S-Adenosylmethionine: A Wolf in Sheep's Clothing, or a Rich Man's Adenosylcobalamin?

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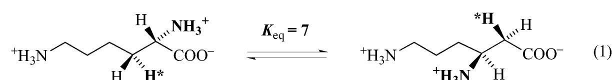
I. Introduction

S-Adenosylmethionine (SAM) has long been known as the biological methylating agent, because it serves as the methyl group donor in methylations of DNA, hormones, neurotransmitters, and signal transduction systems. Methylation of nucleophiles is an intrinsic chemical property of SAM as a sulfonium ion that has been exploited in biology by the action of enzymes that bring SAM into contact with nucleophilic groups of substrates. As such, methylation by SAM may be regarded as chemically well precedented.

Recently, however, a new class of enzymatic reactions of SAM has come to light that in the beginning were unprecedented and far more chemically intriguing and novel than methylation. SAM appeared to be initiating radical-based reactions at the active sites of enzymes, much in the way that adenosylcobalamin initiates radical chemistry.[1,2] In these reactions, SAM appeared to be, in the time-worn metaphor, "a wolf in sheep's clothing". In the reaction of lysine 2,3-aminomutase, SAM seemed to play the role of adenosylcobalamin in the coenzyme B_{12}-dependent rearrangements, and for this reason it became known by a then new metaphor as "a poor man's adenosylcobalamin".[3,4] Today, SAM is recognized as the initiator of a greater diversity of radical reactions than adenosylcobalamin itself. Like adenosylcobalamin, SAM initiates difficult rearrangements involving the internal transfer of unreactive hydrogen atoms, and like adenosylcobalamin, it initiates the reduction of ribonucleotides. Unlike adenosylcobalamin, however, SAM also initiates the repair of DNA, the insertion of sulfur into unreactive C−H bonds, and the introduction of glycyl radicals into the polypeptide chains of a class of enzymes that use this radical to catalyze a variety of reactions. Thus, SAM has a greater scope than adenosylcobalamin in catalyzing radical reactions at enzymatic sites. For this reason, we now propose still another metaphor for SAM as a radical initiator, "a rich man's adenosylcobalamin".

In the late 1960s and early 1970s, H. A. Barker studied the metabolism of lysine by *Clostridium* species and discovered a novel enzyme, which he named lysine 2,3-aminomutase.[5] This enzyme catalyzed the first step in the metabolism of lysine to acetate.[6,7] The reaction itself was novel, the interconversion of L-lysine and L-β-lysine according to eq 1.



Another novel aspect was that enzyme activity required coenzymes that had never before been

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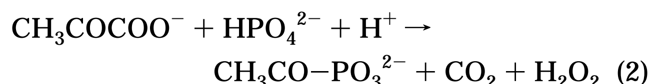
Perry Allen Frey was born in Plain City, Ohio, on November 14, 1935. After graduation from high school, he enrolled in The Ohio State University to study chemistry. After his first year at the university, he was drafted into the U.S. Army for two years. He returned to Ohio State in 1956 and graduated with a B.Sc. degree in chemistry in 1959. He worked as a chemist at the Sanitary Engineering Center in Cincinnati, Ohio, for four years and at the same time studied chemistry at the University of Cincinnati. He received his Ph.D. degree in biochemistry in 1968 from Brandeis University and studied as a postdoctoral fellow under Professor Frank H. Westheimer at Harvard University. In 1969, he became an Assistant Professor of Chemistry at The Ohio State University, where he rose to the rank of Professor of Chemistry in1979. In 1981, he moved to the University of Wisconsin–Madison and was named the Robert H. Abeles Professor of Biochemistry in 1993. His research interests are centered on the mechanisms of action of enzymes and coenzymes.

Olafur Th. Magnusson was born in Hafnarfjordur, Iceland, in 1970. He received his B.Sc. in biochemistry at the University of Iceland in 1995. After studying the cold-adaptation of serine proteases at the Science Institute in Reykjavik, Iceland, with Prof. M. M. Kristjansson, he moved to the University of Wisconsin–Madison in 1996, where he did his Ph.D. studies with Prof. Perry A. Frey. His thesis work concerns the characterization of radical intermediates in the SAM and B_{12} enzymes lysine 2,3-aminomutase and diol dehydrase, respectively. Olafur is now a Miller Fellow at the University of California at Berkeley, where he works with Prof. Judith P. Klinman toward the elucidation of the biosynthesis of pyrroloquinoline quinone (PQQ). His other interests include hiking, mountain climbing, and playing soccer with his 2-year-old son.

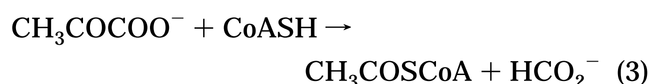
associated with enzyme catalysis of an isomerization reaction. The interconversion of α- and β-lysine was analogous to coenzyme B_{12}-dependent rearrangements, but it did not require adenosylcobalamin or any other corrinoid for activity. Lysine 2,3-aminomutase displayed activity only under anaerobic conditions, it contained pyridoxal-5′-phosphate (PLP) and iron, and addition of ferrous ions and PLP increased its activity. The enzyme absolutely required the addition of SAM to catalyze the reaction. This discovery ushered SAM into the world of novel enzymatic rearrangements that later came to be recognized as radical-based reactions. It took 17 years for Barker's original finding to be appreciated as a quantum leap in the field of radical enzymology.[2,8]

In the late 1960s and early 1970s, research on the metabolism of pyruvate led to the recognition of "clastic" reactions. The phosphoroclastic reaction of pyruvate required phosphate and led to acetyl phosphate, hydrogen peroxide, and carbon dioxide (eq 2).



$$CH_3COCOO^- + HPO_4^{2-} + H^+ \rightarrow CH_3CO{-}PO_3^{2-} + CO_2 + H_2O_2 \quad (2)$$

The enzyme is pyruvate oxidase [pyruvate:oxygen 2-oxidoreductase (phosphorylating) EC 1.2.3.3], a flavoprotein that requires thiamine pyrophosphate (TPP) as a coenzyme. In a distinct process, briefly known as the "thioclastic" reaction (eq 3), pyruvate was cleaved by coenzyme A (CoA) to form acetyl-CoA and formate.[9] This enzyme was difficult to assay and characterize. In cellular extracts it seemed to be activated by TPP and SAM. Because the carboxyl group was removed from pyruvate, it seemed natural that TPP would be required, as it was for pyruvate decarboxylase, pyruvate dehydrogenase, and many pyruvate oxidoreductases. However, purification studies showed that the effect of TPP was on another protein that seemed to increase the activity of the enzyme catalyzing reaction 3, and this effect could be replaced by the imposition of strict anaerobic conditions and the addition of Co^{2+} and a reducing agent.[10] Thus, the effect of TPP appeared to be to bring about anaerobic conditions through the action of a TPP-dependent enzyme in the extract. The purified enzyme catalyzing reaction 3 was named pyruvate formate-lyase [EC 2.3.1.54], and its activation required a second protein, SAM, ferrous ion, and a reducing agent. The second protein was eventually found to be a SAM-dependent activase that introduced a radical site into pyruvate formate-lyase.[1]



$$CH_3COCOO^- + CoASH \rightarrow CH_3COSCoA + HCO_2^- \quad (3)$$

II. SAM and Catalysis of Radical Reactions

A. Lysine 2,3-Aminomutase (LAM)

1. Molecular Properties

Lysine 2,3-aminomutase from *Clostridium subterminale SB4* is a hexamer of identical subunits with an overall molecular weight of 280 000 and a subunit molecular weight of 47 000.[5,11,12] Lysine 2,3-aminomutase from *Bacillus subtilis* is a tetramer of identical subunits with an overall molecular weight of 220 000 and a subunit molecular weight of 55 000.[13] The coenzyme requirements are the same for both enzymes, and the amino acid sequences are very similar, about 68% identical. The *Clostridium* enzyme is about 100 times as active as the *Bacillus* enzyme. Both enzymes function under anaerobic

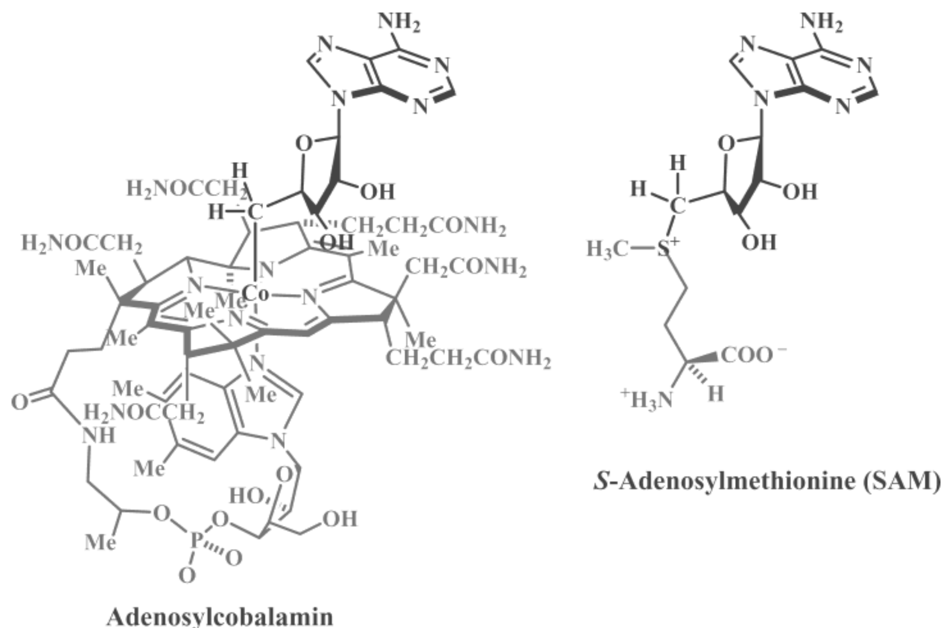


Figure 1. Structural comparison of adenosylcobalamin with *S*-adenosylmethionine.

conditions; however, the *Clostridium* enzyme is more sensitive to oxygen than the *Bacillus* enzyme.

2. The Radical Mechanism

The reaction catalyzed by lysine 2,3-aminomutase follows the pattern of adenosylcobalamin-dependent enzymatic reactions in that it proceeds as a 1,2-migration of an unreactive hydrogen atom concomitant with the reverse migration of a group on the adjacent carbon, in this case the 2-amino group. The hydrogen and amino group do not exactly exchange positions because their migrations proceed with inversion of configuration, as shown in eq 1.[14] The movement of hydrogen from C2 to C3 proceeds without exchange with protons of solvent water, so hydrogen in the substrate is retained in the product. However, the reaction mechanism allows for both intermolecular and intramolecular hydrogen transfer.[8] These facts are exactly as expected for an adenosylcobalamin-dependent rearrangement reaction.

