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Adenosyl Radical: Reagent and Catalyst in Enzyme Reactions

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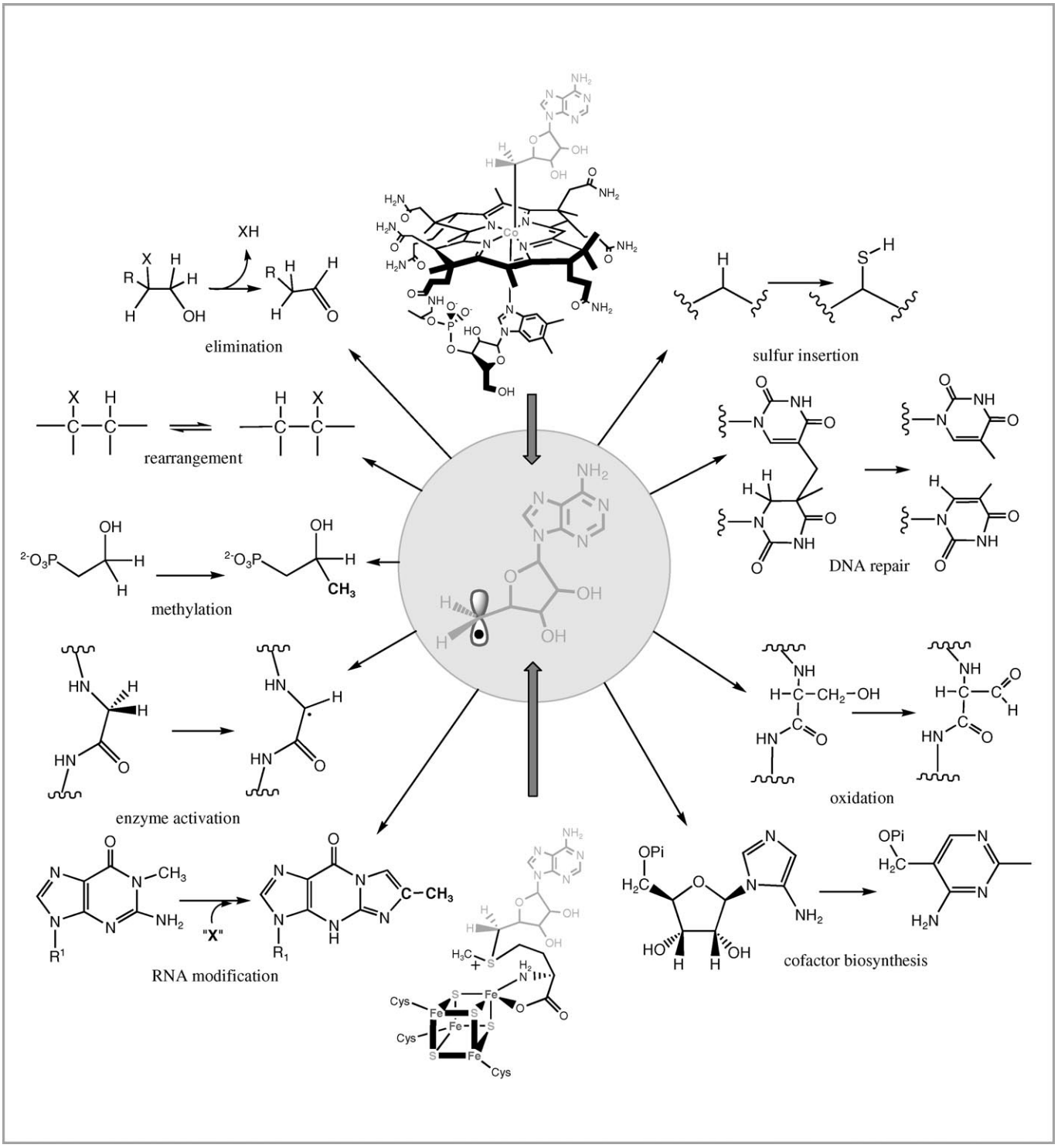
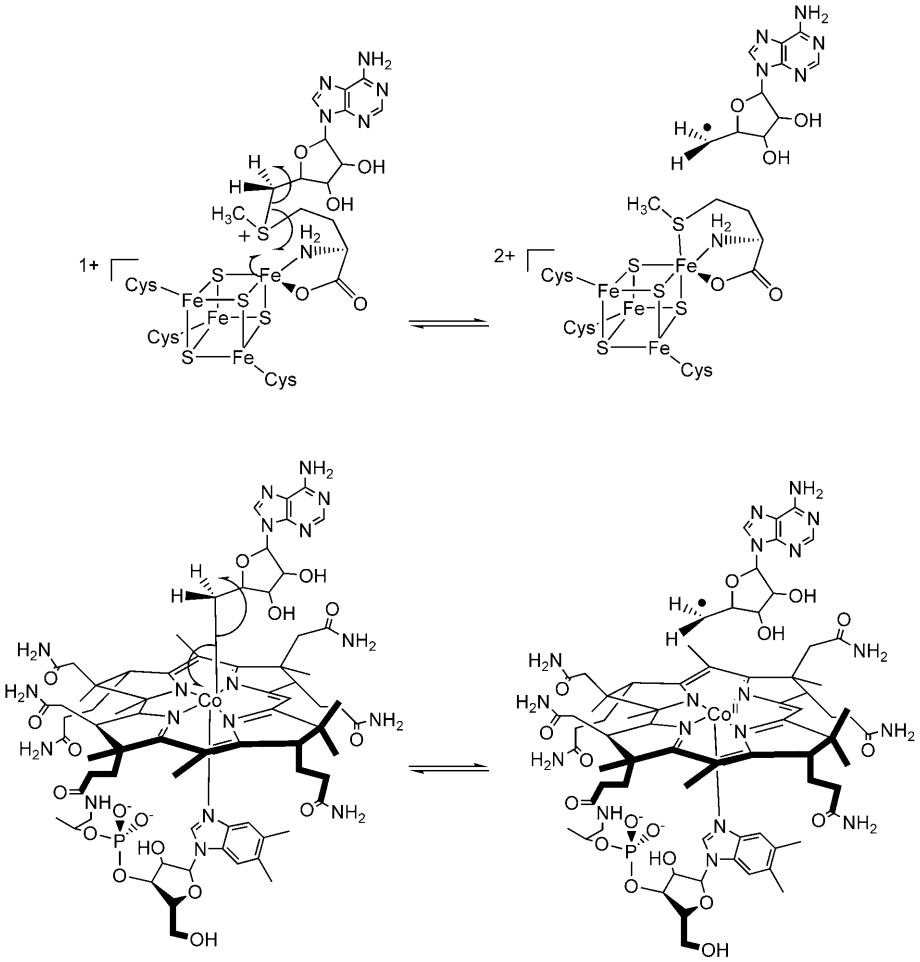


Adenosine is undoubtedly an ancient biological molecule that is a component of many enzyme cofactors: ATP, FADH, NAD(P)H, and coenzyme A, to name but a few, and, of course, of RNA. Here we present an overview of the role of adenosine in its most reactive form: as an organic radical formed either by homolytic cleavage of adenosylcobalamin (coenzyme B_{12}, AdoCbl) or by single-electron reduction of *S*-adenosylmethionine (AdoMet) complexed to an iron–sulfur cluster. Although many of the enzymes we discuss are newly discovered, adenosine's role as a radical cofactor most likely arose very early in evolution, before the advent of photosynthesis and the production of molecular oxygen, which rapidly inactivates many radical enzymes. AdoCbl-dependent enzymes appear to be confined to a rather narrow repertoire of rearrangement reactions involving 1,2-hydrogen atom migrations; nevertheless, mechanistic insights gained from studying these enzymes have proved extremely valuable in understanding how enzymes generate and control highly reactive free radical intermediates. In contrast, there has been a recent explosion in the number of radical-AdoMet enzymes discovered that catalyze a remarkably wide range of chemically challenging reactions; here there is much still to learn about their mechanisms. Although all the radical-AdoMet enzymes so far characterized come from anaerobically growing microbes and are very oxygen sensitive, there is tantalizing evidence that some of these enzymes might be active in aerobic organisms including humans.

1. Introduction

5′-Deoxyadenosyl radical (Ado•) serves two functions in biochemical reactions. Firstly, it is an extremely powerful single-electron oxidant that can remove a hydrogen atom from the least reactive of molecules.[1] This allows cells to catalyze difficult oxidation reactions under anaerobic conditions that would otherwise require the oxidizing power of activated oxygen species, as exemplified by the oxidations catalyzed by cytochrome P450 enzymes. Secondly, it can function as a catalyst by reversibly abstracting a hydrogen atom from the substrate: this allows enzymes to exploit the reactivity of free radicals to catalyze reactions that would be difficult or impossible to effect by ionic chemistry.[2] As illustrated in Scheme 1, there are two biological mechanisms for generating Ado•: homolytic cleavage of adenosylcobalamin (coenzyme B_{12}, AdoCbl), which results in cob(II)alamin and Ado•, and single-electron reduction of *S*-adenosylmethionine (AdoMet) complexed to a reduced iron–sulfur cluster, which yields Ado•, methionine, and the oxidized iron–sulfur cluster. Whereas AdoCbl always serves as a cofactor, Ado• generated from AdoMet may be used catalytically as a true cofactor, but more often is consumed as a cosubstrate.



Scheme 1. Generation of adenosyl radicals. Top: radical generation by 1-electron reduction of AdoMet complexed with a [4Fe–4S] cluster; bottom: radical generation by homolysis of the Co–C bond of AdoCbl.

There have been significant advances in understanding the mechanisms and biological roles of this group of enzymes in the last few years, especially the discovery of many new radi-

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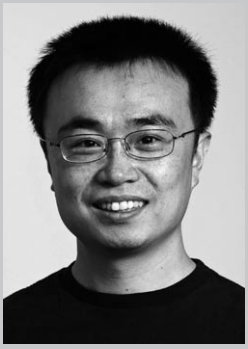
cal-AdoMet enzymes. Here we present an overview of adenosyl radical enzymes, in particular contrasting radical-AdoMet and AdoCbl-dependent enzymes, and discuss whether radical-AdoMet enzymes might be active in animals as well as anaerobic microbes. We also refer the reader to several recent reviews that discuss various aspects of adenosyl radical biochemistry in more detail than space permits here.[1–15]

Neil Marsh received his undergraduate education at Christ's College, Cambridge University. He remained at Cambridge for his Ph.D. studies under the guidance of Prof. Peter Leadlay in the Department of Biochemistry and then studied as a post-doctoral fellow with Prof. Craig Townsend at Johns Hopkins University, Baltimore. He returned to Cambridge to initiate his own research program as a Royal Society University Research Fellow. In 1995 he moved to the University of Michigan where he is currently Professor of Chemistry and Biological Chemistry. His research interests include the mechanisms by which enzymes generate and control free radicals and the use of fluorinated amino acids to modulate the structure, stability and biological activity of proteins and peptides.



Lei Li received his Ph.D. from Johns Hopkins University under the guidance of Prof. Kenneth D. Karlin in 2005, studying copper–dioxygen-complex-mediated aliphatic C–H bond and DNA nucleobase oxidation. He then moved to Prof. Neil Marsh's laboratory at the University of Michigan, investigating the reaction mechanism of benzylsuccinate synthase, a glycyl radical enzyme involved in toluene degradation. In 2008, he was the recipient of an NIH Pathway to the Independence Award. In 2009, he started his independent career as an assistant professor in the Department of Chemistry at Indiana University-Purdue University Indianapolis. His research focuses on the mechanistic elucidation of oligonucleotide modifications mediated by radical-AdoMet enzymes.

