coli* revealed that the proteins harbored the canonical CX₃CX₂C RS motif [42]. Interestingly, the authors indicated that the MiaB proteins had no obvious SAM binding site, and suggested that MiaB activity might be involved in thiolation but not methylation. Moreover, they identified a highly conserved region that had the potential to act as a pyridoxal 5'-phosphate binding site [42].

MiaB proteins from *E. coli* (MiaBEc) and *Thermotoga maritima* (MiaBTm) have been isolated and characterized. MiaBEc is a monomer of 474 amino acids and has a molecular mass of 53,649 Da [42,48], while MiaBTm is composed of 443 amino acids, has a molecular mass of 50,710 Da, and is also monomeric [49]. MiaB was originally thought to contain only one [4Fe–4S] cluster per polypeptide, but similarly to BS and LS, it has six conserved cysteine residues, three of which reside in the RS cluster motif, and three of which (Cys10, Cys46, and Cys79 for MiaBTm) might be available to bind a second Fe/S cluster. Indeed, recent spectroscopic studies in combination with site-directed mutagenesis and quantitative analyses for iron and sulfide indicate that the active form of MiaBTm contains two [4Fe–4S] clusters that possess similar UV–visible absorption, resonance Raman, and Mössbauer spectroscopic properties, but different redox properties. In addition, the reduced forms of the clusters display slightly different EPR spectra [50]. The original failure to recognize the second cluster resulted from the method employed to reconstitute the Fe/S clusters, which involved chelation of excess iron by treatment of the protein with EDTA, and subsequent gel-filtration chromatography. Apparently the EDTA had access to one of the Fe/S clusters and destroyed it [43,48,49].

The MiaB reaction diverges somewhat from that of BS and LS, in that it contains two distinctly different types of SAM-dependent activities in one polypeptide. Although two equiv of SAM are proposed to be expended per equiv of $\text{ms}^2\text{i}^6\text{A}$ formed—as is observed in BS and LS—one equiv is converted to 5'-dA, while the other equiv is proposed to be converted into S-adenosylhomocysteine (SAH) as a result of the protein's SAM-dependent methyltransferase activity. Although it had been speculated that methylation might be catalyzed by an additional hypothetical enzyme in the pathway, denoted MiaC, purified MiaB was able to catalyze transfer of radioactivity from S-adenosyl-L-[methyl- ^3H]methionine to the tRNA product, $\text{ms}^2\text{i}^6\text{A}$ [42,43]. Similarly to BS and LS, abortive formation of 5'-dA is believed to occur during turnover, since the ratio of 5'-dA to $\text{ms}^2\text{i}^6\text{A}$ was found to be ~1.5 to 1 rather than the predicted 1 to 1 [43]. Like BS and LS, evidence suggests that MiaB itself acts as the immediate sulfur donor in its reaction. When an apo form of the protein was reconstituted with iron and selenide and then incubated under turnover conditions, 2-methylseleno- N^6 -(isopentenyl)adenosine ($\text{mse}^2\text{i}^6\text{A}$) was observed [43]. A plausible mechanism of catalysis by MiaB would involve abstraction of a hydrogen atom from C-2 of i^6A , followed by attack of the substrate radical on one of the bridging μ -sulfido ligands of the [4Fe–4S] cluster that is bound by Cys10, Cys46, and Cys79 (MiaBTm). The sulfur atom in this intermediate might be methylated directly, or subsequent to its protonation and dissociation of the product from the cluster. At present it is not known whether thiolation and methylation take place at the same active site, or whether methylation of a sulfur atom in the relevant [4Fe–4S] cluster takes place before sulfur insertion.