The fact that adenosylcobalamin plays no role in the action of lysine 2,3-aminomutase led to a search for another coenzyme that would mediate hydrogen transfer in place of the vitamin B_{12} coenzyme. SAM incorporates an adenosyl moiety in common with adenosylcobalamin, as illustrated in Figure 1. SAM and adenosylcobalamin have little else in common in terms of chemical properties. Nevertheless, the possible role of the adenosyl moiety of SAM in catalysis of hydrogen transfer was investigated. The classic demonstration of hydrogen transfer by adenosylcobalamin involves conducting the reaction with [*adenosyl*-5′-^{3}H]adenosylcobalamin and determining whether tritium appears in the product and substrate.[15] The corresponding experiment with lysine 2,3-aminomutase and [*adenosyl*-5′-^{3}H]SAM resulted in the appearance of tritium in both L-lysine and L-β-lysine at equilibrium.[2] All of the tritium associated with enzyme-bound [*adenosyl*-5′-^{3}H]SAM was transferred to the substrate and product, and excess SAM in solution did not participate in this process.[8] These experiments prove that the adenosyl moiety of SAM mediates hydrogen transfer in the lysine 2,3-aminomutase reaction, just as adenosylcobalamin does in coenzyme B_{12}-dependent enzymatic processes. Because of the many chemical differences between SAM and adenosylcobalamin, fundamentally different chemistry must underlie the identical function of the adenosyl moieties in these coenzymes. We shall discuss the chemical differences in due course.

The similarities in the action of SAM in lysine 2,3-aminomutase and adenosylcobalamin in B_{12}-dependent enzymes suggested that the mechanism of the interconversion of lysine and β-lysine would have elements in common with B_{12}-dependent reactions. Inasmuch as adenosylcobalamin initiates radical mechanisms by serving as a source of the 5′-deoxyadenosyl radical,[16−21] it seemed that SAM might be the source of this same radical in the action of lysine 2,3-aminomutase. This hypothesis presented significant questions about the chemistry by which SAM would be cleaved to a radical. Moreover, the presence and apparent involvement of PLP introduced a further complication. PLP was known to stabilize carbanions and had never before been implicated in a radical mechanism.

Deferring the problem of the chemistry for SAM cleavage, it proved possible to formulate a hypothetical radical mechanism that explained the roles of both SAM and PLP.[2] This mechanism is shown in Figure 2, in which the chemistry for the cleavage of SAM to the 5′-deoxyadenosyl radical is not specified, and all intermediates remain bound to the active site throughout the catalytic cycle. In the working hypothesis, lysine is bound at the active site through its α-amino group as a conventional external aldimine to PLP. Abstraction of the 3-*pro-R* hydrogen from the lysyl side chain by the 5′-deoxyadenosyl radical leads to the substrate radical **1** and 5′-deoxyadenosine. Radical **1** undergoes a rearrange-

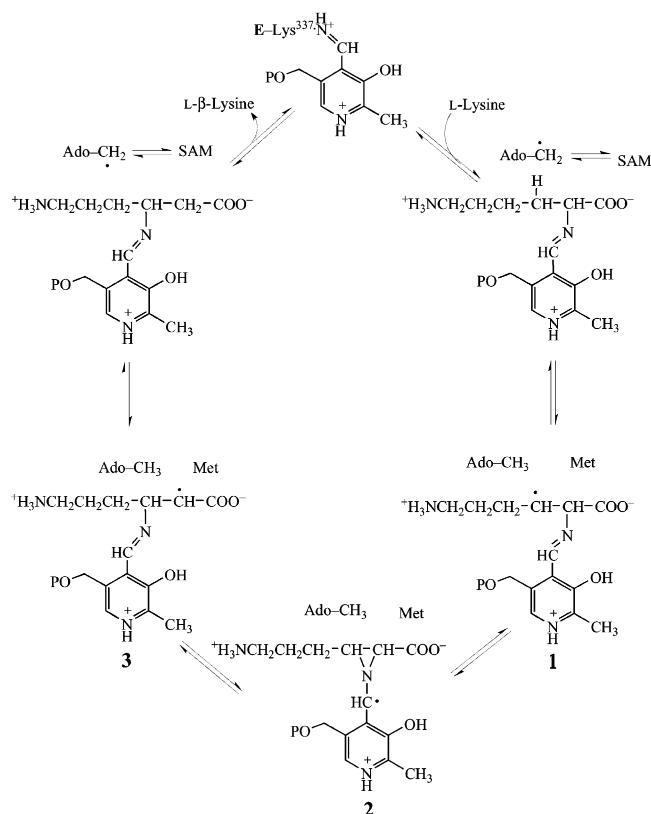


Figure 2. Radical mechanism of lysine 2,3-aminomutase.

ment to the product radical **3** in two steps. Pairing of the unpaired electron in **1** with a π-electron of the imine bond forms a cyclic intermediate radical **2**, an azacyclopropylcarbinyl radical, in which the unpaired electron resides on the aldehydic C4′ of PLP. This is a quasi-symmetric species that can react with cleavage of either C−N bond in the aziridine ring. In the forward direction, the C2−N bond is cleaved to form the product radical **3**, in which the unpaired electron resides on C2 of the lysyl side chain. Abstraction of a hydrogen atom from the methyl group of 5′-deoxyadenosine by radical **3** produces the external PLP aldimine of β-lysine and regenerates the 5′-deoxyadenosyl radical.

The mechanism in Figure 2 has survived critical tests of its validity and may be accepted as proven in principle. Radical **3**, the product radical, was identified and characterized spectroscopically and kinetically as the predominant radical intermediate in the reaction of L-lysine.[22−25] The primary tools were electron paramagnetic resonance (EPR) and electron spin−echo envelope modulation (ESEEM) spectroscopy of reaction mixtures frozen at 77 K in the initial steady-state of the conversion of L-lysine to L-β-lysine. Rapid mix−freeze quench EPR validated its kinetic competence. Radical **3** is the only radical stable enough to be observed by EPR when L-lysine is the substrate. Its stability can be attributed to delocalization of the unpaired electron by the carboxyl group.

In the reaction of an alternative substrate, 4-thia-L-lysine, the analogue of the substrate radical **1** was identified by the same methods employed to characterize radical **3** in the reaction of L-lysine. The alternative substrate was employed for this purpose because in its reaction the radical **1** analogue was stabilized by the adjacent 4-thia group, making it the most stable radical intermediate in the steady state.[26,27] The key to the identification of the analogue of radical **1** is stabilization of the unpaired electron on C3 by the delocalizing effect of the nonbonding electrons in the 4-thia group of 4-thia-L-lysine. As a substrate, 4-thia-L-lysine reacts with a value of k_{cat} about 1% of that for L-lysine. The two react with comparable values of K_m. The reaction of 4-thia-L-lysine is practically irreversible because the rearrangement product is unstable and spontaneously decomposes to formyl acetate and β-mercaptoethylamine.

The substrate analogue *trans*-4,5-dehydro-L-lysine (*trans*-4-dehydrolysine) provides another means of stabilizing the unpaired electron on C3. *trans*-4-Dehydrolysine reacts in place of L-lysine as a suicide substrate to form the allylic analogue of radical **1** in Figure 2 at the active site.[28] The allylic analogue of radical **1** is too stable to abstract a hydrogen atom from 5′-deoxyadenosine, and so the reaction is halted at the stage of the allylic analogue of radical **1** in the mechanism of Figure 2. This transformation into the complex of the allylic analogue of radical **1** has proven to be useful in studies of the mechanism of SAM cleavage (see below).

The 5′-deoxyadenosyl radical in Figure 2 is too high in energy to exist in the active site of an enzyme at concentrations detectable by EPR spectroscopy. However, the unsaturated analogue, 3′,4′-anhydro-5′-deoxyadenosin-5′-yl (anhydroadenosyl radical) is an allylic radical that is stable enough to be observed by EPR spectroscopy. The corresponding SAM analogue, 3′,4′-anhydroadenosylmethionine (*an*SAM), activates lysine 2,3-aminomutase to about 0.25% of the activity observed with SAM.[29,30] The anhydroadenosyl radical is the predominant radical in the steady state of the reaction when *an*SAM activates lysine 2,3-aminomutase.

In the characterization of radical intermediates by EPR spectroscopy, the primary tools for structural analysis are isotopically labeled substrates with specifically placed heavy isotopes. In the case of the anhydroadenosyl radical, deuterium and carbon-13 labeling of *an*SAM led to spectra that characterized the anhydroadenosyl radical. Deuterium labeling was particularly effective for this purpose. The nuclear hyperfine splitting constant for a deuterium nucleus spin-coupled to a π-radical is about one-sixth of that for a hydrogen nucleus.[31] Consequently, deuterium substitution typically narrows and simplifies the EPR spectra of organic radicals. Figure 3 illustrates this effect in the characterization of the anhydroadenosyl radical at the active site of lysine 2,3-aminomutase. Each deuterium substitution around the ribose ring decreases the apparent number of splittings in the spectrum, proving that the unpaired electron resides on the ribose ring. The simulations prove that the spin is localized approximately equally on C5′ and C3′, as expected for the allylic radical. The simulations further show that the dihedral angle between the axis of the radical orbital and the C2′(H) is 35°, fixing the conformation of the anhydroribose ring.[30]

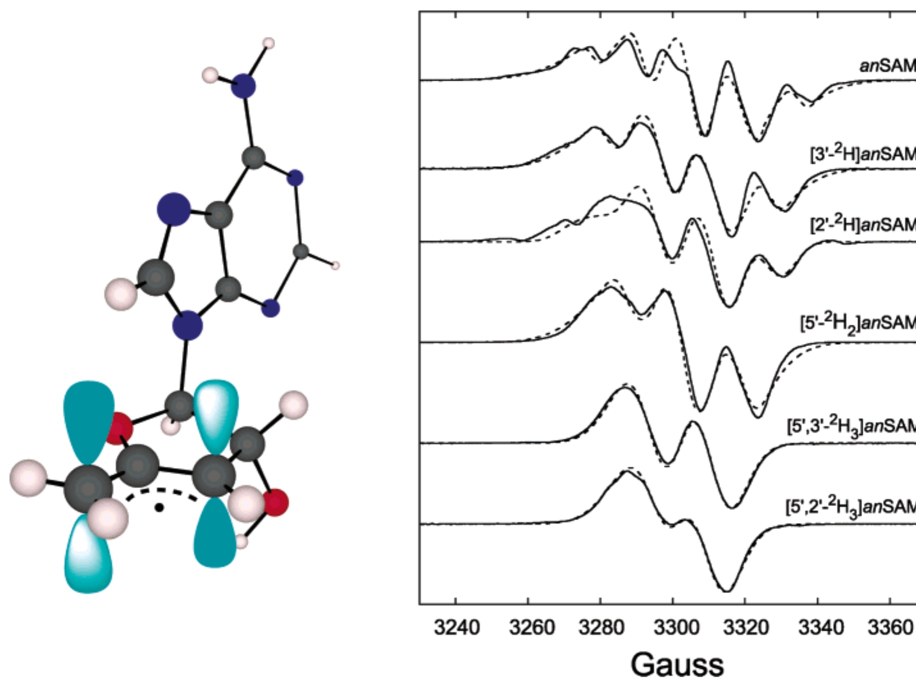


Figure 3. EPR spectroscopic characterization of the anhydroadenosyl radical at the active site of lysine 2,3-aminomutase. Solid lines are the experimental spectra. Dashed lines are computer-simulated spectra.