Dustin Patterson received his BS in Chemistry with a specialization in Biochemistry from Bowling Green State University, Ohio. He is currently a graduate student at the University of Michigan working in Prof. Neil Marsh's research group. His research projects include characterizing the structure of the glycyl radical enzyme benzylsuccinate synthase and the development of new strategies to assemble supramolecular protein complexes.

The first member of this class of enzymes to be identified was glutamate mutase, an AdoCbl-dependent enzyme involved in the fermentation of glutamate by various bacteria, which was discovered by H. A. Barker and colleagues in the late 1950s.[16–19] Notably, this discovery provided a specific biochemical function for vitamin B_{12}, which is the precursor to AdoCbl. In humans the requirement for vitamin B_{12} derives, in part, from the AdoCbl-dependent enzyme methylmalonyl-CoA mutase, which is involved in odd-chain fatty acid metabolism.[14,20] The radical nature of these reactions was first postulated by Abeles through work on dioldehydratase.[21,22] The first radical-AdoMet enzyme, lysine-2,3-aminomutase (LAM), was not discovered until 20 years later,[23] also by Barker, who noted the similarity of the reaction to the rearrangements catalyzed by AdoCbl-dependent aminomutases. Another 20 years would pass before the second radical-AdoMet enzyme would be discovered through studies in J. Knappe's laboratory on pyruvate formate-lyase (PFL),[24] an enzyme that converts pyruvate to formate and acetyl-CoA as part of the anaerobic metabolism of glucose in *E. coli*. PFL was the first-discovered member of a group of enzymes that contain a radical centered on the α-carbon of the specific protein glycyl residue that is required in the catalytic mechanism. Knappe and colleagues showed that the glycyl radical in PFL is installed by a specific activase enzyme that uses an adenosyl radical, derived from AdoMet, to abstract a hydrogen atom from gly734 of PFL.[25]

Since then studies in numerous laboratories have identified radical-AdoMet enzymes that participate in a remarkably wide range of chemical transformations; representative examples that are discussed in this review are summarized in Table 1. These efforts were greatly aided by a sequence comparison study, published in 2001 that, based on the CX_3CX_2C motif shared by the known radical-AdoMet enzymes, identified a further 600 enzymes that can utilize AdoMet in this manner[26] (today the sequence database contains around 3000 putative radical-AdoMet enzymes[5]). Although some of the sequences were homologues of known radical-AdoMet enzymes from different bacteria, and many more sequences were from completely unknown proteins, some were from known proteins for which a connection to radical-AdoMet chemistry had not been made. Here the sequence motif provided the clue needed to guide biochemical studies elucidating the role of AdoMet in the enzyme reactions. Recent findings suggest that the radical-AdoMet family could be even larger: in 2006, an elongator subunit Elp3 from *Methanocaldococcus jannaschii* was found to contain a [4Fe–4S] cluster coordinated by a CX_4CX_2C sequence. This cluster is able to bind AdoMet and generate 5′-deoxyadenosine probably via a similar sulfur–carbon bond-cleavage chemistry.[27] Additional evidence has been obtained on ThiC, which also contains a CX_4CX_2C motif, that reductive cleavage of AdoMet to generate Ado$^{\bullet}$ is a feature of the mechanism.[28,29] These findings indicate that the CX_3CX_2C motif might not be the definitive sequence for this enzyme superfamily. Furthermore, the Elp3 subunits from *Saccharomyces cerevisiae*, *Schizosaccaromyces pombe*, and human were found to

Table 1. Summary of AdoCbl and radical-AdoMet-dependent enzymes discussed in this review.

Enzyme	Function	Ref.
Adensylcobalamin-dependent radical enzymes		
lysine-5,6-aminomutase	aminomutase	[2], [36]
ornithine-4,5-aminomutase	aminomutase	[2]
glutamate mutase	carbon skeleton mutase	[17], [19], [80]
methylmalonyl-CoA mutase	carbon skeleton mutase	[20], [32], [75]
isobutyryl-CoA mutase	carbon skeleton mutase	[9], [76]
2-methyleneglutarate mutase	carbon skeleton mutase	[9], [77]
ribonucleotide reductase	eliminase/reductase	[36], [69]
diol dehydratase	eliminase	[21], [34]
ethanolamine deaminase	eliminase	[13], [67]
glycerol dehydratase	eliminase	[13], [67]
***S*-Adenosyl-L-methionine-dependent radical enzymes**		
Using AdoMet as catalyst		
lysine-2,3-aminomutase	aminomutase	[23], [52]
glutamate 2,3-aminomutase	aminomutase	[15]
arginine 2,3-aminomutase	aminomutase	[15]
spore photoproduct lyase	lyase	[47], [49], [50]
DesII	eliminase	[53], [68]
Using AdoMet as co-substrate		
anaerobic ribonucleotide reductase activase	glycyl radical generation	[101], [103], [105]
pyruvate formate-lyase activase	glycyl radical generation	[25], [42]
benzylsuccinate synthase activase	glycyl radical generation	[106], [108]
glycerol dehydratase activase	glycyl radical generation	[110], [111]
4-hydroxyphenylacetate decarboxylase activase	glycyl radical generation	[102]
biotin synthase	sulfur insertion in biotin	[115], [116]
lipoyl synthase	sulfur insertion in lipoate	[122], [123]
MiaB	methylthiolation of tRNA	[124], [125]
RimO	methylthiolation of protein	[130]
HydE and HydG	metal cofactor biosynthesis	[133], [136]
coproporphyrinogen III oxidase	oxidative decarboxylation	[40], [138]
formylglycine synthase	alcohol oxidation	[139], [141]
BtrN	alcohol oxidation	[142]
Fom3	methylation	[146]
ThiH	thiamine biosynthesis	[148], [150]
ThiC	thiamine biosynthesis	[28], [29], [152]
MoaA	molybdopterin synthesis	[42], [154]
TWY1	wybutosine tRNA biosynthesis	[156]
Elp3	transcription factor	[27], [164]
viperin	unknown	[168], [169]

possess a CX_9CX_2C motif.[27] This suggests that more, as yet unrecognized enzymes, may utilize this radical-AdoMet chemistry. In contrast, AdoCbl-dependent enzymes appear to be relatively rare; only ten are known so far.[12] No new enzymes have been definitively identified in over 20 years, although the recently reported AdoCbl-dependent 2-hydroxyisobutyryl-CoA mutase[30] might be a distinct enzyme from isobutyryl-CoA mutase. Their reactions are restricted to either isomerization or elimination reactions. Although a B_{12}-binding motif has been described,[31,32] it is not shared by all enzymes, nor is it unique to AdoCbl enzymes because it also occurs in some cobalamin-dependent methyl transferases; this has made identifying new AdoCbl enzymes in sequence databases difficult.

2. Similarities and Differences between Radical-AdoMet and AdoCbl Enzymes

The most significant difference between AdoCbl and radical-AdoMet enzymes is their oxygen sensitivity. Whereas AdoCbl enzymes are not especially oxygen sensitive, all radical-AdoMet enzymes studied to date must be handled under rigorously anaerobic conditions to maintain their activity. Reactive oxygen species rapidly oxidize and destroy the iron–sulfur clusters in these enzymes. Oxygen sensitivity might have provided the pressure for the evolution of AdoCbl-dependent enzymes; however, as discussed below, radical-AdoMet enzymes can be stable in the microenvironment of a cell even under aerobic conditions.

Although a detailed discussion of their structures is beyond the scope of this review, both classes of enzyme are built on variations of the β/α-barrel (TIM barrel) scaffold. The structures of representative members of the two classes of enzymes are compared in Figure 1. All AdoCbl enzymes for which structures are known comprise "complete" barrels—eight-stranded barrels for methylmalonyl-CoA mutase,[33] diol dehydratase,[34] glutamate mutase,[35] and lysine-5,6-aminomutase,[36] and a ten-stranded barrel for ribonucleotide reductase.[37] Radical-AdoMet enzymes exhibit more structural diversity: the catalytic domains of biotin synthase, ThiC, and HydE are complete eight-stranded barrels[29,38,39] whereas HemN, LAM, MoaA, and PFL activase are six-stranded $(\beta/\alpha)_6$ "3/4" barrels[40–43] in which the fold is opened out to facilitate entry of the substrate.[3,5] This opening out is most marked for PFL-activase, which must accommodate a protein substrate within its active site.[42]

Lastly we note that, intriguingly, both AdoMet and cobalamin, as methylCbl (MeCbl), also function as methyl donors, indeed in MeCbl-dependent methionine synthase AdoMet can act as a methyl donor to cob(II)alamin under some conditions.[12] In both classes of enzymes, methyl transfer involves ionic reactions, rather than radical chemistry; in the cobalamin-dependent methylases cobalt cycles between +3 (methylated) and +1 (unmethylated) oxidation states. This illustrates a further similarity between the intrinsic reactivity of sulfonium ions and organocobalt complexes that has been exploited by these two classes of enzymes.

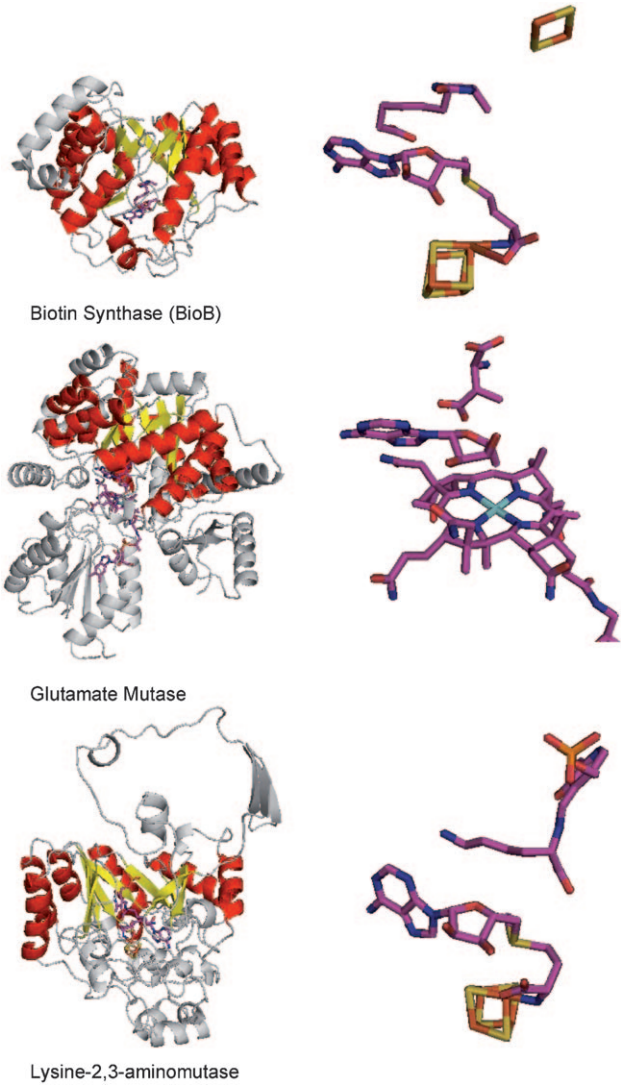


Figure 1. Comparison of the structures of AdoCbl and radical-AdoMet enzymes. Left: the structures of biotin synthase ($(\beta/\alpha)_8$ complete barrel), glutamate mutase ($(\beta/\alpha)_8$ complete barrel) and lysine-2,3-aminomutase ($(\beta/\alpha)_6$ 3/4 barrel); the structure of the core barrel domain is highlighted in red and gold. Right: details of the cofactor and substrate interactions for each protein.