Conclusion and future prospects

Sulfur insertion reactions catalyzed by RS enzymes represent a new and novel mechanism for anaerobic C–H bond functionalization, which is reminiscent of oxygen-dependent enzymes that catalyze hydroxylation reactions using the combination of dioxygen and a metal-containing cofactor [3]. Under anaerobic conditions the oxidant is the 5'-dA• generated from reductive cleavage of SAM, while the activated functional group appears to derive from some form of an Fe/S cluster. The use of an Fe/S cluster to deliver the functional group appears to be a judicious strategy for addition of a sulfur atom to a carbon-centered radical. Since the sulfur atom has a complete octet of electrons, it would need to donate an electron to an appropriate acceptor in the process. In Fe/S proteins, the coordination of sulfide to iron atoms should allow facile electron transfer via an inner-sphere mechanism. The lack of enthusiasm for the use of Fe/S clusters in this manner derives principally from the presumption that the enzymes would kill themselves after only one turnover. However, it is becoming apparent that Fe/S cluster assembly and disassembly is a dynamic process in biology, and the many proteins that participate in rebuilding clusters under various conditions are being brought to light.

Indeed, *E. coli* BS has recently been shown to be catalytic in vivo, and turnover renders it susceptible to proteolysis [51]. The model in Figure 4 would therefore suggest that destruction of the [2Fe–2S] cluster would generate regions of unstructured polypeptide that might be recognized by certain proteases. In *Saccharomyces cerevisiae*, the proteins Isa1 and Isa2, which are involved in Fe/S cluster formation, were found to be important for the function of biotin synthase but not for de novo synthesis of its Fe/S clusters. The authors suggest that they function to regenerate the [2Fe–2S] cluster during catalysis [52].

BS, LS, and MiaB are by no means the only RS proteins that catalyze sulfur insertion. In fact, evidence suggests that new reactivity is on the horizon [4]. For example, RS proteins that participate in the biosynthesis of the H-cluster of the [FeFe]-hydrogenase have been identified, and have been suggested to be involved in generating a unique non-protein dithiolate ligand that has putatively been assigned as a dithiopropene or di(thiomethyl)amine [53–55]. Clearly, a new day is dawning with regard to the novel chemistry that involves old cofactors (Fe/S clusters and SAM) in new roles.

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References and Recommended Reading

- of special interest
 - of outstanding interest
1. Frey PA, Hegeman AD, Reed GH. Free radical mechanisms in enzymology. *Chem Rev* 2006;106:3302–3316. [PubMed: 16895329]
 2. Frey PA, Magnusson OT. S-Adenosylmethionine: a wolf in sheep's clothing, or a rich man's adenosylcobalamin? *Chem Rev* 2003;103:2129–2148. [PubMed: 12797826]
 3. Frey PA. Importance of organic radicals in enzymic cleavage of unactivated carbon-hydrogen bonds. *Chem Rev* 1990;90:1343–1357.
 - 4 •. Sofia HJ, Chen G, Hetzler BG, Reyes-Spindola JF, Miller NE. Radical SAM, a novel protein superfamily linking unresolved steps in familiar biosynthetic pathways with radical mechanisms: functional characterization using new analysis and information visualization methods. *Nucleic Acids Res* 2001;29:1097–1106. [PubMed: 11222759] This paper is the first detailed bioinformatics study that recognized that proteins that use S-adenosylmethionine as the source of a 5'-deoxyadenosyl radical constituted a superfamily.
 5. Chin KC, Cresswell P. Viperin (cig5), an IFN-inducible antiviral protein directly induced by human cytomegalovirus. *Proc Natl Acad Sci USA* 2001;98:15125–15130. [PubMed: 11752458]
 6. Ching YP, Qi Z, Wang JH. Cloning of three novel neuronal Cdk5 activator binding proteins. *Gene* 2000;242:285–294. [PubMed: 10721722]
 7. Chen D, Walsby C, Hoffman BM, Frey PA. Coordination and mechanism of reversible cleavage of S-adenosylmethionine by the [4Fe-4S] center in lysine 2,3-aminomutase. *J Am Chem Soc* 2003;125:11788–11789. [PubMed: 14505379]
 8. Krebs C, Broderick WE, Henshaw TF, Broderick JB, Huynh BH. Coordination of adenosylmethionine to a unique iron site of the [4Fe-4S] of pyruvate formate-lyase activating enzyme: a Mössbauer spectroscopic study. *J Am Chem Soc* 2002;124:912–913. [PubMed: 11829592]
 9. Walsby CJ, Hong W, Broderick WE, Cheek J, Ortillo D, Broderick JB, Hoffman BM. Electron-nuclear double resonance spectroscopic evidence that S-adenosylmethionine binds in contact with the catalytically active [4Fe-4S]⁺ cluster of pyruvate formate-lyase activating enzyme. *J Am Chem Soc* 2002;124:3143–3151. [PubMed: 11902903]
 10. Walsby CJ, Ortillo D, B WE, Broderick JB, Hoffman BM. An anchoring role for FeS clusters: chelation of the amino acid moiety of S-adenosylmethionine to the unique iron site of the [4Fe-4S]

