Published in final edited form as:

Nat Prod Rep. 2018 July 18; 35(7): 615–621. doi:10.1039/c7np00058h.

Following the electrons: Peculiarities in the catalytic cycles of Radical SAM enzymes

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Abstract

Radical SAM enzymes use *S*-adenosyl-L-methionine as an oxidant to initiate radical-mediated transformations that would otherwise not be possible with Lewis acid/base chemistry alone. These reactions are either redox neutral or oxidative leading to certain expectations regarding the role of SAM as either a reusable cofactor or the ultimate electron acceptor during each turnover. However, these expectations are frequently not realized resulting in fundamental questions regarding the redox handling and movement of electrons associated with these biological catalysts. Herein we provide a focused perspective on several of these questions and associated hypotheses with an emphasis on recently discovered radical SAM enzymes.

Graphical abstract

1 Introduction

Radical SAM enzymes (in so far as the present discussion is concerned) catalyze the reductive homolysis of *S*-adenosyl-L-methionine (SAM) by an active site [4Fe-4S]¹⁺ cluster to generate the equivalent of a 5'-deoxyadenosyl radical (see Fig. 1).^{1–3} With a few notable exceptions to be discussed, completion of the full two-electron reduction of SAM to L-methionine and 5'-deoxyadenosine yields a substrate (or enzyme) radical during the initial phase of the catalytic cycle. This endows radical SAM enzymes with the ability to catalyze

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6 Conflicts of interest

There are no conflicts to declare.

oxidative or redox-neutral transformations that would otherwise be out of reach in a biological system if only Lewis acid/base catalysts were available.^{4,5} These enzymes are thus responsible for a diverse range of chemical reactions by utilizing SAM essentially as an oxidant.^{6,7}

However, close attention to the movement of electrons during the catalytic cycles of these enzymes reveals a number of peculiarities. For example, after reductively priming a radical SAM enzyme to the activated $[4\text{Fe-4S}]^{1+}$ state, most oxidative transformations would not be expected to require an additional reductant beyond the substrate itself, and yet current reports suggest that they do. The dehydrogenase BtrN⁸ is a well-characterized example of this phenomenon (see Fig. 1). Likewise, redox neutral transformations such as the dehydration reaction catalyzed by AprD^{9–11} (see Fig. 1) in principle should be able to utilize SAM catalytically; however, there are relatively few examples where this has been shown to be the case. These oddities suggest that fundamental aspects of the chemistry and *in vitro* handling of radical SAM enzymes remain to be fully addressed.

In this *Viewpoint*, we step back and consider the field of radical SAM enzymology as a whole with the intent of highlighting the aforementioned idiosyncrasies. An emphasis is placed on both well established radical SAM enzymes as well as more recently discovered examples. Hypotheses to explain some of these phenomena are suggested as potential avenues for future investigation. However, the *Viewpoint* is not intended to serve as an introduction to the field or a survey of detailed mechanisms of catalysis. For this purpose, the reader is directed to several excellent and comprehensive reviews that are already available.^{7,12–15}

2 Oxidations

Some of the earliest radical SAM enzymes to be studied in detail employ SAM as a formal two-electron oxidant as shown in Figs. 1 & 2.⁶ For example, the BtrN-catalyzed dehydrogenation of 2-deoxy-*scyllo*-inosamine during the biosynthesis of 2-deoxystreptamine is a two-electron oxidation that results in the consumption of one molecule of SAM per turnover.⁸ Likewise, introduction of a tetrahydrothiophene ring to dethiobiotin catalyzed by BioB corresponds to a four-electron oxidation^{16,17} as does the two consecutive oxidative decarboxylations of coproporphyrinogen-III catalyzed by HemN (see Fig. 2).¹⁸ Hence, two molecules of SAM are consumed per completed reaction of BioB and HemN. Without an alternative oxidant, the stoichiometric consumption of SAM in systems such as these is *obligatory*. Furthermore, while the product radical must be oxidized at the end of each catalytic cycle and the catalytic $[4\text{Fe-4S}]^{2+}$ cluster reduced, mounting evidence suggests these two half-reactions are not directly coupled.