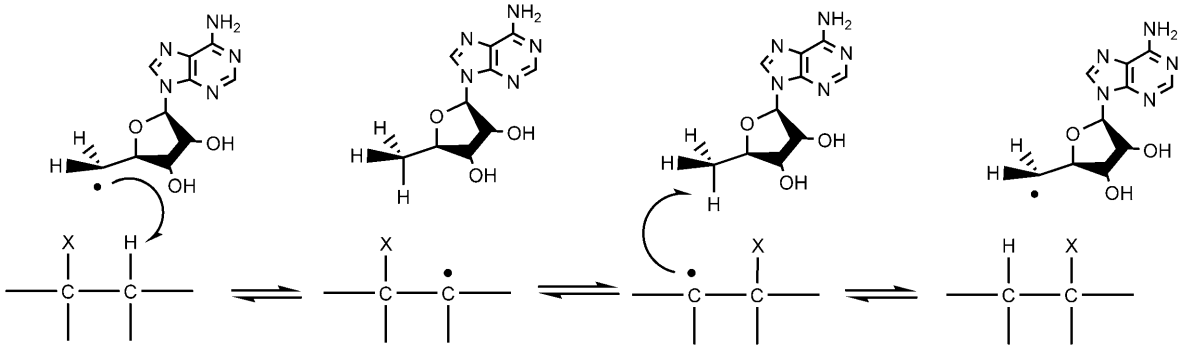
3. Adenosyl Radical Formation

For AdoCbl-dependent enzymes Ado$^\bullet$ is generated through enzyme-catalyzed homolysis of a cobalt–carbon bond, and in all cases this is a reversible process.[2,14,15] For radical-AdoMet enzymes homolytic cleavage of the sulfur–carbon bond of AdoMet (BDE $\geq$ 60 kcal mol^{-1}) is not energetically feasible. Rather, reductive cleavage must occur to produce methionine and the 5′-deoxyadenosyl radical.[44] This is achieved by a unique [4Fe–4S]$^+$ cluster that is coordinated by the 3 cysteine residues of the CX_3CX_2C motif. The fourth iron lacks a cysteine ligand, which allows AdoMet to coordinate to the cluster through the amino and carboxyl groups of the cofactor.[45,46] Next, an electron from the cluster directly reduces the sulfonium ion to the neutral radical that spontaneously fragments to generate Ado$^\bullet$ and methionine (Scheme 1). For most radical-AdoMet enzymes, the Ado$^\bullet$ radicals generated this way are subsequently used to oxidize the substrate, and the overall process is irreversible. However, three enzymes are known that generate Ado$^\bullet$ reversibly to catalyze their respective reactions that involve no overall change in the oxidation state of the substrate; these are LAM, spore photoproduct lyase, and DesII, which is involved in desosamine biosynthesis.[47–53]

An important aspect of the mechanisms of both AdoCbl and radical-AdoMet enzymes is that the energetics of forming Ado$^\bullet$ are extremely unfavorable. Ado$^\bullet$ is such a reactive species that it has never been directly observed in an enzyme, although it is generally accepted as the key intermediate in the mechanisms of both classes of enzymes. However, FT-EPR studies on the nonenzymic photolysis of AdoCbl have provided spectroscopic evidence for the formation of Ado$^\bullet$.[54] In free solution the bond dissociation energy of AdoCbl is 32 kcal mol^{-1},[55] representing a considerable obstacle to forming radicals. Yet in response to substrate binding, AdoCbl readily undergoes homolytic cleavage and substrate-based radicals accumulate on the enzyme during turnover; this implies that the equilibrium constant for homolysis is now close to unity.[56–59] A similar problem faces radical-AdoMet enzymes. Reduction of AdoMet to Ado$^\bullet$ and methionine is estimated to require a redox potential of -1.8 V,[60] however the reduction potential of the [4Fe–4S]$^{+/2+}$ cluster in LAM is -0.43 V.[61] The -1.4 V difference corresponds to an unfavorable free energy for forming Ado$^\bullet$ from AdoMet that is also about 32 kcal mol^{-1}.

How AdoCbl and radical-AdoMet enzymes overcome this energetic challenge remains poorly understood. For AdoCbl enzymes it has been generally assumed that a protein-induced distortion of the coenzyme, presumably triggered by substrate binding, weakens the Co–C bond sufficiently to promote homolysis; that is, the enzyme uses binding energy to offset the unfavorable bond dissociation energy. However, attempts to verify this hypothesis experimentally have failed to find evidence for any significant structural distortion of the coenzyme or change in the electronic or vibrational properties of the Co–C bond upon binding to the cognate enzymes.[62–65] In contrast, recent studies on LAM have shown that the effects of AdoMet binding, and to a lesser extent lysine, dramatically shifts the reduction potential of the [4Fe–4S] cluster so that ΔE° is $\sim$0.4 V and electron transfer is much more favorable.[66]

The mechanisms for generating Ado$^\bullet$ and the biochemical reactions that Ado$^\bullet$ participates in can largely be considered separately. For example, the amino group migrations catalyzed by the radical-AdoMet-dependent α-aminomutases (lysine-, glutamate-, and arginine-2,3-aminomutases) and AdoCbl-dependent ω-aminomutases (ornithine-4,5- and lysine-5,6-aminomutases) are chemically identical even though the coenzymes are different. Radical-AdoMet enzymes appear to catalyze a much wider range of reactions than AdoCbl enzymes; these may be broadly classified into reactions in which Ado$^\bullet$ is used catalytically and those in which Ado$^\bullet$ is consumed as an oxidizing agent.



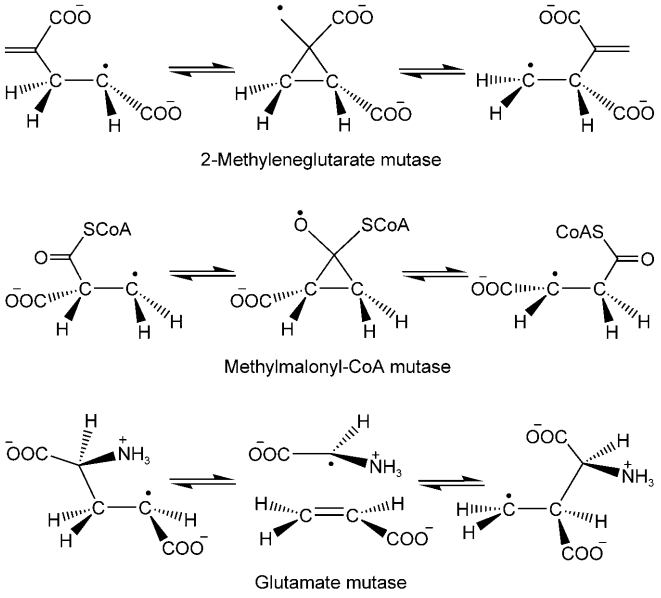
Scheme 2. A minimal mechanism for the 1,2-rearrangements catalyzed by adenosyl radical enzymes, here X may be OH, NH_2 or a carbon-containing fragment.

4. Catalytic Reactions of Adenosyl Radical

All AdoCbl-dependent enzymes use Ado• catalytically and, with the exception of AdoCbl-dependent ribonucleotide reductase, catalyze reactions involving the 1,2-rearrangement of a hydrogen atom with an electron-withdrawing group, X, as illustrated in Scheme 2. X may be either NH_2-, HO-, or a carbon-containing fragment so that a carbon skeleton rearrangement occurs.[2,13,15,67] Three radical-AdoMet enzymes, LAM, DesII, and spore photoproduct lyase (SPL), catalyze reactions that are mechanistically similar to the AdoCbl-dependent enzymes, and for which there is evidence from isotope-exchange experiments to support the regeneration of AdoMet after turnover.[50–52,68] Presumably glutamate 2,3-aminomutase and arginine 2,3-aminomutase, which are closely related to LAM, operate very similarly.

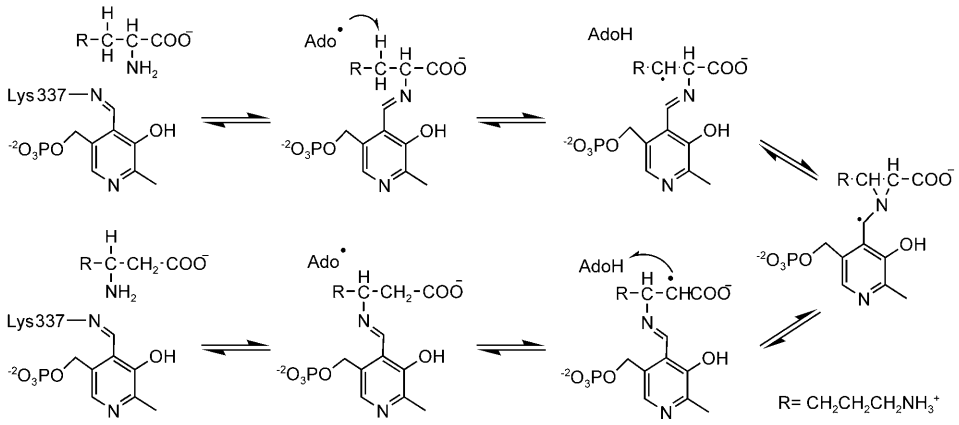
The key step in all these reactions is abstraction by Ado• of a non-acidic hydrogen atom from the substrate, which is thus activated towards rearrangement. This step is best understood for AdoCbl enzymes, for which it has been shown that hydrogen abstraction is coupled to homolysis of the coenzyme cobalt–carbon bond.[57–59] Thus the energetic cost of forming Ado• is offset, in part, by forming a much more stable substrate-based radical. In an interesting experiment using a ribonucleotide reductase in which the catalytic cysteine residue was mutated, it was shown that epimerization of AdoCbl that was stereospecifically deuterium-labeled at the 5′-carbon could occur in the presence of an allosteric activator, thereby providing evidence for the transient formation of Ado•.[69] Detailed studies involving the measurement of pre-steady-state kinetic isotope effects and computational experiments on methylmalonyl-CoA mutase and glutamate mutase have demonstrated that hydrogen transfer between the substrate and Ado• occurs through quantum tunneling rather than classical motion,[70–73] as is increasingly observed for many enzymes catalyzing hydrogen transfer reactions.[74]

Once formed, the mechanisms by which the substrate radicals rearrange are dependent on the nature of the migrating chemical group. The carbon-skeleton-rearranging enzymes have attracted particular interest as these reactions have no ready counterparts in conventional organic chemistry. For the reactions catalyzed by methylmalonyl-CoA mutase,[14,75] isobutyryl-CoA mutase,[76] and 2-methyleneglutarate mutase[9,77] the interconversion of substrate and product radicals can occur through a cyclopropylcarbenyl radical intermediate (Scheme 3), a mechanism supported by model chemical reactions.[78,79] In contrast, the rearrangement catalyzed by glutamate mutase[18,19,80] cannot occur through this type of intermediate and it has been shown experimentally that the mechanism involves fragmentation of the glutamyl radical to form a glycyl radical and acrylate followed by recombination to form the methylaspartyl radical.[81]



Scheme 3. Mechanisms for the carbon skeleton rearrangements catalyzed by the AdoCbl-dependent enzymes 2-methyleneglutarate mutase, methylmalonyl-CoA mutase, isobutyryl-CoA mutase, and glutamate mutase.