cluster of pyruvate formate-lyase activating enzyme. *J Am Chem Soc* 2002;124:11270–11271. [PubMed: 12236732]

11. Layer G, Moser J, Heinz DW, Jahn D, Schubert WD. Crystal structure of coproporphyrinogen III oxidase reveals cofactor geometry of Radical SAM enzymes. *EMBO J* 2003;22:6214–6224. [PubMed: 14633981]
- 12 ••. Berkovitch F, Nicolet Y, Wan JT, Jarrett JT, Drennan CL. Crystal structure of biotin synthase, an S-adenosylmethionine-dependent radical enzyme. *Science* 2004;303:76–79. [PubMed: 14704425]
This paper describes the X-ray structure of biotin synthase. Although it was not the first radical SAM protein to have its structure determined, it was the first determined with all substrates and cofactors bound.
13. Hänzelmann P, Schindelin H. Crystal structure of the S-adenosylmethionine-dependent enzyme MoaA and its implications for molybdenum cofactor deficiency in humans. *Proc Natl Acad Sci USA* 2004;101:12870–12875. [PubMed: 15317939]
14. Lepore BW, Ruzicka FJ, Frey PA, Ringe D. The x-ray crystal structure of lysine-2,3-aminomutase from *Clostridium subterminale*. *Proc Natl Acad Sci USA* 2005;102:13819–13824. [PubMed: 16166264]
15. Jarrett JT. The novel structure and chemistry of iron-sulfur clusters in the adenosylmethionine-dependent radical enzyme biotin synthase. *Arch Biochem Biophys* 2005;433:312–321. [PubMed: 15581586]
16. Lotierzo M, Tse Sum Bui B, Florentin D, Escalettes F, Marquet A. Biotin synthase mechanism: an overview. *Biochem Soc Trans* 2005;33:820–823. [PubMed: 16042606]
17. Marquet A, Bui BT, Florentin D. Biosynthesis of biotin and lipoic acid. *Vitam Horm* 2001;61:51–101. [PubMed: 11153271]
18. Parry RJ. Biosynthesis of some sulfur-containing natural products. Investigations of the mechanism of carbon-sulfur bond formation. *Tetrahedron* 1983;39:1215–1238.
19. Cosper MM, Jameson GNL, Hernández HL, Krebs C, Huynh BH, Johnson MK. Characterization of the cofactor composition of *Escherichia coli* biotin synthase. *Biochemistry* 2004;43:2007–2021. [PubMed: 14967041]
20. Hewitson KS, Baldwin JE, Shaw NM, Roach PL. Mutagenesis of the proposed iron-sulfur cluster binding ligands in *Escherichia coli* biotin synthase. *FEBS Lett* 2000;466:372–376. [PubMed: 10682863]
21. Hewitson KS, Ollagnier-de Choudens S, Sanakis Y, Shaw NM, Baldwin JE, Münck E, Roach PL, Fontecave M. The iron-sulfur center of biotin synthase: site-directed mutants. *J Biol Inorg Chem* 2002;7:83–93. [PubMed: 11862544]
22. Broach RB, Jarrett JT. Role of the [2Fe–2S]²⁺ cluster in biotin synthase: mutagenesis of the atypical metal ligand arginine 260. *Biochemistry* 2006;45:14166–14174. [PubMed: 17115711]
- 23 •. Gibson KJ, Pelletier DA, IM Turner S. Transfer of sulfur to biotin from biotin synthase (BioB protein). *Biochem Biophys Res Commun* 1999;254:632–635. [PubMed: 9920791] This paper provides the first in vitro evidence that the sulfur atom in biotin derives from an iron–sulfur cluster on the protein.
- 24 •. Tse Sum Bui B, Florentin B, Fournier F, Ploux O, Méjean A, Marquet A. Biotin synthase mechanism: on the origin of sulphur. *FEBS Lett* 1998;440:226–230. [PubMed: 9862460] This paper provides the first in vitro evidence that the sulfur atom in biotin derives from an iron–sulfur cluster on the protein.
- 25 ••. Ugulava NB, Sacanell CJ, Jarrett JT. Spectroscopic changes during a single turnover of biotin synthase: destruction of a [2Fe–2S] cluster accompanies sulfur insertion. *Biochemistry* 2001;40:8352–8358. [PubMed: 11444982] This paper provides the first evidence that the active form of biotin synthase contains both a [2Fe–2S] cluster and [4Fe–4S] cluster, and that the sulfur in biotin derives from the [2Fe–2S] cluster.
- 26 ••. Jameson GNL, Cosper MM, Hernández HL, Johnson MK, Huynh BH. Role of the [2Fe–2S] cluster in recombinant *Escherichia coli* biotin synthase. *Biochemistry* 2004;43:2022–2031. [PubMed: 14967042] This paper describes the use of rapid kinetics methods coupled with Mössbauer, EPR, and UV-visible spectroscopies to show that the [2Fe–2S] cluster decays faster than biotin is