Table 1. Oxidation States of the Iron−Sulfide Center in Lysine 2,3-Aminomutase

oxidation state	EPR (*g* value)	origin/significance
$[4Fe\text{-}4S]^{3+}$	2.007	minor component of purified enzyme; not functional in catalysis
$[3Fe\text{-}4S]^{+}$	2.015	oxidation product of $[4Fe\text{-}4S]^{3+}$; not functional in catalysis
$[4Fe\text{-}4S]^{2+}$	EPR silent	major component of purified enzyme; reduction of $[4Fe\text{-}4S]^{3+}$ with RSH
$[4Fe\text{-}4S]^{+}$	1.96	SAM-dependent reduction with dithionite; catalytic form

3. The [4Fe-4S] Center and Formation of the 5′-Deoxyadenosyl Radical

We now consider the question of how SAM and *an*SAM are reversibly cleaved into the 5′-deoxyadenosyl and anhydroadenosyl radicals, respectively, at the active site of lysine 2,3-aminomutase. In the steady state of the overall reaction of L-lysine, the only recoverable cleavage products from SAM are methionine and 5′-deoxyadenosine.[32] This is because the predominant radical is the product radical **3**, which has been derived from the 5′-deoxyadenosyl radical by hydrogen abstraction. Thus, the SAM cleavage reaction initially produces the 5′-deoxyadenosyl radical and methionine, a process that requires an electron. The source of this electron is an iron−sulfur center in lysine 2,3-aminomutase and other enzymes of this class.

Lysine 2,3-aminomutase contains a [4Fe-4S] center that serves as the source of an electron in the reductive cleavage of SAM.[33,34] Table 1 lists the four species of iron−sulfur centers that have been observed in lysine 2,3-aminomutase, together with information about their EPR properties and their relationship to the function of the enzyme. Two species do not have any relevance to the function of this enzyme but are significant for the information they provided in early studies. When purified in less than perfectly anaerobic conditions, the enzyme contains variable amounts of an iron−sulfide center that displays an EPR signal centered at $g = 2.007$.[33] Gentle oxidation produced the $[3Fe\text{-}4S]^{+}$ center ($g = 2.015$), which could be definitively identified by EPR spectroscopy.[33] This transformation strongly implied that the $g = 2.007$ signal represented a [4Fe-4S] center at the 3+ oxidation state. The $[4Fe\text{-}4S]^{3+}$ center could be reduced by addition of either glutathione or dihydrolipoate to an EPR-silent form, the $[4Fe\text{-}4S]^{2+}$ center, which was not itself capable of supporting enzymatic activity. The addition of SAM and dithionite after reduction of the iron−sulfide center to the 1+ state activated lysine 2,3-aminomutase. The $[3Fe\text{-}4S]^{+}$ form could also be activated by treatment first with ferrous ion and dihydrolipoate, followed by SAM and dithionite. Purification of lysine 2,3-aminomutase under strict anaerobic conditions yields enzyme with $[4Fe\text{-}4S]^{2+}$ as the major form.

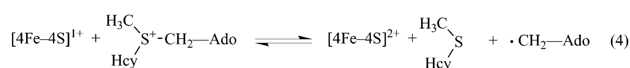
Reduction of the $[4Fe\text{-}4S]^{2+}$ center to $[4Fe\text{-}4S]^{+}$ takes place when both SAM and dithionite are added, and this activates lysine 2,3-aminomutase. Reduction to $[4Fe\text{-}4S]^{+}$ is signaled by the appearance of the characteristic EPR signal at $g = 1.96$ when observed at 11 K, and this reduction absolutely depends on the presence of SAM or *S*-adenosylhomocysteine (SAH).[34] While SAH supports the reduction by dithionite, it does not activate lysine 2,3-aminomutase.

The ease with which mild oxidizing conditions lead to loss of iron and formation of the $[3Fe\text{-}4S]^{+}$ center suggests the possibility that one iron is not ligated to a cysteine residue of the protein. A precedent is aconitase, which also contains a [4Fe-4S] center that readily loses one iron to form the [3Fe-4S] center and can be reconstituted with ferrous iron and a reducing agent.[35−37] The [4Fe-4S] center in aconitase has only

three cysteine ligands, and the fourth ligand site is occupied by carboxyl and hydroxyl ligands from the substrate. That LAM has only three cysteine ligands for the [4Fe-4S] center is further supported by the amino acid sequence deduced from the nucleotide sequence of the gene cloned from *C. subterminale SB4*.[12] The cysteine motif in the [4Fe-4S] binding site is ...**C**SMY**C**RH**C**TRRR..., very similar or identical to motifs that appear in at least 14 proteins identified in bacterial genomes and assigned as homologues of lysine 2,3-aminomutase. The amino acid sequences of the homologues did not reveal the presence of a fourth conserved cysteine residue, further indicating the absence of a fourth cysteine ligand.

Other SAM-dependent enzymes, including pyruvate formate-lyase activase, anaerobic ribonucleotide reductase activase, biotin synthase, and lipoyl synthase, incorporate analogous cysteine motifs following the pattern ...**C**XXX**C**XX**C**..., with no conserved fourth cysteine.[38] A search of databases for sequences incorporating this motif plus a glycine-rich SAM binding motif characteristic of SAM-dependent enzymes unveiled more than 600 proteins in bacteria, plants, and animals.[39] These proteins included lysine 2,3-aminomutase, anaerobic ribonucleotide reductase activase, pyruvate formate-lyase activase, biotin synthase, lipoyl synthase, benzoylsuccinate synthase activase, and many other proteins involved in coenzyme and antibiotic synthesis. They were named the radical SAM superfamily. Little is known about other members of the family, but one of them forms the subject of the following section on spore photoproduct lyase.

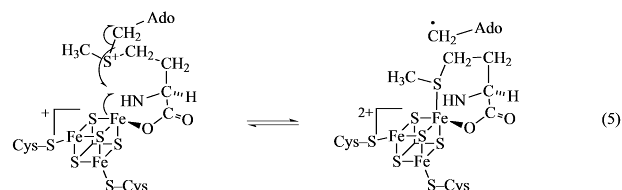
The mechanism by which SAM is reductively cleaved in its reversible reaction with the $[4Fe\text{-}4S]^+$ center is of considerable interest. Unlike the weak Co−C5′ bond in adenosylcobalamin, which generates the 5′-deoxyadenosyl radical, the C−S bonds in SAM are strong ($\sim$ 60 kcal mol^{-1} BDE), and the bond linking the adenosyl moiety to sulfur cannot be cleaved homolytically simply by binding to the enzyme. Electron transfer from the $[4Fe\text{-}4S]^+$ center to SAM must somehow be involved. The question of the electron-transfer mechanism is the subject of intensive research. The overall process may be described by eq 4, which specifies the bond cleavage in SAM



and change in oxidation state of the [4Fe-4S] center but not the mechanism of the reaction. Mechanisms that have been considered for lysine 2,3-aminomutase as well as other SAM-dependent radical enzymes include the following: (1) Outer-sphere electron transfer from the $[4Fe\text{-}4S]^+$ center to SAM to form a SAM-radical, which then undergoes fragmentation to the 5′-deoxyadenosyl radical and methionine.[40] (2) Adenosylation of a thiolate ligand in $[4Fe\text{-}4S]^+$ to form methionine and an *S*-adenosyl iron−sulfur center, followed by fragmentation of this center to the 5′-deoxyadenosyl radical and $[4Fe\text{-}4S]^{2+}$.[41] (3) Adenosylation of iron in the $[4Fe\text{-}4S]^+$ to form methionine and an *Fe*-adenosyl iron−sulfur center, followed by fragmentation of this center to the 5′-deoxyadenosyl radical and $[4Fe\text{-}4S]^{2+}$.[1] (4) Inner-sphere electron transfer from the $[4Fe\text{-}4S]^+$ center to the sulfonium center of SAM, accompanied by ligation of iron to sulfur of methionine and concomitant cleavage of the S−C5′ bond of SAM and formation of the 5′-deoxyadenosyl radical.[42]

Experiments employing X-ray absorption spectroscopy (XAS) support mechanism 4 above, inner-sphere electron transfer with concomitant ligation between iron and methionine and formation of the 5′-deoxyadenosyl radical.[43] These experiments depended on the use of the selenium analogue of SAM, *Se*-adenosylselenomethionine (*Se*SAM), to activate lysine 2,3-aminomutase. *Se*SAM activates the enzyme nearly as well as SAM and potentiates the formation of the same substrate-based radicals as does SAM. However, the replacement of sulfur by selenium introduces selenium XAS as a spectroscopic probe. Recalling that the suicide inactivator *trans*-4-dehydrolysine transforms lysine 2,3-aminomutase into the allylic analogue of the complex of radical **1** in Figure 2, the substitution of *Se*SAM for SAM would generate selenomethionine and 5′-deoxyadenosine at the active site. Selenium XAS then provides information about the ligation of selenium in this complex. In the experiments, selenium XAS clearly revealed a scatterer at a distance of 2.7 Å, and this was assigned as Fe in the iron−sulfur cluster.[43] The observation of Fe−Se ligation in these experiments implicated mechanism 4) above.

Electron nuclear double resonance (ENDOR) results with *S*-adenosyl-L-[2-^{15}N]methionine, *S*-adenosyl-L-[1-^{13}C]methionine, and *S*-adenosyl-L-[1-^{17}O]-methionine show that the α-amino and α-carboxyl groups of SAM are ligated to iron in the [4Fe-4S] cluster (C. Walsby, D. Chen, B. Hoffman, and P. A. Frey, manuscript in preparation). Taking into account the XAS results with *Se*SAM and the ENDOR results, it is therefore likely that the SAM cleavage takes place at an iron site by a process related to the hypothetical transformation in eq 5, in which the C−S cleavage occurs in concert with electron transfer.



Whether the mechanism of electron transfer and SAM cleavage is best described as the concerted process of eq 5 or whether additional steps intervene cannot be concluded with certainty. The concerted mechanism seems to offer advantages in harvesting energy from the ligation of methionine by lowering the activation barrier to transferring an electron to the sulfonium ion. The midpoint reduction potential for electron transfer to a sulfonium ion is very negative.[44] In any case, the equilibrium of eq 5 is likely to lie far to the left, and the observation of radicals in the lysine 2,3-aminomutase reaction relies on the potential to form delocalized, stable radicals.

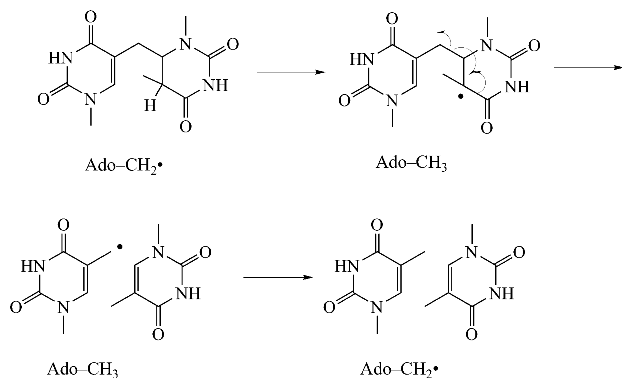


Figure 4. Hypothesis for the mechanism of thymine dimer repair by spore photoproduct lyase.