The aminomutases, whether AdoMet or AdoCbl-dependent, require pyridoxal phosphate as a cofactor; the best understood is LAM, the mechanism for which is shown in Scheme 4. EPR experiments on LAM employing isotopically labeled substrates and substrate analogues of lysine that preferentially stabilize the different radicals formed during the course of the rearrangement have allowed each of the substrate radical species



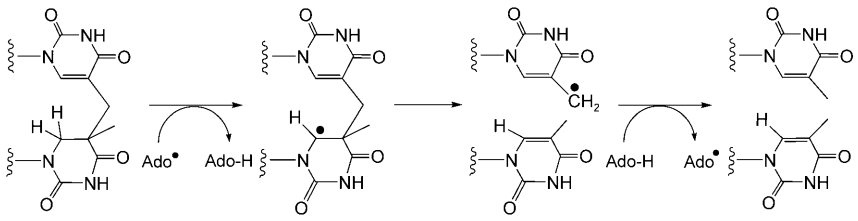
Scheme 4. Mechanism for the 1,2-rearrangement catalyzed by AdoMet-dependent lysine-2,3-aminomutase; a similar mechanism could be drawn for AdoCbl-dependent lysine-5,6-aminomutase or ornithine-4,5-aminomutase.

proposed in the mechanism to be identified.[82–84] During the reaction, the α-amino group of lysine forms an external aldimine with pyridoxal phosphate,[85] rendering the nitrogen sp^2 hybridized. This allows the 1,2-nitrogen migration to occur through a cyclic azacyclopropylcarbinyl radical intermediate transition state in which nitrogen is bonded to both C-2 and C-3 of lysine and the unpaired electron is situated on the 4′-carbon of pyridoxal and stabilized by the adjacent π system.

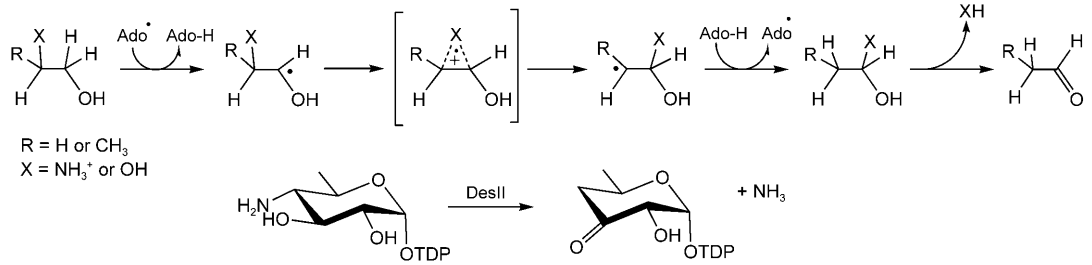
The AdoCbl-dependent diol dehydratase, glycerol dehydratase, and ethanolamine deaminase[13,22,67,86] catalyze elimination reactions that first involve a 1,2-migration of OH or NH_3^+ followed by dehydration or deamination of the resulting 1,1-diols or amino alcohols to give aldehydes.[87,88] Here it appears that the charge state of the migrating oxygen or nitrogen atom is important, with theoretical studies pointing to a favorable migration pathway involving a positively charged cyclic transition-state structure (Scheme 5).[89,90] The amino group of ethanolamine, of course, is readily protonated, whereas diol dehydratase contains a potassium ion in the active site to which the hydroxyl groups of the substrate coordinate that, presumably, can supply the necessary positive charge. A similar mechanism can be drawn for the deamination reaction catalyzed by the radical-AdoMet enzyme DesII (Scheme 5) as part of the biosynthesis of the aminosugar D-desosamine.[53] Recent labeling studies[68] suggest that AdoMet might function catalytically in the deamination reaction, which involves no change in the oxidation state of the substrate.

As mentioned above, the rearrangement mechanisms of several of these enzymes have been investigated by using computational techniques.[91–95] These support the energetic feasibility of the proposed reaction pathways and have also highlighted the importance of the protonation state of the functional groups adjacent to organic radicals in stabilizing the various intermediates.

The reaction catalyzed by spore photoproduct lyase appears very different from those catalyzed by other adenosyl-radical-dependent isomerases, although mechanistically it is quite similar.[48] The enzyme requires only a catalytic quantity of AdoMet for activity and transfer of tritium from AdoMet to thymine has been demonstrated, supporting a catalytic role for Ado• in this reaction.[50,51] A plausible mechanism, shown in Scheme 6, involves abstraction of the C-6 hydrogen from the thymine dimer, which then undergoes fragmentation to generate one thymine and the thymine monomer radical. Transfer of hydrogen back from 5′-dA forms the second thymine and regenerates Ado•.



Scheme 6. Mechanism for the resolution of thymidine dimers in DNA catalyzed by AdoMet-dependent spore photoproduct lyase.



Scheme 5. Mechanism for the radical elimination reactions catalyzed by AdoCbl-dependent ethanolamine ammonia lyase and diol dehydratase. The reaction catalyzed by AdoMet dependent DesII, shown below, is thought to occur by a similar mechanism.

The stereochemistry and kinetics of the reaction have also recently been investigated by using a "minimal" dinucleotide substrate comprising the crosslinked thymine dimer, and it was shown that the enzyme specifically repairs the 5*R* stereoisomer of the spore photoproduct.[47,96,97] However, with this substrate the mechanism appears to be altered. AdoMet appears to be consumed stoichiometrically, no transfer of deuterium between AdoMet and thymine was observed, and there is a significant contribution from the uncoupled reaction of AdoMet with the enzyme.[98] The source of the hydrogen atom in the resolved thymine dimer product is unclear. Possibly, this redirection of radical chemistry arises because the small, unnatural substrate is incorrectly oriented in the active site.

Lastly we note that AdoCbl-dependent ribonucleotide reductase is unique among B_{12} enzymes in *not* catalyzing an isomerization. Here AdoCbl is used to reversibly generate an active-site cysteinyl residue.[69] The cysteine residue, in turn, abstracts the 3′-H adjacent to the site of reduction and this activates the 2′-OH to become a good leaving group (Scheme 7). In this respect the chemistry is very similar to that for the 1,2-migration of OH catalyzed by diol dehydratase. Indeed, this essential radical chemistry, including the cysteinyl radical, is conserved in both the aerobic tyrosyl radical-dependent and anaerobic glycyl radical-dependent ribonucleotide reductases.[99–101]

5. Irreversible Reactions of Adenosyl Radical

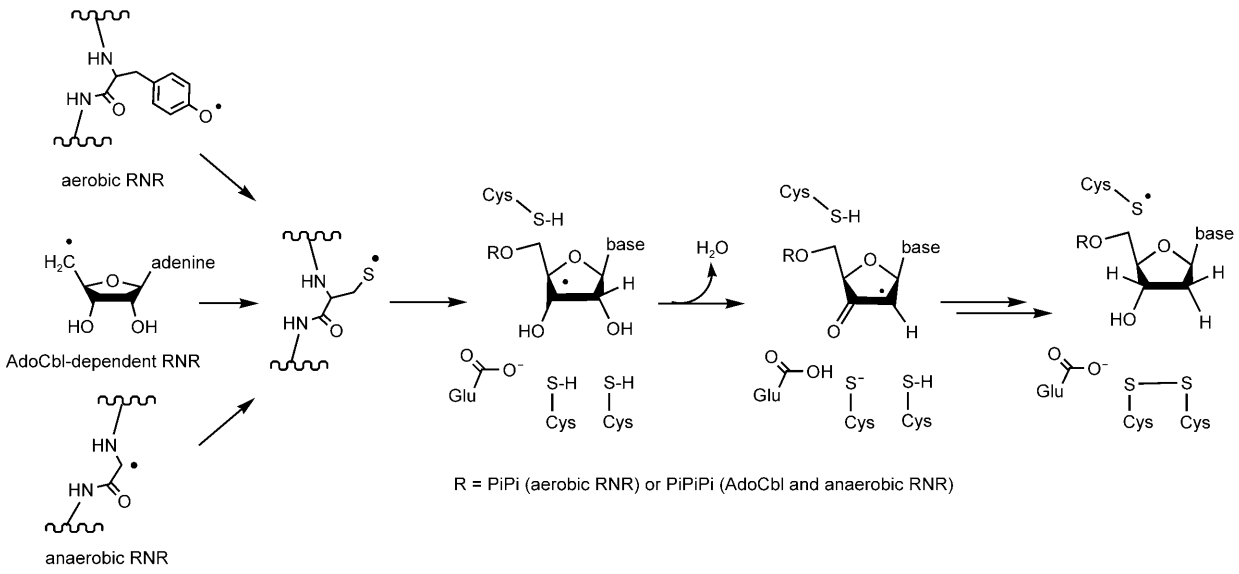
These constitute a much larger mechanistic class of Ado•-dependent reactions that are solely AdoMet-dependent. One reason for this might be that AdoMet is much less expensive, metabolically, to synthesize than AdoCbl and thus better suited for reactions in which it is consumed as a reagent. These enzymes catalyze a remarkable variety of reactions in which Ado• functions as a powerful oxidizing agent. To facilitate our discussion we have grouped them according to the type of chemical reactions they catalyze.

5.1 Glycyl radical-generating enzymes

Mechanistically, these are the simplest radical-AdoMet reactions. These enzymes use Ado• to abstract a hydrogen atom from a glycine residue on the protein backbone of a cognate member of the glycyl radical family of enzymes, thereby generating a glycyl radical that is required in the catalytic mechanism of the enzyme. The glycyl residue provides a "storage" site for the radical; during catalysis it is transferred to an active site cysteine residue and thence to the substrate. So far, all the glycyl radical enzymes identified catalyze various reactions, shown in Scheme 8, that enable bacteria to grow under anaerobic conditions.[102]

Pyruvate formate-lyase (PFL) was the first-discovered and is the best-characterized member; it catalyzes the conversion of pyruvate to acetyl-CoA and formate during the fermentation of glucose.[24,103] Anaerobic ribonucleotide reductase[103–105] is required by *E. coli* for anaerobic growth because the aerobic enzyme requires oxygen to generate the tyrosyl radical needed for activity. Benzylsuccinate synthase catalyzes an unusual radical addition reaction of toluene to the double bond of fumarate as the first step in the fermentation of toluene by various sulfate and nitrate-reducing bacteria.[106–109] 4-Hydroxyphenylacetate decarboxylase catalyzes the formation of *p*-cresol in various Clostridia.[102] Lastly, glycerol dehydratase catalyzes the conversion of glycerol to 3-hydroxypropanal, interestingly the same reaction is also catalyzed by an AdoCbl-dependent enzyme.[110,111] This, again, highlights the interchangeability of the mechanisms for generating radicals.