Many of the radical SAM enzymes that catalyze two-electron oxidations are structurally characterized by a “SPASM” or “twitch” domain.^{13,19} Located at the C-terminus, the SPASM domain is responsible for the binding of two auxiliary $[4\text{Fe-4S}]$ clusters distinct from the catalytic $[4\text{Fe-4S}]$ cluster, whereas the truncated twitch domain only binds one auxiliary cluster.¹³ Examples include BtrN,^{8,20,21} the anaerobic sulfatase maturing enzymes (anSMEs), which oxidize serine and cysteine residues in protein substrates to

aldehydes,^{22–25} AlbA/CteB, which introduce thioether linkages to peptide substrates,^{26–29} as well as PqqE³⁰ and StrB/SuiB,^{31,32} which catalyze oxidative cyclizations (see Fig. 2 for reactions). Crystal structures of anSME,²⁵ BtrN,²¹ SuiB³² and CteB²⁹ (see Fig. 3) indicate that in those cases where the reaction is indeed a net oxidation, the substrate is sufficiently close to an auxiliary cluster for it to serve as an electron acceptor even if direct coordination is not involved.

These observations have led to the hypothesis that oxidative catalytic cycles in general proceed without direct reduction of the catalytic cluster by the product radical (e.g., see BtrN in Fig. 1). This is somewhat counterintuitive given that direct reduction is observed in some systems with nonphysiological substrates.³³ Instead, the left-over electron is transferred to an *external* redox system (e.g., ferredoxin) via an alternative route such as the auxiliary clusters in the twitch/SPASM enzymes. This hypothesis has been supported by experiments with the anSME from *Clostridium perfringens*, where multiple turnovers require the presence of an external electron acceptor even after reductive priming of the catalytic cluster.³⁴

A similar pattern is seen with BioB and LipA, which are examples of radical SAM oxidases that do not possess twitch/SPASM domains. LipA is involved in the biosynthesis of the lipoyl cofactor and catalyzes introduction of the thiol groups at C6 and C8 (see Fig. 2).³⁵ As in the case of BioB, this is a net four-electron oxidation and two molecules of SAM must be consumed with each completed reaction.^{16,17,36} Though they lack twitch/SPASM domains, LipA and BioB have, respectively, one [4Fe-4S] or [2Fe-2S] auxiliary cluster distinct from the catalytic [4Fe-4S] cluster. These auxiliary clusters are believed to serve dual roles as both electron acceptors (as in the case of twitch/SPASM enzymes) as well as sulfur donors during the reactions.^{37,38} Consequently, no more than one or two turnovers are observed during *in vitro* assay of these enzymes.^{39,40} Very recently, it has also been demonstrated that the *E. coli* iron-sulfur cluster carrier protein NfuA is able to interact with LipA and reconstitute the auxiliary [4Fe-4S] cluster under turnover conditions.⁴¹ This result represents a significant breakthrough and opens the door to additional studies as to how the reducing equivalents are effectively cycled back to the catalytic cluster from the remnants of the auxiliary cluster in order to return the entire system to its activated resting state.

Another recent example of oxidative chemistry is the reaction catalyzed by the B₁₂-dependent radical SAM enzyme OxsB during biosynthesis of the antiviral agent oxetanocin A.⁴² The OxsB-catalyzed reaction is ostensibly the contraction of a five-membered ribose ring in its 2'-deoxyadenosine phosphate substrate to a four-membered oxetane ring in the corresponding product. While the ring-contraction is itself redox neutral, the reaction is coupled with a two-electron oxidation that introduces an aldehyde to the product (see Fig. 2).⁴³ Consequently, net reduction of SAM is required and may serve to compensate for the thermodynamically unfavorable ring-contraction. However, OxsB does not contain an auxiliary iron-sulfur cluster, and the fate of the final unpaired electron in the product radical is presently unclear. One possibility is that the cobalamin cofactor may serve in some manner as an electron conduit in lieu of an auxiliary cluster; however, other mechanistic possibilities for the cobalamin are easy to envision.⁴³

To date, very few studies have been conducted to determine just what happens to the unpaired electron in the product radical during net oxidations catalyzed by radical SAM enzymes. However, preliminary work suggests that in general this left-over reducing equivalent is not directly transferred back to the catalytic cluster. Instead, it appears to take a more circuitous route via a second redox system that works together with the radical SAM enzyme as part of a larger catalytic system. Whether this is a consequence of the redox properties of the catalytic versus the auxiliary clusters, offers additional control in the handling of energetic free radicals, provides some flexibility with respect to the timing of changes in the oxidation state of the catalytic cluster or is simply what happened to work first during the evolution of these enzymes remains an unanswered and outstanding question.