Such radicals include all those so far observed: radical **3** in Figure 2 derived from lysine, the 4-thia-L-lysine analogue of radical **1** in Figure 2, the corresponding allylic radical derived from *trans*-4-dehydrolysine, and the anhydroadenosyl radical.

B. Spore Photoproduct Lyase

A 41-kDa iron–sulfur protein from *Bacillus subtilis*, a member of the radical SAM superfamily known as spore photoproduct lyase, repairs methylene-bridged thymine dimers in DNA.[45,39] The reaction proceeds in the dark, unlike the reaction of the photolyase that repairs fused-ring thymine ring dimers in DNA. Experiments with spore photoproduct lyase showed that it contains a [4Fe-4S] center, requires SAM for activity, and cleaves SAM into 5′-deoxyadenosine and methionine in the course of repairing thymine dimers.[46] Experiments indicate that the reduced form of the iron–sulfur center is $[4Fe\text{-}4S]^+$ and that this is oxidized to the EPR-silent 2+ form upon cleavage of SAM.

Repair of bridged thymine dimers labeled with tritium led to tritium labeling in SAM, and dimer repair with activation by [5′-^{3}H]SAM led to tritiated thymine.[47] All evidence points to the elegant mechanism shown in Figure 4 for the repair of methylene-bridged thymine dimers by spore photoproduct lyase. The reversible cleavage of SAM by reaction with the $[4Fe\text{-}4S]^+$ center produces the 5′-deoxyadenosyl radical, which abstracts C6(H) from the thymine dimer to form the dimer radical and 5′-deoxyadenosine. Radical fragmentation generates one thymine and the thymine monomer radical, which abstracts a hydrogen from 5′-deoxyadenosine to form the second thymine and regenerate the 5′-deoxyadenosyl radical. The mechanism is compatible with and supported by the results of chemical model experiments.[48]

III. SAM and the Activation of Radical Enzymes

A. Pyruvate Formate-Lyase

1. Properties of PFL and Characterization of the Glycyl Radical

Pyruvate formate-lyase (PFL) catalyzes the reaction of coenzyme A with pyruvate to produce acetyl-CoA and formate (eq 3). The reaction is fully reversible with turnover numbers of 770 and 260 s^{-1} at 30 °C for the forward and backward reactions, respectively.[49] Purified PFL from *Escherichia coli* is a homodimer with a molecular mass of 170 kDa and contains no metals or other cofactors.[50,51] The enzyme is catalytically inactive when isolated under aerobic conditions, and activation is accomplished only under anaerobic conditions by the action of another enzyme, named pyruvate formate-lyase activating enzyme (PFL-activase).[10,49] Knappe and co-workers were able to show that activation of PFL entails the generation of a stable free radical on the enzyme,[1] which amounts to one radical spin per dimer.[52] The radical is, however, highly susceptible to oxygen and displays a half-life of only 10 s in air-saturated buffer at room temperature.[1] Oxygen-mediated destruction of the radical in PFL leads to fission of the polypeptide chain, generating 82- and 3-kDa fragments, respectively.[53] The mechanism of inactivation by dioxygen has been studied by Kozarich and associates, who demonstrated the involvement of transient sulfinyl (RSO•) and peroxyl (ROO•) radicals in that process.[54,55] Their results have recently been substantiated by computational work[56] and will not be discussed further.

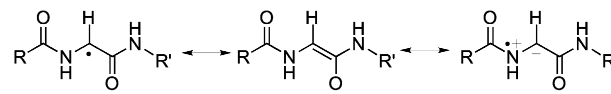


Figure 5. Resonance structures for the captodative stabilization of glycyl radicals.

Wagner et al. established the nature of the stable radical in PFL. By performing EPR experiments with isotopically labeled forms of PFL and by examining the O_2 cleavage products by mass spectrometry and amino acid sequencing, they could assign the radical to the α-carbon of Gly734.[53] This discovery was the first proven case of a protein-derived glycyl radical involved in enzymatic catalysis, but more cases were to be discovered, as will be described in the following sections of this review. The EPR spectrum of the glycyl radical displayed a prominent doublet ($A_{iso} = 15$ G) centered at $g = 2.0037$, which was attributed to a hyperfine coupling to the remaining α-hydrogen.[53] The structural assignment was corroborated by various isotopic labeling experiment using auxotrophic strains of *E. coli*—in particular, the EPR spectrum of [2-^{13}C]glycine-substituted PFL displayed marked increases in signal width and complexity.[53] Simulations of EPR spectra of the glycyl radical have shown that approximately 55% of the spin density is localized on the α-carbon.[57] This property emphasizes the inherent stability of glycyl radicals due to the combined effects of an adjacent electron donor (amide nitrogen) and an electron acceptor (glycine carbonyl) by what is known as the captodative effect.[58] This effect is illustrated by the resonance structures shown in Figure 5. However, both experimental[57] and theoretical[59] work has shown that additional spin delocalization occurs, which is due to the adjacent peptide bonds in the molecule.

An intriguing property of the glycyl radical in PFL is that the α-hydrogen of Gly734 exchanges with solvent, as is apparent from EPR experiments done in D_2O buffer, which resulted in the disappearance

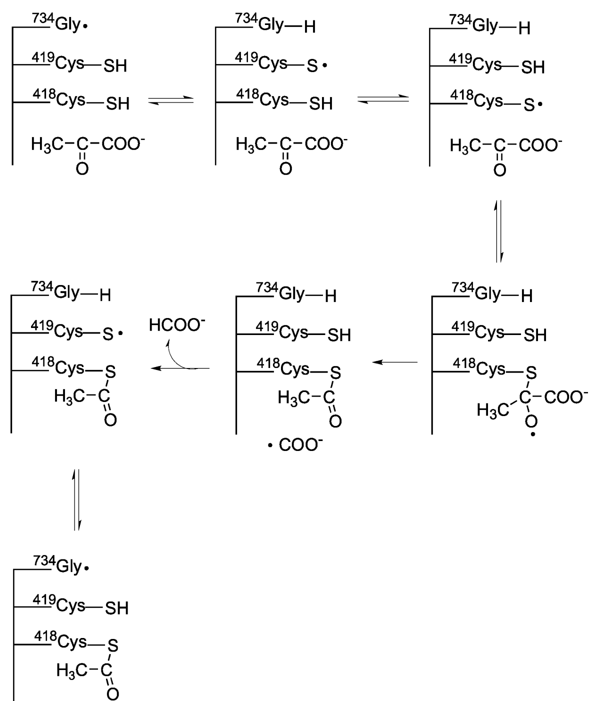
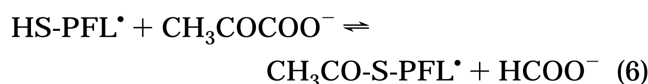


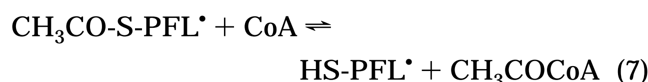
Figure 6. Radical mechanism for the acetylation reaction of pyruvate formate-lyase.

of the 15-G doublet splitting.[52,53] The exchange process is relatively rapid, with a half-life of ~5 min at 0 °C. By a combination of site-directed mutagenesis and mechanism-based inactivation studies, Kozarich and associates were able to show that the exchange process is facilitated by Cys419.[60] Mutation of this residue abolished both the activity of PFL and the solvent exchange of the α-hydrogen of Gly734. Further studies with the mechanism-based inhibitor mercaptopyruvate allowed for the detection of sulfur-based radicals and established the roles of both Cys419 and Cys418 in the catalytic mechanism of PFL.[61]

Although the catalytic mechanism of PFL is not fully understood, the basic premise is that the reaction proceeds by a ping-pong mechanism with an isolable *S*-acetylated enzyme intermediate, as shown in eqs 6 and 7.[49]



$$\text{HS-PFL}^{\bullet} + \text{CH}_3\text{COCOO}^- \rightleftharpoons \text{CH}_3\text{CO-S-PFL}^{\bullet} + \text{HCOO}^- \quad (6)$$



$$\text{CH}_3\text{CO-S-PFL}^{\bullet} + \text{CoA} \rightleftharpoons \text{HS-PFL}^{\bullet} + \text{CH}_3\text{COCoA} \quad (7)$$

One possible mechanism for the acetylation half-reaction is presented in Figure 6. The glycyl radical is propagated via Cys419 to the adjacent cysteine residue. Cys418 becomes acetylated upon radical attack on C2 of pyruvate. The resulting tetrahedral radical adduct collapses into the thioester on Cys418 and a formyl radical, which can abstract a hydrogen atom from Cys419 and ultimately regenerate the resting glycyl radical. The role of the two critical cysteine residues is based on the crystal structure of PFL with the substrate analogue oxamate bound in the active site, where Cys418 is in close proximity to

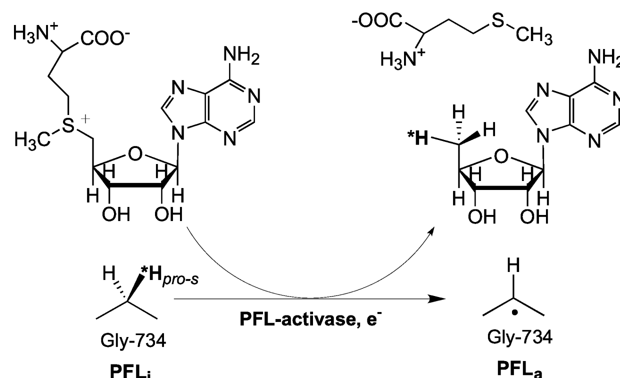


Figure 7. Activation of PFL, catalyzed by the action of PFL-activase.