The paradigm for the radical-generating partner enzyme is PFL-activase, experiments on which established many features



Scheme 7. Radicals required for the mechanism of ribonucleotide reductase (RNR) are generated differently by the aerobic (tyrosyl radical), anaerobic (glycyl radical) and AdoCbl-dependent enzymes. However the mechanism for ribonucleotide reduction, involving radical elimination of the 2′-OH group, is the same for all classes of enzyme.

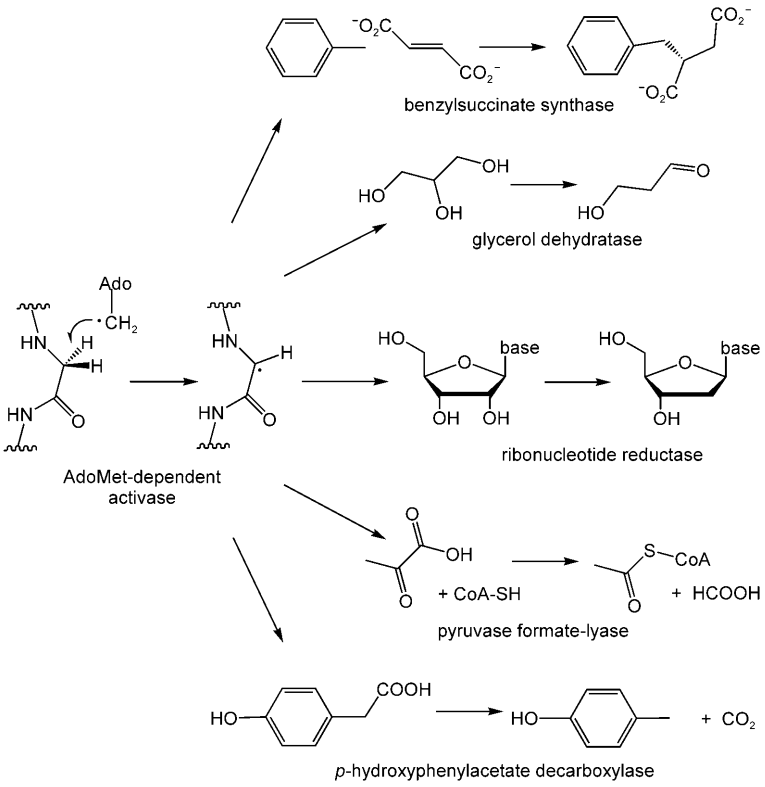
of the mechanism common to radical-AdoMet enzymes;[46, 112, 113] most recently the crystal structure has been solved.[42] Studies from Knappe's laboratory initially showed that short peptides encompassing the sequence containing the catalytic glycine residue were substrates for the activase.[25, 114] This suggested that the glycyl radical resides on a mobile loop that can interact with both the activase and active site of their cognate enzyme. The activases appear to be specific to their corresponding enzymes, for example, the PFL and anaerobic ribonucleotide reductase enzymes from *E. coli* do not show cross reactivity, and this specificity allows the activity of the parent enzyme to be regulated by the cognate activase.

5.2 Sulfur-inserting enzymes

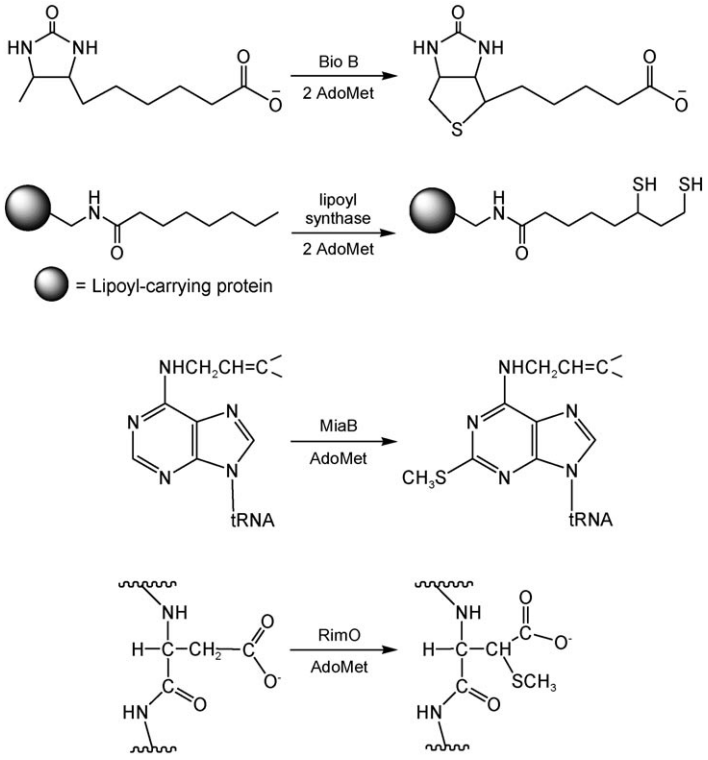
Four radical-AdoMet enzymes are now known that catalyze reactions involving the insertion of sulfur atom(s) into C–H bonds during the biosynthesis of biotin, lipoic acid, and in the modification of tRNA and ribosomal proteins (Scheme 9). The best characterized is biotin synthase (BioB), which catalyzes the formation of biotin from dethiobiotin as the last step in biotin biosynthesis.[10] The reaction, shown in Scheme 10, takes place in two steps whereby adenosyl radicals generated from AdoMet are used to oxidize first the terminal carbon and then the internal carbon of dethiobiotin.[115] The organic radicals that are generated as intermediates react with sulfur to form the carbon–sulfur bonds.[116] The sulfur atom is supplied by a separate [2Fe–2S] cluster in the enzyme;[117, 118] this destroys the cluster so that, in vitro at least, the enzyme never undergoes more than one turnover. However, it is reported that in vivo the enzyme can undergo multiple turnovers so that, presumably, the cluster is rebuilt by intrinsic cysteine desulfurases.[119]

Lipoyl synthase catalyzes the formation of lipoyl acyl carrier protein from octanoyl acyl carrier protein by an analogous mechanism to biotin synthase (Scheme 9).[7, 120] However, in this case two sulfur atoms are inserted. The sulfur donor is thought to be a second [4Fe–4S] cluster present in the enzyme, with both sulfur atoms coming from the same protein.[121, 122] The sulfur atoms appear to be inserted in a stepwise manner with the C-6 sulfur inserted first,[123] although there is some contradictory evidence in the literature.[7]

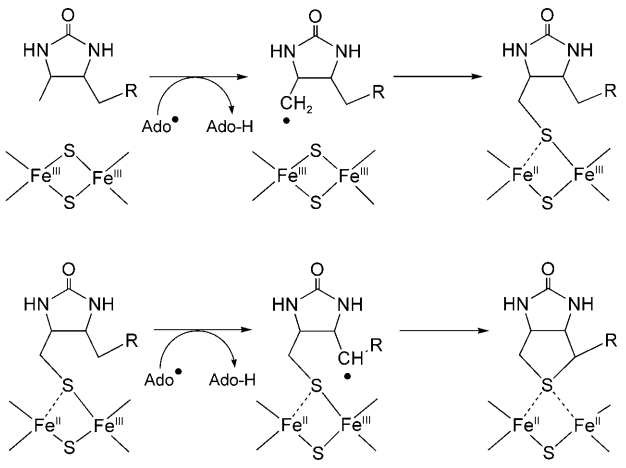
MiaB catalyzes the methylthiolation of a hypermodified adenosine base, i^6A, (Scheme 9)[124–126] found in tRNA to yield 2-methylthio-N^6-isopentenyladenosine (ms^2i^6A). Apart from the interesting chemistry, which is not well understood, it is significant in occurring in both prokaryotes and eukaryotes (wheat germ).[127] In a few bacteria, such as *Salmonella typhimurium* and in the wheat germ, the product 2-methylthio-N^6-iso-



Scheme 8. Overview of the reactions catalyzed by glycyl radical enzymes. Each enzyme has a cognate radical-AdoMet activase responsible for generating the glycyl radical.



Scheme 9. The sulfur insertion reactions catalyzed by biotin synthase, lipoyl synthase, MiaB, and RimO.



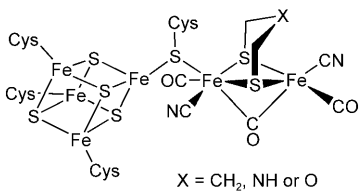
Scheme 10. Mechanism for the sulfur-insertion reactions catalyzed by biotin synthase.

pentenyladenosine is further hydroxylated by an enzyme MiaE[128] to yield 2-methylthio-N^6-(*cis*-hydroxyisopentenyl) adenosine (ms^2io^6A) by using O_2 as the oxidant; this provides the first clue that radical-AdoMet enzymes might also function under aerobic conditions.[129]

The most recently discovered sulfur-inserting enzyme is RimO.[130] It catalyzes the methylthiolation of Asp-88 of the ribosomal protein S12 (Scheme 9), a unique post-translational modification that occurs in *E. coli* and several other bacteria. RimO has extensive sequence similarity to MiaB, suggesting a closely related mechanism, but little else is known about the enzyme.

Lastly, radical-AdoMet enzymes also play a role in the biosynthesis of the Fe–Fe hydrogenase cofactor. Various microbes possess hydrogenases that allow them to metabolize hydrogen, either as a source of electrons or as an electron sink during fermentation or photosynthesis.[131] One class of enzymes, the Fe–Fe hydrogenase, contains an unusual metallocofactor (Scheme 11) in which a [4Fe–4S] cluster is linked through a bridging cysteine residue to a dinuclear iron cluster. The ligands to the di-iron cluster are CN and CO, and an unusual dithiol-bridging ligand -SCH_2XCH_2S- that was discovered when the X-ray structure of the protein was solved;[132] "X" might be CH_2, NH, or O, the exact chemical composition was unclear.

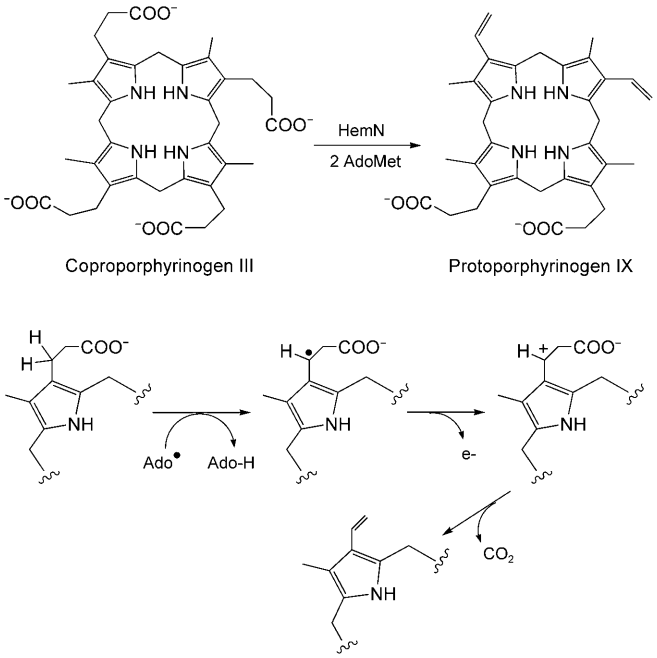
Three maturation proteins are required for the assembly of the di-iron cluster, HydE, HydF, and HydG. Two of these, HydE and HydG, are radical-AdoMet enzymes[133] whereas hydF is a GTPase.[134] The X-ray structure of HydE has recently been determined, confirming its identity as a radical-AdoMet enzyme with a fold similar to biotin synthase.[39] However, the precursors for the dithiolate ligand and the substrate for HydE remain unknown. It has been generally assumed that HydE and HydG are involved in sulfur insertion chemistry akin to that catalyzed by biotin synthase, which is why we discuss them here. However, it has recently been shown that HydG can catalyze the cleavage of tyrosine to give dehydroglycine,[135] a reaction that also occurs in the biosynthesis of thiamine pyrophosphate (discussed below). On the basis of this observation it was suggested that dehydroglycine could be a precursor to the dithiolate ligand if "X" were NH. Recent EPR spectroscopy studies on this intriguing metallo-cofactor also suggest that "X" is, in fact, nitrogen.[136]



Scheme 11. The structure of the Fe–Fe hydrogenase cofactor. Two radical-AdoMet enzymes, HydE and HydG, are involved in the biosynthesis of the bridging dithiolate moiety.