3 Redox neutral transformations

Radical SAM chemistry is also associated with several redox neutral transformations, some of which are summarized in Fig. 4. As with all radical SAM-catalyzed reactions, these transformations begin with the initial reduction of SAM that leads to formation of a substrate radical intermediate. In all redox neutral reactions, however, the final product radical must be *reduced* back to the original oxidation state of the substrate rather than oxidized as discussed above. The *reverse* of SAM homolysis is sufficiently fast in the confines of an enzyme active site that it can serve as a catalytically competent reaction step.⁴⁴ Therefore, it is not surprising that a number of radical SAM enzymes catalyzing redox-neutral transformations such as lysine-aminomutase^{45,46} and sporephotoproduct lyase⁴⁷ utilize SAM catalytically. What is surprising, however, is that recent and past examples suggest that regeneration of SAM with each turnover appears to be the exception and not the rule—at least *in vitro*.

The radical SAM lyase NosL from *Streptomyces actuosus* is a case in point. NosL rearranges tryptophan to 3-methyl-2-indolic acid eliminating formaldehyde and ammonia in the process (see Fig. 4).⁴⁸ The mechanism of NosL has garnered considerable attention, because initial oxidation of the substrate involves H-atom abstraction from the α -amino functionality rather than a carbon center.^{49–51} Somewhat less emphasized, however, is the fact that the overall reaction is redox neutral and still appears to proceed with the net reduction of SAM to 5'-deoxyadenosine and methionine. Part of this can be attributed to the reduction of SAM uncoupled from substrate turnover,⁴⁸ which is a frequently encountered oddity of radical SAM chemistry in its own right. However, this does not quite explain the correlated increase in SAM consumption in the presence of substrate.⁴⁸ Given that the elimination of formaldehyde and ammonia from tryptophan should be a thermodynamically favorable process, it is not immediately obvious as to why SAM would necessarily be consumed with each turnover. New insight, however, comes from a report published while this *Viewpoint* was under review and suggests that cyanide is produced rather than formaldehyde and ammonia.⁵² If correct, this would make the NosL-catalyzed reaction an oxidation of tryptophan such that the net reduction of SAM is in fact the *expected* outcome as discussed in the previous section.

Another example of this type of chemistry with several interesting features is AprD4. AprD4 together with AprD3 is responsible for the reduction of paromamine to lividamine during the

One possible explanation for these seemingly wasteful processes is suggested by recent studies of QueE from *Bacillus subtilis*. This radical SAM enzyme is responsible for the ring contraction that converts 6-carboxy-5, 6, 7, 8-tetrahydropterin (CPH₄) to 7-carboxy-7-deazaguanine (CDG) during the biosynthesis of 7-deazapurine.^{56,57} The reaction results in the elimination of ammonia from CPH₄ and the introduction of a C=C double-bond to the contracted five-membered ring rendering the overall process redox neutral (see Fig. 4). As in the case of NosL, the catalytic cycle of QueE may involve the formation of a nitrogen-centered radical and results in the formation of 5'-deoxyadenosine as a product. However, careful stoichiometric measurements have shown that net reduction of SAM takes place in competition with its regeneration.⁵⁶