C2 of pyruvate (3.3 Å) and Cys419 is located 3.7 Å from the α-carbon of Gly734.[62] Studies with the suicide inactivator, methacrylic acid, have provided further support for the roles of Cys418 as a thiyl nucleophile and Cys419 as a H-atom mediator.[63] The second half-reaction could, in principle, be formulated by either homolytic or heterolytic chemistry. Theoretical work has shown that the attack of a thiyl radical to the thioester intermediate is energetically favored over attack by a thiolate.[64]

2. PFL-Activase: The Role of SAM and the Iron–Sulfur Cluster

The formation of the glycyl radical of PFL is catalyzed by PFL-activase, a 28-kDa monomeric protein. The reaction is dependent on SAM, pyruvate, and an external electron source, which can be supplied either by NADPH via the flavodoxin/flavodoxin reductase system or by chemical reductants, such as dithionite or 5-deazaflavin.[10,50] The enzyme was also reported to contain a covalently bound chromophore and required exogenous iron for activity.[50] During the reaction, one equivalent of SAM is cleaved to yield 5′-deoxyadenosine and methionine, as depicted in Figure 7.[1,65] SAM is therefore used as a cosubstrate in this reaction, which differs from its role as a true coenzyme as in the cases of LAM and spore photoproduct lyase. By using [2-^{2}H]-Gly-labeled PFL, Knappe and co-workers demonstrated the stoichiometric incorporation of deuterium into 5′-deoxyadenosine, thereby providing a direct link between the cleavage of SAM and H-atom transfer from Gly734 via the 5′-deoxyadenosyl radical.[66] Furthermore, by using short peptides that mimicked the Gly radical site in the protein, they were able to show that the reaction proceeds by stereospecific abstraction of the *pro-S* hydrogen of Gly73.[66] Further evidence for the intermediacy of the 5′-deoxyadenosyl radical was provided by chemical trapping, using an octapeptide substrate containing a dehydroalanine residue in lieu of the reactive glycine.[67] By characterizing the product of the reaction by mass spectrometry and 2D NMR, the researchers were able to show that C5′ of the adenosyl moiety was linked to the *β*-carbon of the dehydroalanine residue (Figure 8).

A recombinant form of PFL-activase was first characterized by Kozarich and co-workers.[68] However, the protein needed to be refolded from insoluble

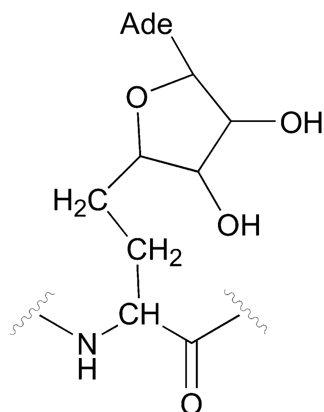


Figure 8. Chemical trapping of the 5′-deoxyadenosyl radical. An octapeptide (Succ−R−V−P−ΔA−Y−A−V−R−NH_2) containing a dehydroalanine (ΔA) residue was used as a substrate for PFL.

inclusion bodies, displayed ∼20-fold lower activity than the wild-type protein, and did not exhibit any visible chromophores. Subsequent work in other laboratories has led to the production of soluble and more active recombinant protein. Broderick and associates showed that PFL-activase is an iron−sulfur protein.[69] The cluster states were probed by UV−vis, variable-temperature magnetic circular dichroism (VTMCD), EPR, and resonance Raman spectroscopies. The results showed the presence of both $[2Fe\text{-}2S]^{2+}$ and $[4Fe\text{-}4S]^{2+}$ in the as-isolated enzyme, which underwent complete conversion to the cuboidal [4Fe-4S] state upon reduction by dithionite.[69] Like in the case of lysine 2,3-aminomutase,[34] the 1+ oxidation state of the cluster could be attained only in the presence of SAM. Further studies, where adherence to strict anaerobicity were followed, showed that as-isolated PFL-activase could display a wide variety of Fe−S cluster states. Using Mössbauer spectroscopy, cuboidal $[3Fe\text{-}4S]^{+}$, $[4Fe\text{-}4S]^{2+}$, $[2Fe\text{-}2S]^{2+}$, and linear $[3Fe\text{-}4S]^{+}$ clusters were detected.[70] The most recent preparations, however, yield as-isolated protein that almost exclusively contains a $[4Fe\text{-}4S]^{2+}$ cluster.[71]

Quantitative 5-deazaflavin photoreduction of the [4Fe-4S] cluster to the 1+ oxidation state in the absence of SAM has yielded important information about the role of the Fe−S cluster in PFL-activase. In these experiments, it was shown that a direct correlation existed between the formation and subsequent oxidation of the $[4Fe\text{-}4S]^{+}$ cluster in PFL-activase and glycyl radical formation in PFL.[72] PFL-activase was photoreduced for time intervals between 0 and 60 min. After addition of excess SAM, one half of each sample was used to obtain the amount of EPR-active $[4Fe\text{-}4S]^{+}$ cluster, whereas PFL was added to the other half and the glycyl radical spin was quantified by EPR (Figure 9). In samples containing PFL, the EPR signal from the $[4Fe\text{-}4S]^{+}$ cluster could not be detected, indicating complete oxidation to the EPR-silent $[4Fe\text{-}4S]^{2+}$ state. The concentrations of spin for the two paramagnetic species were shown to be identical, which supports the notion that the $[4Fe\text{-}4S]^{+}$ cluster is the source of the electron needed for the reductive cleavage of SAM and subsequent generation of the glycyl radical in PFL.[72]

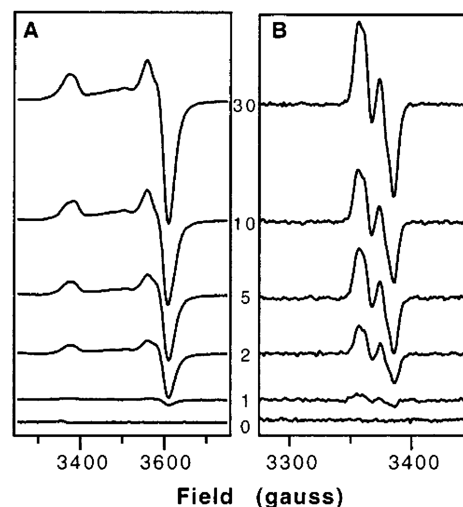


Figure 9. EPR spectra of photoreduced PFL-activase before and after addition of PFL. (A) Spectra of the $[4Fe\text{-}4S]^{+}$ for different times (0−30 min) after addition of SAM. (B) Spectra of the glycyl radical produced upon addition of PFL to the samples in A. Spin concentration at each time point is the same (within error) between the spectra in A and B. Reprinted with permission from ref 72. Copyright 2000 American Chemical Society.

PFL-activase contains a conserved Cys motif like other members of the radical SAM superfamily. A sequence alignment for the Fe−S cluster binding domain for the proteins discussed in this review is depicted in Figure 10. Although there is some sequence identity and/or homology between individual proteins, the only strictly conserved residues among all of them are the three cysteines that have the following spacing: C−X_3−C−X_2−C. As mentioned previously, a glycine-rich region, which is believed to be involved in SAM binding, is also present in all the putative members of the radical SAM superfamily.[39] The role of potential cysteine ligands to the Fe−S cluster in PFL-activase has been explored by mutagenesis experiments. PFL-activase contains six Cys residues; single mutations of each of these residues to Ser showed that only the conserved cysteines (C29, C33, C36) were essential for activity and cluster assembly, whereas the other mutations resulted in proteins that displayed wild-type activity.[73] It should be mentioned that the PFL-activase used in these studies was prepared as the apoenzyme followed by reconstitution of the Fe−S cluster under anaerobic conditions. The use of only three Cys ligands to the [4Fe-4S] cluster in PFL-activase may, in fact, be extrapolated to all members of the radical SAM superfamily and is undoubtedly related to the unique use of this cluster in radical formation. Strong evidence for incomplete cysteinyl coordination by mutagenesis and/or spectroscopic experiments has been observed for lysine 2,3-aminomutase, anaerobic ribonucleotide reductase activase, and biotin synthase, as discussed elsewhere in this review.

The nature of the fourth ligand to the Fe−S cluster in PFL-activase has recently been addressed. Using a dual-iron-isotope method, Krebs et al. were able to show by Mössbauer spectroscopy that PFL-activase has a unique iron site.[74] This was accomplished by reconstituting a ^{56}Fe-containing $[3Fe\text{-}4S]^{+}$ form of the

LAM	132	L	L	I	T	D	M	C	S	M	Y	C	R	H	C	T	R	R	R	F	150
SPL	86	I	P	F	A	T	G	C	M	G	H	C	H	Y	C	Y	L	Q	T	T	104
PFL-activase	24	I	T	F	F	Q	G	C	L	M	R	C	L	Y	C	H	N	R	D	T	42
ARR-activase	20	V	L	F	V	T	G	C	L	H	K	C	E	G	C	Y	N	R	S	T	38
BSS-activase	23	T	I	F	L	K	G	C	P	L	R	C	P	W	C	H	N	P	E	T	41
BioB	47	S	I	K	T	G	A	C	P	Q	D	C	K	Y	C	P	Q	T	S	R	65
LipA	48	M	I	L	G	A	I	C	T	R	R	C	P	F	C	D	V	A	H	G	66

Figure 10. Sequence alignment of the Fe−S cluster binding motif in the radical SAM enzymes. Conserved residues are shown with a gray background. LAM, lysine 2,3-aminomutase from *C. subterminale*; SPL, spore photoproduct lyase from *B. subtilis*; PFL-activase, pyruvate formate-lyase activase from *E. coli*; ARR-activase; anaerobic ribonucleotide reductase activase from *E. coli*; BSS-activase; benzylsuccinate synthase activase from *T. aromatica*; BioB, biotin synthase from *E. coli*; LipA, lipoate synthase from *E. coli*.

enzyme with ^{57}Fe in the presence of dithiothreitol (DTT) to give the $[4Fe\text{-}4S]^{2+}$ form of the enzyme. Selective incorporation of ^{57}Fe into a single site of the cluster was established by Mössbauer spectroscopy. Such a phenomenon has also been observed for aconitase, which contains one "labile" iron in its [4Fe-4S] cluster—a site where substrate binding occurs.[35] Interestingly, upon addition of SAM to PFL-activase containing one equivalent of ^{57}Fe, a significant change in the spectral parameters of the Mössbauer spectrum was observed, especially in the isomer shift, which went from $\delta = 0.42$ mm/s without SAM to $\delta = 0.72$ mm/s in the presence of SAM.[74] Such an increase in the isomer shift is indicative of an increase in coordination number and/or coordination of more ionic ligands. On the basis of these data, the authors concluded that SAM was coordinated to the $[4Fe\text{-}4S]^{2+}$ cluster via either the carboxylate of SAM or one of the hydroxyls of the ribose ring of the cofactor. Coordination by the sulfur atom was considered unlikely due to the large shift in the isomer shift—a conclusion that is different than the one reached by Cosper et al. in their XAS study on lysine 2,3-aminomutase, as mentioned earlier.[43] However, it must be noted that in the XAS study, SAM underwent cleavage to selenomethionine and 5′-deoxyadenosine, and coordination of selenomethionine was observed. In the studies of PFL-activase, SAM was not cleaved.

Most recently, a collaborative effort between Broderick, Hoffmann, and their associates, using Q-band ENDOR spectroscopy, has provided compelling evidence for a N/O chelate between SAM and the unique iron site in the $[4Fe\text{-}4S]^{+}$ cluster in PFL-activase.[75] Using SAM labeled with ^{17}O and ^{13}C in the carboxylate group and ^{15}N in the α-amino group, respectively, these investigators obtained ENDOR spectra that clearly demonstrate Fe ligation of both an oxygen from the carboxylate group and the α-nitrogen.[75] The magnitudes of the hyperfine interactions compare favorably with data obtained for aconitase with bound citrate/isocitrate[76,77] (^{17}O/^{13}C) and to Rieske [2Fe-2S] centers, which have His coordination[78] (^{15}N). These data are consistent with a N/O five-membered ring chelate to the unique iron site of the cluster in PFL-activase (Figure 11), which the authors propose could serve as an anchor for correct positioning of the coenzyme for the ensuing electron-transfer reaction.