5.3 2-Electron oxidation reactions

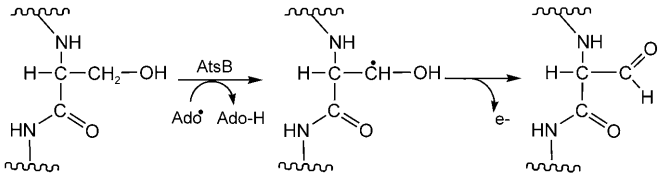
Several enzymes are known that catalyze "simple" 2-electron oxidations by using AdoMet and an electron acceptor. Interestingly, these enzymes have all been identified through sequence similarities that placed them in the radical-AdoMet superfamily. Coproporphyrinogen III oxidase (HemN) catalyzes the oxidative decarboxylation of the two propionate groups of the A and B rings of coproporphyrinogen III to form protoporphyrinogen IX, shown in Scheme 12, in anaerobic organisms[137] as part of the heme anaerobic biosynthetic pathway (in aerobic organisms an oxygen-dependent enzyme is used). It is the best-characterized member of this group and its crystal structure has been solved.[40] The mechanism involves abstraction of hydrogen from the β-position of the propionate side-chain



Scheme 12. The radical oxidative decarboxylation reaction catalyzed by HemN during the anaerobic biosynthesis of heme.

to form a radical that is stabilized by conjugation to the π-system of the pyrole ring and which has been observed by EPR.[138] Further oxidation of this radical by a one-electron acceptor (the physiological electron acceptor is as yet unknown) would yield an allylic carbocation that would facilitate the decarboxylation and formation of the vinyl group (Scheme 12). It is also plausible that fragmentation of the radical to form the vinyl group and formate radical, which is subsequently oxidized to CO_2 could occur.

Formylglycine synthase (AtsB)[139–141] catalyzes the post-translational oxidation of a conserved cysteine or serine to the corresponding aldehyde to form formylglycine in sulfatase enzymes that hydrolyze sulfate esters (Scheme 13). Again, this



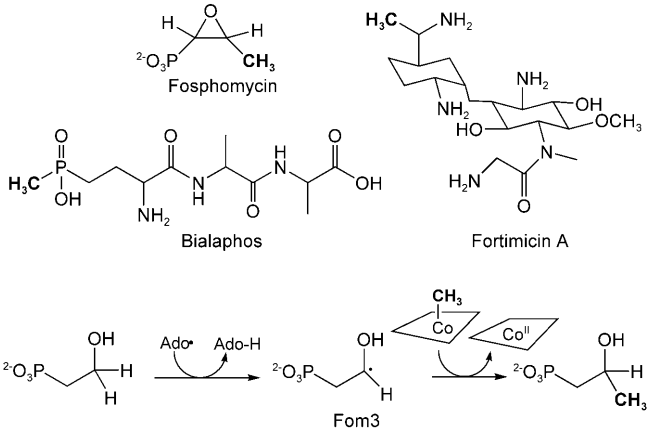
Scheme 13. The oxidation of the active-site serine in sulfatase to formylglycine catalyzed by AtsB under anaerobic conditions.

enzyme is the anaerobic counterpart of an oxygen-dependent enzyme that operates under aerobic conditions. In solution the aldehyde is predominately hydrated, and nucleophilic attack of one of the hydroxyl groups on the sulfate ester results in transfer of the sulfate group to the enzyme; subsequent elimination of the sulfate occurs to regenerate the formyl group. The mechanism of AtsB is proposed to involve hydrogen abstraction from the β-carbon of serine by Ado• and subsequent one-electron oxidation to the aldehyde (Scheme 13), although again the electron acceptor for the second step is not known.

The most recently discovered member of this class of enzymes is BtrN, an enzyme that catalyzes the conversion of 2-deoxy-*scyllo*-inosamine to 2-deoxystreptamine during the biosynthesis of the aminoglycoside antibiotic butirocin. This reaction involves the oxidation of a sugar hydroxyl group to a ketone in a reaction that is mechanistically similar to formylglycine synthase.[142]

5.4 Radical methylation reactions

An increasing number of natural products are now known that contain methyl groups that are introduced by radical-AdoMet enzymes; examples include fosfomycin, bialaphos, and fortimycin A (Scheme 14).[5] These enzymes do not use AdoMet in its conventional methylating role: the sites of methylation are either unreactive carbon atoms or electrophilic sites rather than nucleophilic sites required to react with the electrophilic methyl group of AdoMet. Much of the evidence for these reactions involving radical-AdoMet enzymes is indirect and is built on a combination of sequence comparisons, feeding of labeled precursors, and genetic knock-out and complementation experiments. For the natural products listed above it appears that MeCbl is the source of the methyl group.[143–145]

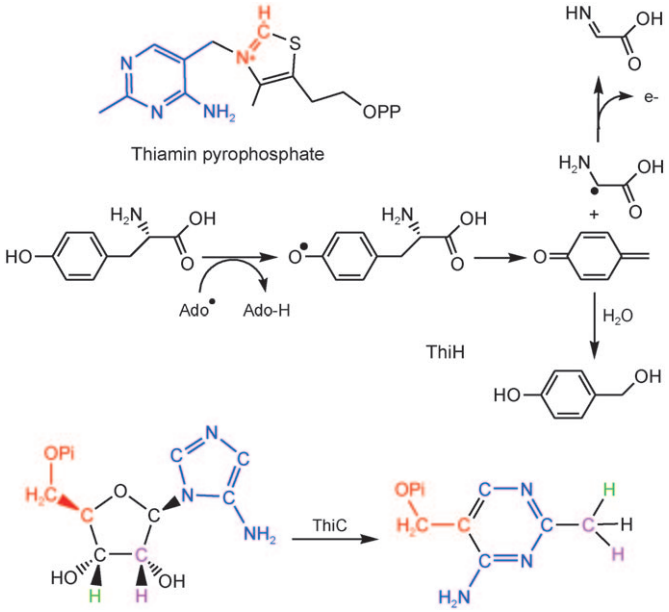


Scheme 14. The natural products shown contain methyl groups (indicated in bold) introduced at non-nucleophilic sites that are derived from MeCbl in radical-AdoMet-dependent reactions. The proposed mechanism for the methylation reaction catalyzed by Fom3 is shown below.

Their mechanisms are not yet well characterized, but a reasonable working model was first proposed for the methylation of hydroxyethyl phosphonate catalyzed by Fom3 in the biosynthesis of fosphomycin.[146] The first step involves removal of a hydrogen atom at the site of methylation by Ado• generated from AdoMet. Next, transfer of the methyl group from MeCbl to the substrate occurs to give the product and cob(II)alamin (Scheme 14). Note that this formally involves transfer of a methyl *radical*. This reaction is quite different from other methyl transfer reactions catalyzed by MeCbl, AdoMet, or methyltetrahydrofolate-dependent enzymes, all of which formally transfer methyl cations. This leaves the problem of remethylation of cob(II)alamin if the reaction is to be catalytic (it is not known whether cobalamin serves as a cofactor or a substrate in these enzymes). This could be accomplished by reductive methylation of cob(II)alamin by AdoMet, as has been established for the "repair" reaction of cobalamin-dependent methionine synthase when cob(I)alamin is inadvertently oxidized to cob(II)alamin.[31]

5.6 Radical-AdoMet enzymes in thiamine biosynthesis

The biosynthesis of thiamine pyrophosphate proceeds through an intriguing set of reactions that differ between bacteria, plants and fungi.[147] The 4-amino-5-hydroxymethyl-2-methylpyrimidine pyrophosphate (HMP-PP) and 5-hydroxyethyl-4-methylthiazole phosphate (THZ-P) moieties of thiamine are synthesized separately and coupled together to form the complete cofactor (Scheme 15). Two radical-AdoMet enzymes, ThiH[148–150] and ThiC,[28,29] are involved in the biosynthesis of thiamine. Interestingly, neither of these enzymes undergoes more than one turnover under in vitro conditions; unlike the sulfur-inserting enzymes, there is no reason to think that reaction involves destruction of the enzyme, and it has been suggested that severe product inhibition by 5′-dA might be responsible for this phenomenon.[151]



Scheme 15. Radical-AdoMet enzymes in thiamine pyrophosphate biosynthesis. Top: the structure of thiamine pyrophosphate; atoms derived from AIR are shown in blue, those derived from tyrosine are shown in red. Middle: mechanism for the formation of dehydroglycine catalyzed by ThiH. Bottom: the reaction catalyzed by ThiC; the color coding indicates where the atoms of the substrate appear in the product, as established by labeling studies.

In *E. coli*, under anaerobic conditions, ThiH, catalyzes the reaction of tyrosine and AdoMet to give dehydroglycine, 4-hydroxybenzyl alcohol, 5′dA and methionine, as shown in Scheme 15. The dehydroglycine formed in these reactions serve as one of the precursors for the THZ-P moiety.[148,149] The reaction is proposed to occur by fragmentation of a tyrosyl radical, initially generated by reaction with Ado•, to form a glycyl radical (Scheme 15), a mechanistic feature common to the rearrangement of glutamate catalyzed AdoCbl-dependent glutamate mutase.[81] The glycyl radical is further oxidized to dehydroglycine (the immediate electron acceptor is unknown), whereas the quinone methide initially formed reacts rapidly with water so that the final product is 4-hydroxybenzyl alcohol. ThiH functions as a complex with ThiG, the enzyme responsible for thiazole ring formation; this might help insure that dehydroglycine, which is unstable, is efficiently incorporated into THZ-P.