An alternative hypothesis, however, is suggested by recent investigations of radical SAM cyclases and epimerases. NikJ and PolH both catalyze the cyclization of enolpyruvyl UMP to uracil octosyl acid 5'-phosphate (U-OAP) during the biosynthesis of nikkomycins and polyoxins, respectively (see Fig. 4).⁵⁹ Likewise, NeoN,⁶⁰ PoyD^{61,62} and YydG⁶³ catalyze

the epimerization of aliphatic carbons in the biosynthesis of neomycin and ribosomally synthesized peptides. Although all of these reactions are redox neutral, stoichiometric amounts of 5'-deoxyadenosine are produced alongside the cyclized or epimerized products. Furthermore, where tested,^{59,60,63} isotope tracer experiments and characterizations of mutant enzymes have indicated that the 5'-deoxyadenosyl radical abstracts a H-atom from the substrate whereas the product radical is reduced via net H-atom transfer from an active site cysteine residue. The resulting cysteinyl radical may then undergo oxidation whereby the odd-electron is transferred to an auxiliary [4Fe-4S] cluster concomitant with formation of a disulfide bond that is subsequently reduced.⁶⁰ Alternatively, the cysteinyl radical may coordinate the auxiliary [4Fe-4S] cluster directly prior to reduction to a thiol via electron transfer without the formation of a disulfide.⁶³ In other words, a mechanism does indeed appear to be in place for the controlled reduction of the product radical in these systems without SAM regeneration.

The net reduction of SAM could provide a mechanism by which epimerization and cyclization reactions can be made to favor the products and modulate flux through a biosynthetic pathway. For example, compared to the amino acid racemases of primary metabolism, which employ Lewis acid-base chemistry, the reactions catalyzed by PoyD/YydG are essentially irreversible due to the net reduction of SAM.^{61,63} However, this does not seem consistent with the reactions catalyzed by NosL, AprD4 and the redox-neutral decarboxylase BlsE^{64,65} among others, where the need for a thermodynamic driving force would not be expected. Furthermore, similar chemistry has also been implicated in the case of spore photoproduct lyase where SAM is indeed regenerated.^{66,67} Therefore, net reduction of SAM may in some cases be nothing more than a byproduct of the regiochemistry of the transformation. In other words, the product radical is simply not properly configured to facilitate H-atom abstraction from 5'-deoxyadenosine and must be safely quenched via some alternative means albeit at the cost of SAM and two reducing equivalents. As relatively little work has been done to characterize the specific fate of product radicals, this remains an open question.

4 Alkylation reactions

In the past five years, it has come to light that several radical SAM enzymes are capable of catalyzing alkylation reactions. While these reactions may be regarded as “one-electron” oxidations when only the oxidation states of the original substrate carbons are taken into account, such a distinction ends up being rather arbitrary given that “new carbons” have been added to reach the product state. Due to this ambiguity, we consider these enzymes separately from those previously discussed, though the basic principles are exactly the same.

Recently there has been an arguably explosive growth in the number of radical SAM methyltransferases (RSMTs) reported. These enzymes operate according to several different mechanisms of catalysis to methylate nonnucleophilic methyl acceptors such as nonacidic carbon and phosphorous centers.^{12,68} The C-methylation of gentamicin X₂ by the B₁₂-dependent RSMT GenK is one example shown in Fig. 4.⁶⁹ In nearly all cases studied so far, there appears to be two molecules of SAM involved in each turnover. The first acts as the methyl donor, though in some cases methylenetetrahydrofolate may serve in its place,⁷⁰

while the second SAM undergoes reduction to form the substrate radical. When the donor-SAM and substrate are treated as a single system, the methylation reaction is redox neutral (see Fig. 4). Thus, the previous discussion regarding redox neutral transformations applies with respect to the redox-active SAM, which in virtually all cases reported to date undergoes net reduction concomitant with methyl transfer. In fact, the only exception is TsrM (also shown in Fig. 4), which catalyzes the C2-methylation of L-tryptophan and appears to use the redox-active SAM catalytically.⁷¹ However, this interpretation is controversial, and the reaction may not involve radical SAM chemistry at all being instead another example of Lewis acid/base chemistry.⁷² The debate over TsrM is instructive, because the equilibrium of SAM-dependent methylation (radical-mediated or not) *in general* favors the products. As in the case of other redox neutral transformations, the benefit (if any) for net reduction of SAM is thus unclear.