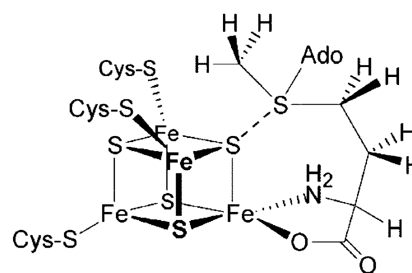


Figure 11. Structure of the N/O chelate of SAM with the unique iron site of the [4Fe-4S] cluster of PFL-activase as deduced by ENDOR spectroscopy. Reprinted with permission from ref 75. Copyright 2002 American Chemical Society.

The same researchers have also used EPR and ENDOR spectroscopies to further probe the interactions between the Fe−S center and SAM in PFL-activase.[71] First, they noted dramatic changes in the EPR spectrum of the photoreduced $[4Fe\text{-}4S]^{+}$ cluster upon addition of SAM, in which a rhombic signal (g = 2.02, 1.94, 1.88) is converted to a more axial signal (g = 2.01, 1.88, 1.87). They were also able to detect direct interactions between the paramagnetic cluster and ^{13}C- and ^{2}H-labeled forms of SAM in the methyl position. By cryoreducing a $[4Fe\text{-}4S]^{2+}$/SAM complex of the enzyme to the 1+ paramagnetic state, they obtained similar results, which showed that the cofactor binds in the same conformation for both oxidation states of the cluster. On the basis of the magnitude and type of hyperfine coupling (isotropic/anisotropic), they estimated that the distance to the closest iron in the cluster was 4−5 Å from the methyl carbon of SAM and 3.0−3.8 Å from the closest methyl hydrogen of SAM, respectively.[71] After further analysis of the data, these investigators also proposed that an interaction between a sulfide in the cluster and the sulfonium ion of SAM (Figure 11) might help facilitate the reductive cleavage of the cofactor via inner-sphere electron transfer.

B. Anaerobic Ribonucleotide Reductase

1. Reaction and Molecular Properties

Ribonucleotide reductases are ubiquitous in nature. These enzymes, which catalyze the essential formation of deoxynucleotides, provide the only pathway for the de novo production of DNA precursors. The enzymes fall into three different categories. The

distinctions are based on the cofactor requirements of the enzymes, but the mechanism of ribonucleotide reduction is believed to be the same for all classes.[79] Class I enzymes are perhaps the most abundant and are found, for example, in eukaryotes and various microorganisms, such as *E. coli* grown under aerobic conditions. They contain a binuclear iron center and a tyrosyl radical cofactor. These enzymes are further divided into class Ia and Ib on the basis of sequence similarity, allosteric properties, and the use of different physiological reductants.[80] Class II ribonucleotide reductases use adenosylcobalamin as a cofactor, where the enzyme from *Lactobacillus leichmanii* serves as a prototype. Anaerobically grown *E. coli* cells produce yet another form of these enzymes, which has been named anaerobic ribonucleotide reductase (ARR). All of these enzymes are thought to use a thiyl radical derived from a cysteine residue on the protein(s) to initiate the reduction process by abstraction of the 3′-hydrogen atom of the ribonucleotide substrates, but the strategy used to generate the thiyl radical differs between the classes. We will not discuss the mechanism of ribonucleotide reduction or the general properties of these enzymes, but excellent reviews are available on those topics.[79,80]

ARR from *E. coli* was first described by Reichard and associates,[81] who had also discovered the aerobic enzyme from *E. coli* 40 years earlier.[82,83] More recently, the enzyme from other organisms, such as bacteriophage T4[84] and *Lactococcus lactis*,[85] have also been studied. In fact, the only crystal structure of a class III reductase is that of the T4 enzyme.[86] However, most of the biochemical work has been done on ARR from *E. coli*, and we will focus our attention on that enzyme.

ARR, which is encoded by the *nrdD* gene, is an α_2-homodimer of 160 kDa.[87] Sequence analysis showed that ARR had some similarity to PFL, especially around the glycine radical site.[87] Subsequent EPR experiments confirmed the presence of an organic radical in ARR,[88] which later was shown to be located on Gly681. The assignment of the radical was based on EPR experiments with ^{2}H- and ^{13}C-Gly-labeled enzyme, in conjunction with site-directed mutagenesis.[89] The radical is a doublet with one major hyperfine coupling to the remaining α-hydrogen of Gly681 (~14 G), which is similar to what is seen in PFL, although further additional splittings are also observed in PFL.[57] In addition, only one glycyl radical per dimer is generated in ARR,[90] which is analogous to what is seen in PFL.[52] As in PFL, exposure to oxygen results in cleavage of the polypeptide chain into two isolable peptide fragments.[87] The site of cleavage at Gly681 was obtained from the mass spectra of these fragments.[91] Unlike PFL, however, the glycyl radical of ARR does not undergo solvent exchange of the remaining α-hydrogen of Gly681.[88]

The role of the glycyl radical is to serve as an initiator of catalysis via the thiyl radical at the active site. As such, the role of the glycyl radical is the same as that of the tyrosyl radical in class I enzymes. The difference is that Gly681 in ARR is reasonably close to the active-site cysteine for a direct H-atom transfer ($C\alpha-S\gamma = 5.2$ Å),[86] whereas the tyrosyl radical in the

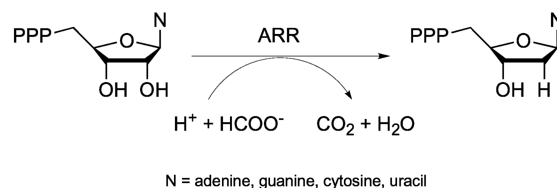


Figure 12. Reaction catalyzed by anaerobic ribonucleotide reductase.

aerobically produced *E. coli* enzyme needs to be propagated over 35 Å to yield the active-site thiyl radical.[92] In a recently determined crystal structure of the class II enzyme, the sulfur of the active-site cysteine (Cys408) is located 10 Å away from the corrinoid cobalt.[93] This structure does not represent the fully active/closed conformation of the enzyme, because Cys408 is still solvent-exposed, and EPR experiments have shown that the thiyl radical is located 5.5−7.5 Å from the cobalt.[94] This is undoubtedly because the protein in this structure does not have a deoxynucleotide effector molecule bound, which is known to increase the rate of Co−C bond homolysis.[95] However, the active-site architectures of the class II and class III enzymes are strikingly alike, where the axial 5′-deoxyadenosyl ligand of B_{12} (shown by modeling) in the class II enzyme and Gly681 in the class III enzyme are situated very similarly with respect to the active-site cysteine.[93]

Interestingly, ARR uses formate as an external reductant,[96] as illustrated by the overall stoichiometry of the reaction (Figure 12). This property differs from class I and II reductases, which use a pair of active-site cysteines as reductants that are regenerated to the thiol form via the thioredoxin/thioredoxin reductase system. The use of formate by ARR provides an important link between ARR and PFL in the anaerobic metabolism of *E. coli*, because formate is one of the products in the reaction catalyzed by PFL. The mechanism by which formate is oxidized to CO_2 is poorly understood. DFT calculations have suggested that a formyl radical may be directly involved in the catalytic mechanism,[97] but further experimental work is required to establish the mechanistic function of formate in ARR.

2. Characterization of ARR-Activase (β_2)

ARR is purified in an inactive dimeric form and requires activation under anaerobic conditions in the presence of SAM, a reducing system consisting of NADPH, flavodoxin, flavodoxin reductase, DTT, K^+, and a protein component named β.[98−100] The β-protein is a dimer encoded by the *nrdG* gene with a subunit molecular mass of 17.5 kDa.[100] Experiments from the Fontecave laboratory suggested that ARR could best be described as an $\alpha_2\beta_2$ tetramer, because of the tight association between the components.[101] However, subsequent work has shown that one equivalent of β_2 is capable of activating several equivalents of α_2, demonstrating that the β-protein is, in fact, a true activating enzyme rather than an integral component of an $\alpha_2\beta_2$ complex.[102]

The presence of Fe−S cluster(s) in the ARR $\alpha_2\beta_2$ complex was noted early on.[88] ARR-activase isolated under aerobic condition from an overexpression sys-

tem is mainly devoid of iron and sulfide. Upon anaerobic reconstitution in the presence of iron and sulfide, uptake of about two equivalents of Fe and S per polypeptide was observed.[101] Under highly reducing conditions, a $[4Fe\text{-}4S]^+$ center was detected by EPR ($g = 2.03, 1.92$), which led the researchers to postulate the formation of this cluster via the bridging of two [2Fe-2S] centers at the subunit interface.[101] Using a combination of various spectroscopic techniques, the reconstituted cluster was, in fact, shown to be a $[2Fe\text{-}2S]^{2+}$ cluster that was converted to the $[4Fe\text{-}4S]^+$ state upon reduction by 5-deazaflavin and light or dithionite.[103] Improvements in the reconstitution process, including stricter anaerobic conditions, have since shown that each β-subunit is capable of binding one [4Fe-4S] cluster, which undergoes degradation to the [2Fe-2S] form upon exposure to air.[102] These results led Fontecave and co-workers to abandon the hypothesis of cluster formation at the subunit interface.[102]

The relative ease of conversion between different Fe–S cluster states appears to be a common theme among enzymes in the radical SAM superfamily. Of those enzymes in the family where the Fe–S clusters have been studied, only lysine 2,3-aminomutase has not displayed the [2Fe-2S] state, although four other cluster forms have been described for that enzyme (see section II.A.3). The lability of these clusters is undoubtedly related to the unique coordination environment, which involves incomplete cysteine ligation, as discussed before. The importance of only three cysteine ligands in ARR-activase was confirmed by site-directed mutagenesis studies. The protein contains five cysteine residues, and only the variants in the $C{-}X_2{-}C{-}X_3{-}C$ motif (C26A, C30A, C33A) were inactive.[104] These variants were, however, able to bind iron and sulfide similar to wild-type, but reduction to the active $[4Fe\text{-}4S]^+$ state could not be reached. Fe–S clusters with only two Cys ligands have not been reported in the literature, and the nature of the third ligand that would substitute for any of the mutated cysteines could not be determined in this study.

The role of SAM in ARR-activase was established in a fashion similar to that described above for lysine 2,3-aminomutase and PFL-activase. Using tritium-labeled SAM in the appropriate positions, the formation of methionine and 5′-deoxyadenosine in a 1:1 ratio was established.[98] In these early experiments, ARR-activase had not been well characterized, and the amount of SAM consumed with regard to the activase was not determined. However, similarities between this reaction and that of PFL-activase were noted, and Figure 7, which describes the activation of PFL, does equally well apply to the activation process of ARR.