produced. It highlights the need to apply these kinds of physical methods to peer deeply into the mechanism of radical SAM proteins.

27. Tse Sum Bui B, Mattioli TA, Florentin D, Bolbach G, Marquet A. Escherichia coli biotin synthase produces selenobiotin. Further evidence of the involvement of the $[2\text{Fe}-2\text{S}]^{2+}$ cluster in the sulfur insertion step. *Biochemistry* 2006;45:3824–3834. [PubMed: 16533066]
28. Lotierzo M, Raux E, Tse Sum Bui B, Goasdoue N, Libot F, Florentin D, Warren MJ, Marquet A. Biotin synthase mechanism: mutagenesis of the YNHNLD conserved motif. *Biochemistry* 2006;45:12274–12281. [PubMed: 17014080]
29. Escalettes F, Florentin D, Tse Sum Bui B, Lesage D, Marquet A. Biotin synthase mechanism: evidence for hydrogen transfer from the substrate into deoxyadenosine. *J Am Chem Soc* 1999;121:3571–3578.
30. Ollagnier-de Choudens S, Mulliez E, Hewitson KS, Fontecave M. Biotin synthase is a pyridoxal phosphate-dependent cysteine desulfurase. *Biochemistry* 2002;41:9145–9152. [PubMed: 12119030]
31. Choi-Rhee E, Cronan JE. A nucleosidase required for in vivo function of the S-adenosyl-L-methionine radical enzyme, biotin synthase. *Chem Biol* 2005;12:589–593. [PubMed: 15911379]
32. Ollagnier-de Choudens S, Mulliez E, Fontecave M. The PLP-dependent biotin synthase from Escherichia coli: mechanistic studies. *FEBS Lett* 2002;532:465–468. [PubMed: 12482614]
33. Tse Sum Bui B, Lotierzo M, Escalettes F, Florentin D, Marquet A. Further investigation on the turnover of Escherichia coli biotin synthase with dethiobiotin and 9-mercaptodethiobiotin as substrates. *Biochemistry* 2004;43:16432–16441. [PubMed: 15610037]
34. Cicchillo RM, Iwig DF, Jones AD, Nesbitt NM, Baleanu-Gogonea C, Souder MG, Tu L, Booker SJ. Lipoyl synthase requires two equivalents of S-adenosyl-L-methionine to synthesize one equivalent of lipoic acid. *Biochemistry* 2004;43:6378–6386. [PubMed: 15157071]
- 35 •. Miller JR, Busby RW, Jordan SW, Cheek J, Henshaw TF, Ashley GW, Broderick JB, Cronan JE Jr, Marletta MA. Escherichia coli LipA is a lipoyl synthase: in vitro biosynthesis of lipoylated pyruvate dehydrogenase complex from octanoyl-acyl carrier protein. *Biochemistry* 2000;39:15166–15178. [PubMed: 11106496] Although the actual substrate for lipoyl synthase was incorrectly identified, this paper describes the first in vitro demonstration of lipoyl synthase activity.