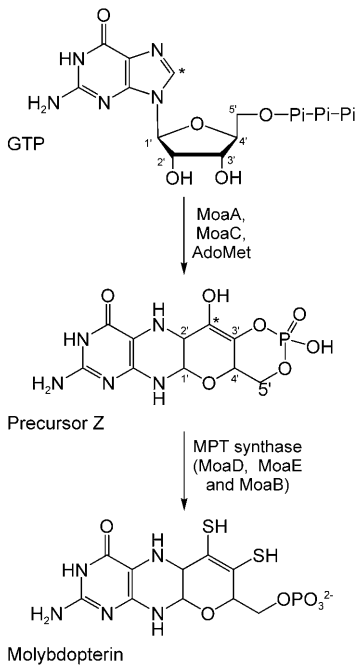
ThiC is involved in the synthesis of the HMP-PP precursor to thiamine pyrophosphate, and was recently demonstrated to be a radical-AdoMet enzyme.[29,152] The enzyme catalyzes the conversion of 5-aminoimidazole ribonucleotide (AIR) to HMP. As Scheme 15 illustrates, this is one of the most enigmatic enzymatic transformations known. Labeling studies established that the C-2′ carbon of the ribose is reduced to the methyl oxidation state and ends up as the methyl group at the C-2 position on the aminoimidazole, and that the C-4′ carbon of the ribose is inserted into the C-4=C-5 double bond of the aminoimidazole to generate the aminopyrimidine. The methyl hydrogens derive from the C-2′ hydrogen, the C-3′ hydrogen, and from the buffer. The fate of the two carbon atoms that are lost from AIR is currently unclear, as is the stoichiometry of the reaction with respect to AdoMet. About three molecules of AdoMet are consumed for each HMP synthesized, suggesting that more than one AdoMet molecule might be required for the reaction, but the extent to which uncoupling of 5′-dA formation occurs (a common problem in radical-AdoMet enzymes) is unclear. There is also an intriguing report that a protein-based radical is formed during the ThiC reaction.[152] An X-ray structure of ThiC has recently been solved, demonstrating that, like other radical-AdoMet enzymes, its core domain is a TIM barrel structure with high structural similarity to biotin synthase.[29] A full understanding of the mechanism of this unique reaction must await further studies.

6. Radical SAM Enzymes in Aerobic Organisms

Due to the oxygen lability of their unique [4Fe–4S] clusters and high reactivity of organic radicals towards dioxygen, the radical-AdoMet enzymes were once thought to be only present in anaerobic organisms. For example, the AdoCbl-dependent ribonucleotide reductase is found in both aerobic and anaerobic organisms; whereas glycyl-radical-dependent ribonucleotide reductase is only found in organisms growing under strictly anaerobic conditions.[153] This view has been challenged by recent discoveries that genes encoding some radical-AdoMet enzymes are present in both bacteria and aerobic eukaryotes, including humans, although as yet no radical-AdoMet enzymes have been biochemically characterized from aerobically growing organisms. Here we highlight four radical-AdoMet enzymes that appear to be involved in mammalian metabolism.

6.1 Molybdenum cofactor biosynthesis

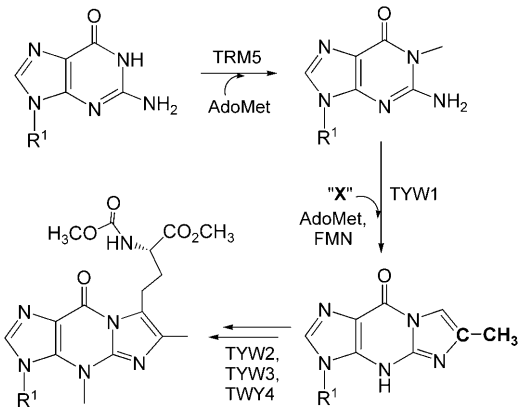
Molybdenum is an essential trace metal that is bound as molybdopterin. In humans, disruption of the molybdopterin biosynthetic pathway leads to pleiotropic loss of molybdoenzymes, and afflicted patients usually die shortly after birth. In the first step, shown in Scheme 16, of the biosynthesis of molybdopterin, GTP is converted to precursor Z through the action of two enzymes MoaA (MOCS1A in humans), which is a radical-AdoMet enzyme, and MoaC.[43,154,155] Biochemical and X-ray crystallographic studies have demonstrated the presence of two [4Fe–4S] clusters in MoaA.[43] The N-terminal cluster binds AdoMet whereas the C-terminal cluster is the GTP binding site. Substrate binding is proposed to bring the ribose moiety into the proximity of Ado• generated through reductive cleavage of AdoMet. However, our understanding of the mechanism by which the enzyme catalyzes this very unusual carbon skeleton rearrangement (which has some general similarity to the ThiC reaction) is minimal; it is unclear whether hydrogen abstraction occurs at either C-2′ or C-3′ of the ribose moiety or at guanine C-8.[155] Nevertheless, it appears highly likely that both prokaryotes and eukaryotes use similar versions of the enzyme that requires radical-AdoMet chemistry.



Scheme 16. The conversion of GTP to precursor Z catalyzed by MoaA and MoaC in a radical-AdoMet-dependent reaction. Precursor Z is then converted to molybdopterin through subsequent reactions catalyzed by MoaB, MoaD, and MoaE.

6.2 Wybutosine biosynthesis and TYW1

TWY1 is a radical-AdoMet enzyme in the modification of eukaryotic phenylalanine tRNA as part of the wybutosine (yW) biosynthetic pathway (Scheme 17).[156] yW is a tricyclic nucleoside found at the 3′-position adjacent to the anticodon of eukaryotic $tRNA^{phe}$. It is believed to enhance codon–anticodon recognition through an increased stacking interaction due to the tricyclic aromatic ring. Its absence might result in increased frame-shifting during translation.[157] yW is missing from $tRNA^{phe}$ in HIV-infected cells; the resulting enhanced frame-shifting is utilized by the HIV virus to synthesize the virus Gag-Pol polyprotein.[158] The Pol protein is the precursor for a number of critical HIV enzymes, including the reverse transcriptase that is essential for the virus activity.[159–161]



Scheme 17. The biosynthetic pathway for wybutosine in $tRNA^{phe}$. The addition of the two-carbon fragment shown in bold in the second step of the pathway is catalyzed by TWY1; the identity of the precursor to these carbons, "X", is currently unknown.

TYW1 catalyzes the formation of the third ring in yW through the reaction of N^1-methylguanine with an unknown substrate "X".[156] Recently, the crystal structures of two archaeal TYW1 homologues were solved.[162,163] Similar to MoaA, TYW1 was found to contain two iron–sulfur clusters. One cluster is the AdoMet-coordinating [4Fe–4S] cluster; however, the nature of the second cluster is uncertain. In one structure it is a [4Fe–4S] cluster, whereas in the other it is a [2Fe–2S] cluster. Despite the structural information, our understanding of this enzyme is at a rudimentary stage, and mechanistic studies are needed to uncover the mechanism of this unusual nucleotide modification.

6.3 Elp3 subunit

Another candidate for a eukaryotic radical-AdoMet protein is the Elp3 subunit, which is one of six subunits of the elongator complex that forms part of the eukaryotic transcription machinery.[164,165] Elp3 contains a well-documented C-terminal histone acetyltransferase (HAT) domain,[166] but in addition to this, a second putative AdoMet domain was recently proposed in this protein.[27] Biochemical studies of an archaeal homologue of Elp3 (from *Methanocaldococcus jannaschii*) confirmed that the CX_4CX_2C motif harbors a [4Fe–4S] cluster and that the protein is able to reductively cleave AdoMet to generate the 5′-deoxyadenosine in the presence of dithionite.[27] These results suggest that Elp3 could be a radical-AdoMet enzyme, even though the biological function of the radical chemistry is unclear.

Elp3 is well conserved from archaea to humans with more than 40% identity between archaeal and eukaryotic sequences, thus the eukaryotic Elp3s are likely to be radical-AdoMet enzymes as well. This raises an intriguing question regarding the sequence motif used to identify potential radical-AdoMet enzymes. The bacterial Elp3 sequences (e.g., *E. coli*, *Bacillus subtilis*, *Clostridium tetani*) contain the established AdoMet CX_3CX_2C motif; whereas the archaeal enzyme has a CX_4CX_2C motif. As revealed by X-ray structural studies, the CX_3CX_2C motif is often located on a flexible loop that should readily accommodate one more amino acid without causing dramatic structural change. Therefore, it is not unreasonable that CX_4CX_2C motif also initiates radical-AdoMet chemistry, as is the case for ThiC.[29] However, eukaryotic (*S. cerevisiae*, *S. pombe*, and human) Elp3s contain a CX_9CX_2C sequence.[27] This suggests that there might be considerable flexibility in the spacing between at least the first two cysteines of the tricysteinyl motif that provide the protein ligands to the [4Fe–4S] cluster and that the potential family of radical-AdoMet enzymes might be even larger than currently thought.

6.4 Viperin

A tentatively identified member of the human radical-AdoMet enzymes is a cellular protein named viperin, discovered

through studies on hepatitis C virus (HCV) infection.[167] Viperin can also be induced by other viruses such as human cytomegalovirus, VSV, dengue virus, yellow fever virus, human polyomavirus JC, and HCV,[167–170] suggesting it is part of a general response to viral infection. HCV infection is a common cause of chronic hepatitis and is currently treated with alpha interferon (IFN-α)-based therapies. Among the 29 interferon-stimulated genes (ISGs) induced by IFN-α, viperin was found to inhibit the replication of HCV through an unknown reaction mechanism. Sequence analysis showed that the protein contains a CX_3CX_2C motif, and mutation of any cysteine within this motif abolishes the protein's antiviral activity. These results have lead to the suggestion that viperin could be a radical-AdoMet enzyme. However, its possible substrates and mode of action currently remain completely unknown.

7. The Problem of Oxygen Sensitivity and Solutions to It

All the radical-AdoMet enzymes so far characterized are extremely oxygen sensitive, primarily because the partially exposed [4Fe–4S] cluster is particularly unstable and easily oxidized. Thus the discovery of genes encoding radical-AdoMet enzymes in aerobic organisms was surprising and suggests that Nature must have adopted some special strategies to allow radical-AdoMet enzymes to function in aerobically respiring cells. Although not much is known at this point on radical-AdoMet enzymes, investigations on the reaction of other iron–sulfur proteins with oxygen have provided some insight into the unusual stability of iron-sulfur proteins in aerobic cells.

7.1. Redox properties of reactive oxygen species (ROS)

O_2 exists predominantly in the triplet state, which is relatively inert. The reduction potential for reducing O_2 to superoxide (O_2^-) is -330 mV,[171] which is quite unfavorable but higher than the reduction potentials of the $[4Fe–4S]^{2+}$ clusters in radical-AdoMet enzymes ($E^\circ = -430$ to -500 mV).[61,126,172] Therefore the reduced $[4Fe–4S]^{+}$ clusters in radical-AdoMet enzymes can be oxidized by O_2 and thus return to their inactive $[4Fe–4S]^{2+}$ state. Although this reaction results in uncoupling of the formation of Ado$^\bullet$ from the consumption of the physiological reductant, it does not destroy the cluster and irreversibly inactivate the enzyme.