Experiments by Fontecave and co-workers demonstrated that the $[4Fe\text{-}4S]^+$ cluster in the activase is the source of electron for the reductive cleavage of SAM.[40] This was the first radical SAM enzyme wherein this process was established. Direct binding experiments showed that SAM binds in a 1:1 stoichiometry to the protein with a K_D of 10 μM, and cleavage of the cofactor produces methionine at a rate

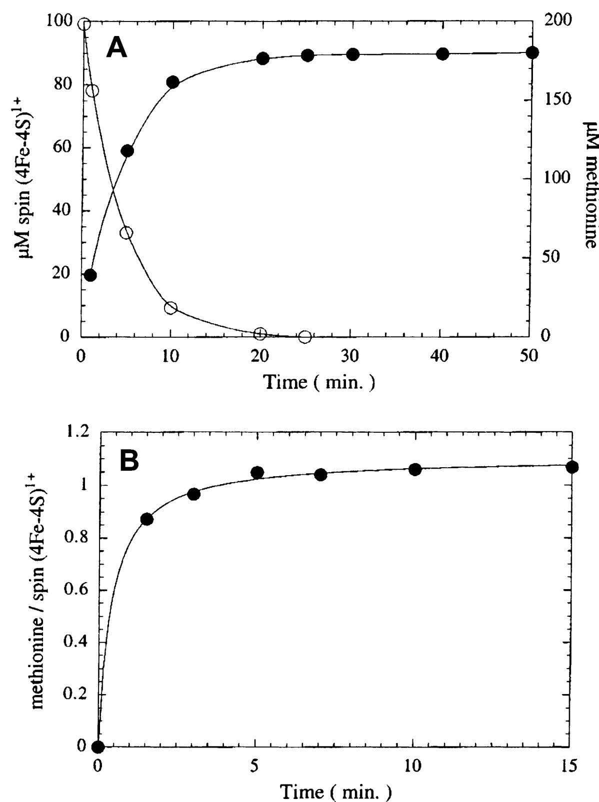


Figure 13. (A) Correlation between methionine production and oxidation of $[4Fe\text{-}4S]^+$ in ARR-activase (β_2). A rate constant of ~0.15 min^{-1} was observed for both processes. Methionine is formed in ~2:1 ratio to the oxidized cluster. (B) Formation of methionine in the $\alpha_2\beta_2$ complex. A ratio of 1:1 between methionine produced and Fe–S cluster oxidized is obtained at the end of the reaction. Reprinted with permission from ref 105. Copyright 2001 American Chemical Society.

that is comparable to the oxidation of the cluster, as monitored by the decrease in the amplitude of the EPR signal of the $[4Fe\text{-}4S]^+$ cluster.[40] These experiments were done using 5′-deazaflavin plus light as a reductant, thereby preventing re-reduction of the cluster by keeping the samples in the dark. Importantly, in analogous experiments with the $\alpha_2\beta_2$ complex, the rate of formation of the glycyl radical was similar to the rate of methionine formation, therefore linking cleavage of SAM directly to the activation of ARR. One puzzling result in this study was that three equivalents of methionine were produced per Fe–S cluster oxidized or glycyl radical formed in experiments with β_2 or $\alpha_2\beta_2$, respectively. In addition, the presence of DTT was absolutely required for the cleavage reaction to occur.

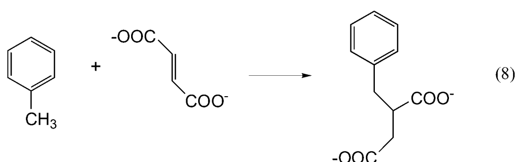
Recent experiments, using more active preparations of enzyme, have addressed these issues. First, it was shown that, in the $\alpha_2\beta_2$ complex, methionine was formed in amounts equal to the $[4Fe\text{-}4S]^+$ present before addition of SAM, as would be expected (Figure 13A).[105] This reaction did not require DTT if care was taken to pre-reduce the enzyme, followed by gel filtration to remove exogenous reductant. A second reaction, involving only the activase (β_2), did require DTT and produced two equivalents of methionine per $[4Fe\text{-}4]^+$ cluster oxidized (Figure 13B). Both processes displayed the same rate constant

(~0.15 min^{-1}), in accordance with previous studies.[40] The unusual stoichiometry of the reaction with β_2 and the absolute requirement for DTT led to the proposal that DTT might play an imminent role in this reaction.[105] In this proposal, DTT would remove the "labile" iron in the cluster, resulting in a "hyper-reduced" $[3Fe\text{-}4S]^{2-}$ cluster that could donate, in succession, two electrons needed to yield two equivalents of methionine. From the stable $[3Fe\text{-}4S]^0$ cluster state, uptake of ferrous iron would give the $[4Fe\text{-}4S]^{2+}$ cluster. Although DTT may possibly interact with the Fe−S cluster, as suggested by perturbations in the EPR spectrum of the $[4Fe\text{-}4S]^+$ cluster,[105] further experiments are clearly required to thoroughly test this interesting hypothesis.

As mentioned previously, flavodoxin/flavodoxin reductase plus NADPH serves as the biologically relevant reducing system for ARR-activase, although in laboratory experiments, chemical reductants such as dithionite and 5′-deazaflavin are routinely employed. Recent experiments have shown that flavodoxin reduction does not lead to a detectable amount of the $[4Fe\text{-}4S]^+$ cluster of β_2, either alone or in the $\alpha_2\beta_2$ complex.[90] Neither the semiquinone (SQ) form nor the fully reduced flavin has a low enough potential to afford this reduction. In fact, the redox potential of the $[4Fe\text{-}4S]^{+/2+}$ couple (−550 mV) was shown to be ~300 mV more negative than that of the SQ flavodoxin couple, although one cannot rule out changes in the redox potential of flavodoxin upon binding to ARR-activase.[90] Interestingly, after addition of SAM, the redox potential of the cluster became slightly more negative (−620 mV), as determined by square-wave voltammetry. These data suggest that activation of ARR is strongly coupled to the subsequent reductive cleavage of SAM and the thermodynamically favorable H-atom abstraction from Gly681. Knowing the redox potentials of the Fe−S clusters in the radical SAM enzymes is important in order to understand the unfavorable reductive cleavage of SAM. Possible mechanisms for this reduction process are discussed elsewhere in this review, but presumably SAM binding to the enzymes would alter the redox potential. In fact, as discussed in section I.A.1, reduction of the [4Fe-4S] cluster in lysine 2,3-aminomutase can be accomplished only in the presence of SAM or SAH. For some of the other enzymes, interactions between the cluster and SAM are apparent, because of the effects on the EPR spectrum of the $[4Fe\text{-}4S]^+$ cluster.[40,71,105]

C. Benzylsuccinate Synthase

Benzylsuccinate synthase (BSS) catalyzes the addition of toluene to fumarate to yield benzylsuccinate, as shown in eq 8. As such, the reaction is novel in



biological systems, because C−C bond formation occurs via carbon addition to a C=C double bond instead of to a C=O double bond or to CO_2. This reaction is the first step in the anaerobic catabolism of toluene in several denitrifying[106−108] and sulfate-reducing[109,110] bacteria. The enzyme, which has been partially purified from two organisms, *T. aromatica*,[111] and *Azoarcus* sp. strain T,[112] is a heterohexamer ($\alpha_2\beta_2\gamma_2$) with subunit molecular masses of 98, 8.6, and 6.6 kDa for the α, β, and γ subunits, respectively. The amino acid sequence of BSS shares homology with PFL and ARR, especially around the glycyl radical site, and it contains a conserved active-site cysteine residue.[108,111] In fact, site-directed mutagenesis studies show that both of the conserved Gly and Cys residues are important for the function of BSS in denitrifying strain T1.[108] The glycyl radical consensus sequence R−V−S/C−G−Y can be found in BSS from different organisms. The only minor exception is the protein from *T. aromatica*, which contains a phenylalanine instead of tyrosine adjacent to the active-site glycine.[111] The existence of a stable free radical in BSS from *Azoarcus* sp. strain T has recently been demonstrated.[113] The radical is a doublet ($A \approx 15$ G) and has all the characteristics of a glycyl radical. Upon incubation of the BSS in D_2O, the EPR signal of the radical becomes a singlet, consistent with H/D exchange of the strongly coupled hydrogen atom.[113] This observation is similar to what occurs in PFL, whereas the glycyl radical in ARR does not undergo solvent exchange.

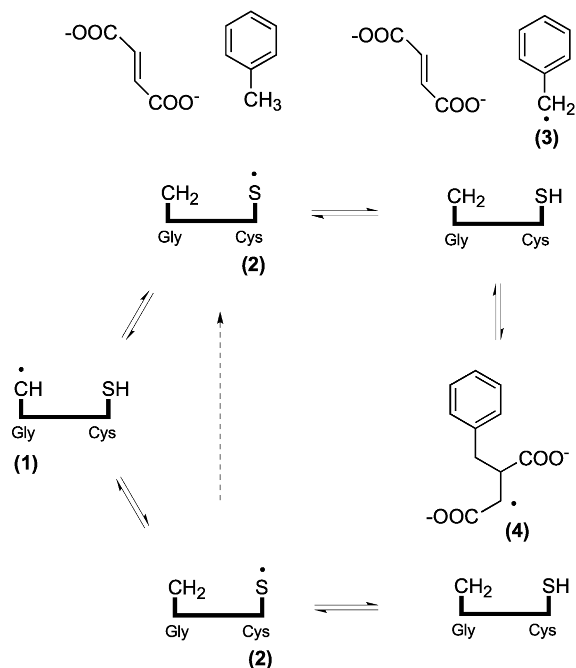


Figure 14. Plausible radical mechanism for the action of benzylsuccinate synthase. The dashed arrow indicates a possible shortcut that excludes regeneration of the glycyl radical at the end of each catalytic cycle.

A plausible mechanism for the BSS reaction is shown in Figure 14. The glycyl radical (**1**) abstracts a hydrogen atom from the active-site cysteine to produce a thiyl radical (**2**). The thiyl radical abstracts a hydrogen atom from the methyl group of toluene, yielding a benzyl radical (**3**). The benzyl radical adds to C2 of fumarate to give a benzylsuccinyl radical (**4**). Product studies have shown that the addition is

stereospecific—occurring at the *re* face of C2 of fumarate.[114] Reabstraction of a H-atom from the cysteine residue yields the product (**5**), which has been shown to fully retain the H-atom that originated from toluene[109,114]—in full agreement with this mechanism. Whether the thiyl radical would be capable of initiating another round of catalysis directly, or whether the glycyl radical is regenerated at the end of each catalytic cycle, is open to question. DFT calculations have shown that both possibilities are energetically feasible and that a homolytic reaction pathway, in general, is thermodynamically very plausible.[115] The same study also found that the rate-limiting step of the reaction is likely to be addition of the benzyl radical to fumarate. Further studies on this interesting enzyme are needed to provide evidence for (or against) the mechanism in Figure 14.

Very little is known about the activase involved in glycyl radical generation, which has not been purified thus far. Genetic analyses of *T. aromatica* have shown that the gene encoding for the putative activase is the first in an operon with the structural genes of BSS.[100] The predicted sequence of the activase gene encodes for a protein of 36 kDa and contains the characteristic Cys motif close to the N-terminus (see Figure 10). Interestingly, the sequence also contains two cysteine clusters matching the typical ferredoxin sequence $C-X_2-C-X_2-C-X_3-C$. The role of these putative clusters in the activase is unknown and will have to await further investigation.