- 36 •. Zhao S, Miller JR, Jiang Y, Marletta MA, Cronan JE Jr. Assembly of the covalent linkage between lipoic acid and its cognate enzymes. *Chem Biol* 2003;10:1293–1302. [PubMed: 14700636] This paper describes the use of both in vivo and in vitro methods to reveal the true identity of the substrate for lipoyl synthase.
37. Cronan JE, Zhao X, Jiang Y. Function, attachment and synthesis of lipoic acid in Escherichia coli. *Adv Microb Physiol* 2005;50:103–146. [PubMed: 16221579]
- 38 ••. Cicchillo RM, Lee KH, Baleanu-Gogonea C, Nesbitt NM, Krebs C, Booker SJ. Escherichia coli lipoyl synthase binds two distinct $[4\text{Fe}-4\text{S}]$ clusters per polypeptide. *Biochemistry* 2004;43:11770–11781. [PubMed: 15362861] This paper describes the use of site-directed mutagenesis coupled with various analytical and spectroscopic methods to establish that lipoyl synthase contains two $[4\text{Fe}-4\text{S}]$ clusters in its active form.
- 39 ••. Cicchillo RM, Booker SJ. Mechanistic investigations of lipoic acid biosynthesis in Escherichia coli: both sulfur atoms in lipoic acid are contributed by the same lipoyl synthase polypeptide. *J Am Chem Soc* 2005;127:2860–2861. [PubMed: 15740115] This paper describes labeling experiments that indicate that the sulfur atoms in lipoic acid are derived from the protein itself, and that both emanate from the same polypeptide.
- 40 •. Douglas P, Kriek M, Bryant P, Roach PL. Lipoyl synthase inserts sulfur atoms into an octanoyl substrate in a stepwise manner. *Angew Chem* 2006;118:5321–5323. This paper describes experiments that indicate that sulfur is inserted at C-6 before C-8 in the biosynthesis of lipoic acid.
41. Hayden MA, Huang IY, Iliopoulos G, Orozco M, Ashley GW. Biosynthesis of lipoic acid: characterization of the lipoic acid auxotrophs Escherichia coli W1485-lip2 and JRG33-lip9. *Biochemistry* 1993;32:3778–3782. [PubMed: 8466915]
42. Esberg B, Leung HCE, Tsui HCT, Björk GR, Winkler ME. Identification of the miaB gene, involved in methylthiolation of isopentenylated A37 derivatives in the tRNA of Salmonella typhimurium and Escherichia coli. *J Bacteriol* 1999;181:7256–7265. [PubMed: 10572129]
- 43 ••. Pierrel F, Douki T, Fontecave M, Atta M. MiaB protein is a bifunctional radical-S-adenosylmethionine enzyme involved in thiolation and methylation of tRNA. *J Biol Chem*

2004;279:47555–47653. [PubMed: 15339930]This paper describes the establishment of methods to assess MiaB-dependent turnover in a complete in vitro system, and shows that this single polypeptide protein catalyzes both sulfur insertion and methylation.