Apart from the RSMTs, the only other radical SAM alkylation reactions that have been studied *in vitro* involve alkylation by the redox-active SAM itself. The first example of this type of chemistry is the conversion of 3-[(1-carboxyvinyl)oxy]benzoate to aminofutalosine by MqnE during biosynthesis of menaquinone (vitamin K) in *Streptomyces coelicolor* (see Fig. 2).⁷³ What makes this reaction especially interesting is that it likely involves direct addition of the 5'-deoxyadenosyl radical to the enol-ether moiety of the substrate rather than H-atom abstraction.⁷⁴ Thus, the reaction is initiated by effectively decreasing the oxidation state of SAM by –1 rather than –2 as is the case in nearly all other instances of radical SAM catalysis. Recently, however, another example of this type of chemistry was described in the case of QueE (see above) with the nonnatural substrate 6-carboxypterin (6-CP).⁷⁵ Both the MqnE and QueE (with 6-CP) reactions involve an oxidative decarboxylation step that again makes the overall reaction an oxidation from the perspective of the substrate with effectively one reducing equivalent (rather than two) transferred from the substrate to SAM (see Fig. 2). This means that one electron must be ejected from the composite 5'-deoxyadenosyl-product radical. However, consistent with the theme of the present discussion, the precise fate of this electron is not clear.

5 Conclusions

Much of the interest in radical SAM enzymes has been focused on the initial steps of the catalytic cycle as well as elucidating the elementary reactions responsible for the radical-mediated rearrangement of the substrate to product. Consequently, relatively little is known regarding how these catalysts are brought back to their initial state. Nevertheless, certain overarching themes are beginning to reveal themselves. First, in many cases, radical SAM enzymes appear to operate as part of larger catalytic systems that may be difficult to reproduce *in vitro* resulting in potentially anomalous behaviour. Second, these larger systems not only reductively prime the radical SAM enzyme but may also participate in ferrying the left-over electron from the product radical back to the catalytic cluster during oxidation reactions. Third, while net reduction of SAM can thermodynamically push reaction equilibria to favor the products in some redox neutral reactions, it is also possible that SAM and two reducing equivalents are simply sacrificed in the service of keeping radical intermediates “under control”. Investigation of these hypotheses, their alternatives

and the implications they make regarding the evolution of these catalysts by natural selection is expected to be a rich source of future insights in the field of enzymology.

Acknowledgments

This work was supported by the National Institutes of Health (GM035906 & GM040541) and the Welch Foundation (F-1511).

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Fig. 1.

Reactions catalyzed by radical SAM enzymes involve either a redox neutral transformation of the nominal substrate or its oxidation. For example, BtrN⁸ catalyzes a dehydrogenation whereas AprD4^{9–11} catalyzes a dehydration. Reduction of SAM to L-methionine (Met) and 5'-deoxyadenosine (5'-dAdo) is a hallmark of the associated catalytic cycles and is depicted here as a stepwise process involving a 5'-deoxyadenosyl radical intermediate (5'-dAdo-CH₂[•]).¹ Oxidation states of the relevant carbon centers are highlighted.

Fig. 2.

Oxidative half-reactions for several radical SAM catalyzed transformations that involve net oxidation of the substrate. The alkylation reaction catalyzed by MqnE is an oxidation from the perspective of the substrate carbons. All centers undergoing oxidation/reduction are labeled with their oxidation states before and after reaction.

Fig. 3. Active site configurations of CteB (PDB 5WGG),²⁹ LipA (PDB 5EXK),³⁷ and anSME (PDB 4K38),²⁵ showing the relative positioning of SAM and the substrate as well as the catalytic and auxiliary Fe-S clusters. The latter is believed to be responsible for oxidizing the product radical.

Fig. 4.

Examples of redox neutral transformations catalyzed by radical SAM enzymes. The oxidation states of all carbon centers undergoing oxidation/reduction are shown; however, the sum of all oxidation states remains the same during the transformation. TsrM and GenK are examples of radical SAM methyltransferases, though the involvement of radical SAM chemistry *per se* in the TsrM reaction is in dispute (see text). The NosL reaction may actually be an oxidation yielding cyanide ($\text{HC(II)}\equiv\text{N}$) as a product rather than formaldehyde and ammonia.⁵²