O_2 could effect further one-electron oxidation to generate the $[4Fe–4S]^{3+}$ cluster, which is very unstable and readily decomposes to a $[3Fe–4S]^{+}$ cluster, releasing a Fe^{2+} ion [Eq. (1)].

$$[Fe_4S_4]^{2+} + O_2 \rightarrow [Fe_3S_4]^{+} + O_2^- + Fe^{2+} \quad (1)$$

Indeed, such a reaction is used by various transcription factors, such as SoxR and FNR (fumarate and nitrate reduction regulatory protein), that sense cellular redox status.[173] FNR in *E. coli* controls the expression of more than 100 genes, particularly those that function in anaerobic respiration.[174] In an O_2-limited environment, FNR binds a $[4Fe–4S]^{2+}$ cluster and promotes the formation of a transcriptionally active dimer. Upon exposure to O_2, it is converted into the non-DNA-binding monomer form with the cluster degraded to a $[2Fe–2S]^{2+}$ state via a $[3Fe–4S]^{+}$ intermediate.[175,176] This oxidation reaction is relatively slow, with a second-order rate constant of $\approx 300\ M^{-1}s^{-1}$ at 25 °C.[177]

In contrast, the reactions of superoxide and hydrogen peroxide with iron–sulfur clusters are much faster and thus present much more of a problem. As shown in Eqs. (2) and (3),

$$[Fe_4S_4]^{2+} + O_2^{\bullet -} + 2H^+ \rightarrow [Fe_3S_4]^{+} + H_2O_2 + Fe^{2+} \quad (2)$$

$$[Fe_4S_4]^{2+} + H_2O_2 + H^+ \rightarrow [Fe_3S_4]^{+} + H_2O + Fe^{2+} + OH^\bullet \quad (3)$$

these reactions are proton-coupled electron-transfer processes. The high reduction potentials of these oxidants mean that the reactions are thermodynamically favored, and both species are capable of irreversibly oxidizing $[4Fe–4S]^{2+}$ clusters to $[3Fe–4S]^{+}$ clusters, releasing Fe^{2+}.

Kinetically, these reactions are favored as well. For example, at 25 °C superoxide oxidizes the [4Fe–4S] clusters in *E. coli* fumarase A, fumarase B, and mammalian aconitase very rapidly, $k_2 = 10^6$ to $10^7\ M^{-1}s^{-1}$[178] and H_2O_2 destroys the [4Fe–4S] cluster in *E. coli* dehydratases with a rate constant of $0.5–1 \times 10^5\ M^{-1}s^{-1}$.[179] Similarly rapid oxidation of the clusters in radical-AdoMet enzymes would certainly occur in vivo if $O_2^{\bullet -}$ and H_2O_2 are not removed quickly.

7.2. Potential strategies to combat oxidative degradation of radical-AdoMet enzymes

7.2.1. Blocking O_2 access to the [4Fe–4S] cluster: As described above, molecular oxygen itself is not very reactive. It appears to require a direct interaction with the [4Fe–4S] cluster before electron transfer occurs. Consequently, blocking O_2 access could greatly improve the cluster stability. For example, in *E. coli* FNR Cys-23, or the iron atom it ligates, is suggested to be a primary target of O_2. Mutating the adjacent Ser24 to a bulky phenylalanine residue partially blocked O_2 access and, consequently, the oxidation of the $[4Fe–4S]^{2+}$ cluster to the $[3Fe–4S]^{+}$ cluster was slowed down by four to five-fold.[180]

A similar strategy might be adopted by Nature to protect the [4Fe–4S] clusters in radical-AdoMet enzymes. The open coordination site of a [4Fe–4S] cluster is even more vulnerable to oxidation by reactive oxygen species in these enzymes. Not surprisingly, purification in air always leads to the formation of $[3Fe–4S]^{+}$ or $[2Fe–2S]^{2+}$ clusters in these enzymes. However, a recent Mossbauer study on pyruvate formate-lyase activase found that after a two-hour overexpression in *E. coli* under aerobic conditions, ~44% of the iron in the cells was in the [4Fe–4S] cluster form, although cluster decomposition products including [2Fe–2S] clusters and noncluster Fe^{2+} and Fe^{3+} ions also existed.[181] The existence of such large amounts of $[4Fe–4S]^{2+}$ in the whole cells is surprising. It was speculated that the cluster might be protected against oxidative damage by coordination of a small molecule, possibly AMP.

7.2.2. Rapid removal of $O_2^{\cdot-}$ and H_2O_2: To combat the more reactive species, $O_2^{\cdot-}$ or H_2O_2, all aerobic organisms maintain high concentrations of superoxide dismutase, which catalyzes the disproportionation of ($O_2^{\cdot-}$) to O_2 and H_2O_2, catalase, which catalyzes the further disproportionation of H_2O_2 to O_2 and H_2O, and peroxidases, which reduces H_2O_2 to water. The importance of these scavenging enzymes is illustrated by the fact that *E. coli* strains devoid of cytoplasmic superoxide dismutase showed a variety of growth defects derived from endogenous ($O_2^{\cdot-}$) under aerobic conditions,[182,183] whereas catalase or peroxidase mutants die rapidly due to the accumulation of micromolar levels of H_2O_2 inside the cell.[179,184]

7.2.3. Specialized cluster assembly pathways: The in vivo Fe–S cluster assembly pathways might also contribute to the ability of radical-AdoMet enzymes to function under aerobic conditions. In vitro, [4Fe–4S] clusters can be regenerated by incubation of enzymes with ferrous iron and sulfide in the presence of a reductant, such as dithiothreitol. In vivo, however, Fe–S cluster biosynthesis requires a surprisingly large number of assembly enzymes.[185–189] To date, three bacterial assembly pathways, namely, the Isc (iron–sulfur cluster), Suf (sulfur formation), and Nif (nitrogen fixation) have been identified.[187,190,191] However, the phylogenetic distribution of these systems is complex. For instance, *E. coli* strains contain both Isc and Suf pathways; whereas organisms such as *Mycobacterium tuberculosis* as well as some archaea only possess the Suf pathway.[187] Nif is dedicated to cluster maturation of nitrogenase in nitrogen-fixing bacteria[192,193] and thus is unlikely to be involved in cluster biosynthesis in radical-AdoMet enzymes.

Isc and Suf pathways can function under both aerobic and anaerobic conditions, although in the latter case the expression of both operons is repressed.[194] This suggests that damage and subsequent turnover of Fe–S clusters during oxygenic growth is responsible for the bulk of the demand for the de novo Fe–S cluster biosynthesis.[187] In *E. coli*, Isc is the housekeeping Fe–S cluster assembly pathway and is likely responsible for all de novo Fe–S cluster biosynthesis under normal growth conditions. Indeed, in vitro the IscA protein was shown to transfer preassembled Fe–S clusters to biotin synthase[195] and coexpression of a plasmid encoding the *Isc* operon from *E. coli* increases the yield of holo-lipoate synthase and ThiC by three–four times.[29,121,196] As discussed below, some Isc homologues appear to be dedicated to radical-AdoMet protein bioassembly in eukaryotes.[186,187,191,197]

The Suf pathway could also contribute to the de novo cluster biosynthesis in radical-AdoMet enzymes, as demonstrated by in vitro studies that showed SufA protein can insert a [4Fe–4S] cluster into biotin synthase.[188,195,198] Although the Suf pathway plays a minor role in *E. coli* under normal grow conditions, it becomes critical in Fe–S bio-assembly during oxidative stress.[199,200] Thus after deletion of both *Isc* and *Suf* genes, *E. coli* strains supplemented with *Suf* genes grew as well as wild-type bacteria upon exposure to H_2O_2,[187,201] whereas strains supplemented with *Isc* genes showed significant growth defects. Thus the Suf pathway could be important for maintaining the Fe–S clusters in radical-AdoMet enzymes during oxidative stress, although no detailed studies have been carried out to test this hypothesis.

Both Isc and Suf pathways are conserved in eukaryotic cells, although the latter is only present in plastids.[202] Isc is found in mitochondria and is involved in the biogenesis of virtually all cellular Fe–S proteins. Even for the cytosolic Fe–S proteins, a currently unknown precursor "X" is exported from the mitochondria to the cytosol via the mitochondrial ISC export apparatus before the cluster is transferred by the cytosolic Fe–S protein assembly machinery into the holo protein.[185,186,203] Recently, a novel member of the mitochondrial ISC assembly system, Iba57p (also termed Caf17), was discovered. By interacting with Isc proteins Isa1 and Isa2, it specifically incorporates Fe–S clusters into aconitase and homoaconitase as well as the radical-AdoMet proteins biotin synthase and lipoic acid synthase.[186,197] Deletion of Iba57p affects only these four proteins in yeast mitochondria; this suggests that Iba57p is a dedicated maturation factor for aconitase and radical-AdoMet enzymes. As Iba57p, Isa1, and Isa2 are conserved in most eukaryotes, as well as some bacterial strains, this Iba57/Isa complex might represent a universal Fe–S assembly machinery specifically for radical-AdoMet enzymes.

7.2.4. In vivo cluster reassembly after oxidative stress is removed: The mechanisms discussed above represent potential protective strategies that Nature may adopt to stabilize the radical-AdoMet enzymes. Additionally, efficient cluster repair mechanisms might also mitigate oxidative stress. For example, when cellular oxidative stress is removed, the intracellular activities of aconitase and 6-phosphogluconate dehydratase, both of which contain oxygen-sensitive Fe–S clusters, rebound to their initial levels in 3–5 min, even when protein synthesis is blocked, implying there is a rapid in vivo protein repair mechanism.[182,204,205] Similarly, as discussed above, exposure of *E. coli* FNR protein to oxygen converts the FNR[4Fe–4S]$^{2+}$ cluster to a [2Fe–2S]$^{2+}$ cluster, a process that decreases DNA binding by FNR. However, once *E. coli* is switched back into an anaerobic environment, a significant amount of [4Fe–4S]$^{2+}$ cluster is regenerated in the cell as observed by Mossbauer spectroscopy.[206] A similar observation was made for PFL activase in *E. coli*. Prolonged incubation of cells containing the overexpressed protein under N_2 gas converted all the [2Fe–2S] clusters and noncluster Fe back to the [4Fe–4S] cluster form.[181] We speculate that the Fe–S assembly machinery might play a role in the cluster regeneration/repair processes as well, however, the mechanistic details remain unknown.

Overall, although no radical-AdoMet enzymes have been biochemically characterized from higher eukaryotes, there is strong evidence that these enzymes are active in aerobic organisms, including ourselves! It is likely that evolutionary pressure has selected for eukaryotic radical-AdoMet enzymes that are less oxygen sensitive than the bacterial enzymes so far characterized. It seems plausible that this could be achieved by a combination of effective scavenging of *intra*-cellular superoxide and hydrogen peroxide, an efficient Fe–S cluster assembly and repair machinery, and protection by the protein of the O_2-labile site.

8. Summary and Outlook

The adenosyl radical serves two biochemical functions: as a reactive catalyst of radical-mediated rearrangement and elimination reactions, and as a powerful one-electron oxidant. AdoCbl-dependent enzymes use Ado$^{\bullet}$ exclusively as a catalyst whereas radical-AdoMet enzymes use Ado$^{\bullet}$ mainly as an oxidant. The scope of the reactions catalyzed by AdoCbl-dependent enzymes is quite narrow and is found primarily in metabolic pathways concerned with bacterial fermentation of various carbon sources. In contrast, radical-AdoMet enzymes are seen to catalyze an increasingly wide range of oxidative reactions in secondary metabolic pathways, many of which involve the biosynthesis of enzyme cofactors.

The challenges for this field include elucidating the mechanisms of these newly discovered radical-AdoMet enzymes, some of which, for example ThiC, catalyze astonishingly complex transformations. The prospect that radical-AdoMet enzymes function in animals is exciting; the challenge here will be to definitively show that any of these putative radical-AdoMet enzyme are both expressed and active in higher eukaryotes. One final question is whether there are other AdoCbl-dependent reactions remaining to be discovered. The availability of genome sequences and bioinformatic techniques were pivotal in uncovering new radical-AdoMet reactions. Will similar analyses using the known AdoCbl enzyme sequences identify new members of this select group of enzymes?

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