44. Barckholtz C, Barckholtz TA, Hadad CM. C–H and N–H bond dissociation energies of small aromatic hydrocarbons. *J Am Chem Soc* 1999;121:491–500.
45. McMillen DF, Golden DM. Hydrocarbon bond dissociation energies. *Ann Rev Phys Chem* 1982;33:493–532.
46. Rosenberg AH, Gefter ML. An iron-dependent modification of several transfer RNA species in *Escherichia coli*. *J Mol Biol* 1969;46:581–584. [PubMed: 4904109]
47. Wettstein FO, Stent GS. Physiologically induced changes in the property of phenylalanine tRNA in *Escherichia coli*. *J Mol Biol* 1968;38:25–40. [PubMed: 4941472]
48. Pierrel F, Björk GR, Fontecave M, Atta M. Enzymatic modification of tRNAs: MiaB is an iron–sulfur protein. *J Biol Chem* 2002;277:13367–13370. [PubMed: 11882645]
49. Pierrel F, Hernandez HL, Johnson MK, Fontecave M, Atta M. Characterization of an extremely thermophilic tRNA-methylthiotransferase. *J Biol Chem* 2003;278:29515–29524. [PubMed: 12766153]
- 50 ••. Hernández HL, Pierrel F, Elleingand E, García-Serres R, Huynh BH, Johnson MK, Fontecave M, Atta M. MiaB, a bifunctional radical-S-adenosylmethionine enzyme involved in the thiolation and methylation of tRNA, contains two essential [4Fe–4S] clusters. *Biochemistry* 2007;46:5140–5147. [PubMed: 17407324]This paper describes the use of site-directed mutagenesis coupled with various analytical and spectroscopic methods to establish that MiaB contains two [4Fe–4S] clusters in its active form.
51. Choi-Rhee E, Cronan JE. Biotin synthase is catalytic in vivo, but catalysis engenders destruction of the protein. *Chem Biol* 2005;12:461–468. [PubMed: 15850983]This is an interesting paper that provides evidence that biotin synthase catalyzes multiple turnover in vivo.
- 52 •. Mühlhoff U, Gerl MJ, Flaeger B, Pirner HM, Balser S, Richardt N, Lill R, Stolz J. The iron–sulfur proteins Isa1 and Isa2 are required for the function but not for the de novo synthesis of the Fe/S clusters of biotin synthase in *Saccharomyces cerevisiae*. *Eukaryot Cell* 2007;6:495–504. [PubMed: 17259550]This is another interesting paper that provides evidence that proteins that are involved in iron–sulfur cluster biosynthesis are required for biotin synthase activity, suggesting that they repair the [2Fe–2S] cluster after turnover.
53. Böck A, King PW, Blokesch M, Posewitz MC. Maturation of hydrogenases. *Adv Microb Physiol* 2006;51:1–71. [PubMed: 17091562]
54. Peters JW, Szilagyi RK, Naumov A, Douglas T. A radical solution for the biosynthesis of the H-cluster of hydrogenase. *FEBS Lett* 2006;580:363–367. [PubMed: 16386249]
55. Rubach JK, Brazzolotto X, Gaillard J, Fontecave M. Biochemical characterization of the HydE and HydG iron-only hydrogenase maturation enzymes from *Thermatoga maritima*. *FEBS Lett* 2005;579:5055–5060. [PubMed: 16137685]

Figure 1.

Formation of methionine and a 5'-deoxyadenosyl radical via the reductive cleavage of S-adenosylmethionine. The iron-sulfur cluster shown is that which is ligated by protein cysteine thiolates that reside in the CX₃CX₂C motif that is common among radical SAM proteins.

Figure 2.

Reactions catalyzed by radical SAM enzymes that are known to involve sulfur insertion. SAM, S-adenosylmethionine; Met, methionine; 5'-dAH, 5'-deoxyadenosine. Hydrogens shown in red are those that are removed by the 5'-deoxyadenosyl radical (A) Biotin synthase reaction (B) Lipoyl synthase reaction (C) MiaB reaction

Figure 3. X-ray structure of biotin synthase (PDB 1R30). (A) Structure of homodimer showing the (α/β)₈ barrel fold. The active site [4Fe-4S] and [2Fe-2S] clusters as well as dethiobiotin and SAM are shown in stick format. (B) View of the active site. Sulfur atoms are shown in yellow. Iron atoms are shown in black. The structure was prepared using the Pymol Molecular Graphics System (<http://www.pymol.org>).

Figure 4.
Working model for catalysis by BS. The 6-*pro-S* hydrogen is shown in red.

First half-reaction

Second half-reaction

Current Opinion in Chemical Biology

Figure 5.Working model for catalysis by LS. The 6-*pro-R* hydrogen is shown in red.