IV. SAM and Sulfur Insertion

A. Biotin Synthase

1. Biosynthesis of Biotin

Biotin is an essential vitamin for humans that is only produced by plants and certain microorganisms. Although biotin is present in only very small amounts in mammalian cells, its function as a coenzyme in various carboxylation reactions is of utmost importance, especially in gluconeogenesis. The genes involved in the biosynthetic pathway of biotin have been described in many organisms, including *E. coli*,[116] *B. subtilis*,[117] *B. sphaericus*,[118] *Saccharomyces cerevisiae*,[119] and higher plants.[120] In *E. coli*, an operon that consists of five genes, *bio*ABFCD, encodes for enzymes that catalyze the last four steps in the pathway—only *bio*C appears to function in an earlier step of biotin synthesis. The four steps from pimeloyl CoA to biotin appear to be common for most bacteria and plants (Figure 15). The last step in this pathway, which is catalyzed by the BioB protein (product of the *bio*B gene), involves insertion of sulfur adjacent to the ureido ring of dethiobiotin. This is the chemically most difficult step in the pathway because it involves activation of unreactive methyl and methylene hydrogens at carbons 9 and 6, respectively. It is also the least understood step in terms of the enzymology of the reaction. Interestingly, before identification of the *bio* operon and isolation of the proteins involved, in vivo labeling studies from the laboratories of Parry and Marquet had provided

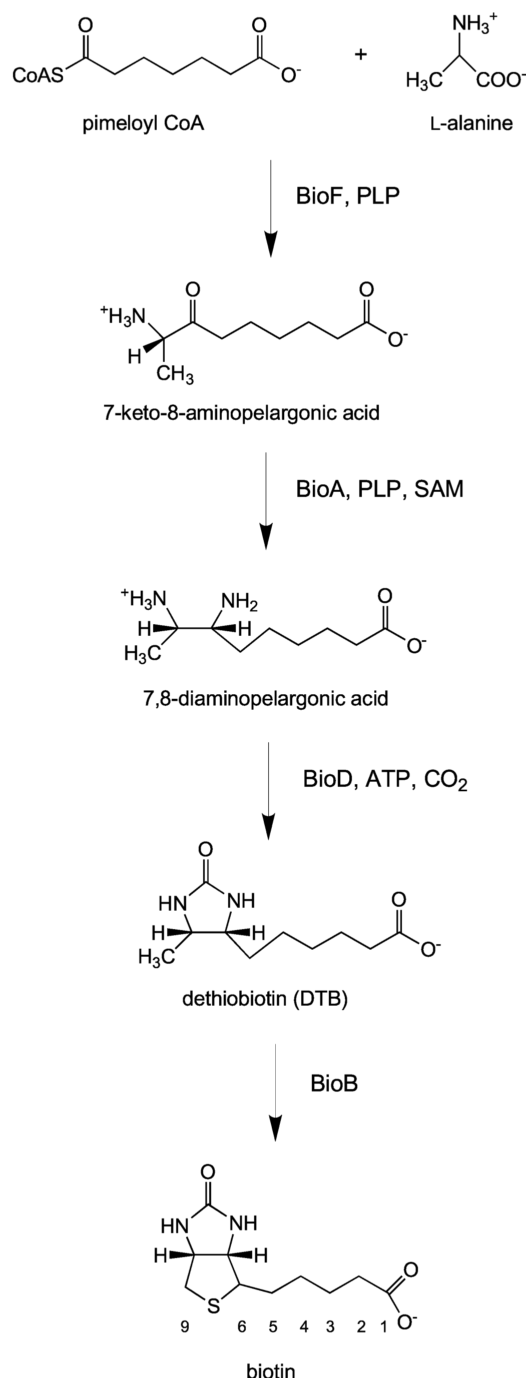


Figure 15. Biosynthetic pathway of biotin. X-ray crystal structures are available for all enzymes except BioB. The carbon atoms of biotin are labeled according to standard nomenclature.

insightful information about the last step of biotin synthesis. These groups showed that one hydrogen atom each from C6 and C9 of dethiobiotin (DTB) were removed in its conversion to biotin.[121-123] In fact, by using DTB stereospecifically tritiated at C6, it was shown that the *pro-S* hydrogen was removed during conversion to biotin.[123] Excellent reviews on the biosynthesis of biotin and other sulfur-containing compounds are available.[124-126]

2. Molecular Properties of BioB and the Role of Cofactors

Biotin formation in cell free extracts of *E. coli* was first measured in 1992. By using a microbiological

assay, Ifuku et al. showed that enzyme activity required SAM, DTB, NADPH, fructose 1,6-bisphosphate, Fe^{2+}, and KCl.[127] Shaw and co-workers developed a radiochemical assay for biotin formation employing ^{14}C-labeled DTB as substrate.[128] Their work with cell-free extracts of BioB required the addition of the following multiple low-molecular components: SAM, cysteine/cystine, thiamine pyrophosphate, Fe^{2+}, NADPH, and either asparagine, aspartate, glutamine, or serine. Enzyme activity was also dependent on the following proteins: flavodoxin, flavodoxin reductase, and a thiamine pyrophosphate-dependent protein. Recombinant BioB from *E. coli* was first isolated by Flint and co-workers.[129] Subsequently, biotin synthase from *B. sphaericus* was purified by the Marquet group.[130] BioB is a homodimer with a native molecular mass of 78 kDa. Interestingly, the reported requirements for biotin synthase activity have varied somewhat between laboratories. The requirement for SAM and DTB has remained unrefuted, whereas the need for other small molecules and protein components has been debated. It should also be noted that in vitro activity measurements of biotin synthase have never resulted in more than a single active-site turnover, which indicates the lack of essential component(s) for sustained catalytic activity (see section IV.A.3).

The role of SAM as a hydrogen atom carrier between substrate and cofactor was firmly established by experiments from the Marquet group. Using tritium-labeled SAM in the appropriate positions and BioB from either *E. coli* or *B. sphaericus*, they were able to show that methionine and 5′-deoxyadenosine were produced in a 1:1 ratio, while both compounds were formed in a 3:1 ratio with respect to biotin.[131] The authors interpretation was that two equivalents of SAM were required for biotin formation, and they attributed the additional consumption of SAM to abortive processes. These researchers went on to show that hydrogen transfer from the substrate into 5′-deoxyadenosine is catalyzed by biotin synthase. Using various deuterium-labeled DTB molecules, followed by mass spectrometry analysis of the labeled 5′-deoxyadenosine, they concluded that two equivalents of SAM were required for cleavage of the C−H bonds at positions 9 and 6 of DTB.[132] Due to the complexity of the amount of deuterium transferred to 5′-deoxyadenosine in these experiments, the authors were not able to distinguish between a direct transfer of hydrogen from the substrate by a 5′-deoxyadenosyl radical and a reaction involving an intermediate protein-based radical. It should be noted that, thus far, a free radical species has not been detected in the biotin synthase reaction, although all the experimental evidence is consistent with a radical-type mechanism.

Shaw and co-workers were able to demonstrate the formation of a chemical intermediate in a crude extract of BioB.[133] Using ^{14}C-labeled DTB as substrate, isolated a compound by HPLC chromatography that showed chromatographic behavior different than that of both DTB and biotin. Importantly, this intermediate could be administered to reaction mixtures of biotin synthase and converted to biotin,

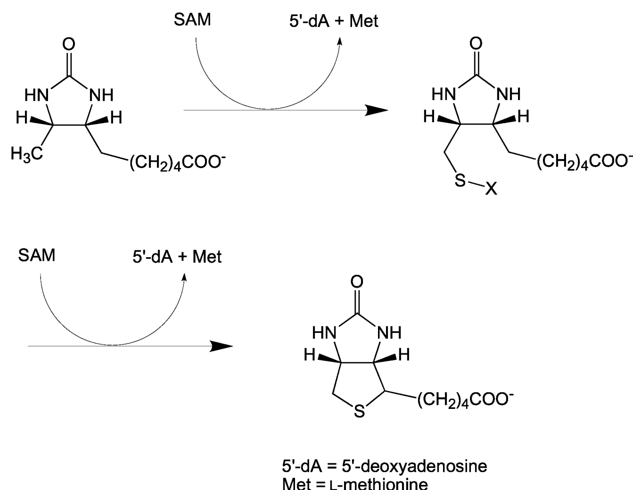


Figure 16. Minimal mechanism for biotin synthase. Two equivalents of SAM are required for activation of the C−H bonds at C9 and C6 of dethiobiotin, respectively.

providing evidence for its chemical competence in the reaction. The stoichiometry of SAM consumption was also established by using a coenzyme with a ^{14}C-label in the methionine moiety. The results were similar to those reported by the Marquet group; however, by analyzing the time-dependent formation of the intermediate, methionine, and biotin, these researchers were able to establish that one equivalent of SAM was consumed to form the intermediate and a second equivalent was needed to produce biotin.[133] Although a structural characterization of this intermediate could not be established, it did contain sulfur derived from ^{35}S-labeled cystine.

A minimal reaction sequence that summarizes the results described above is presented in Figure 16. DTB is converted into a sulfur-containing intermediate, with the concomitant cleavage of SAM into methionine and 5′-deoxyadenosine. In a second step, another molecule of SAM is used to yield biotin. That activation of the C−H bond at C9 occurs before C6 was established by feeding experiments in which 9-mercaptoDTB (X = H in Figure 16) was shown to be transformed into biotin by *B. sphaericus*, whereas the two epimeric C6 thiols were not.[134] Further indirect evidence for the intermediacy of 9-mercaptoDTB in the biotin synthase reaction has also been provided by numerous other studies.[120,135,136] However, whether the intermediate isolated by Shaw and co-workers is 9-mercaptoDTB remains unknown. The experimental results from the Marquet and the Shaw groups firmly established biotin synthase as a new member of the family of enzymes that use SAM to facilitate radical chemistry. The enzyme can, however, be put into a new group as one that catalyzes sulfur insertion and uses SAM as a cosubstrate for that task.

3. Iron−Sulfur Clusters of BioB and the Origin of Sulfur in Biotin

The first purified preparations of biotin synthase from *E. coli* and *B. sphaericus* were reported to contain Fe−S clusters, although no detailed description was provided.[129,130] These preparations, as well as purification schemes developed by other investiga-

tors, have always been performed under aerobic conditions, followed by anaerobic reconstitution of the enzyme. As a consequence, the as-purified protein always contains a nonfunctional form of the Fe−S cluster(s). The first detailed spectroscopic characterization of BioB was performed by Duin et al., which established the presence of a diamagnetic $S = 0$, $[2Fe\text{-}2S]^{2+}$ cluster in each dimer subunit of the as-purified protein, as determined by resonance Raman and VTMCD spectroscopies.[137] Interestingly, a Raman breathing mode at 301 cm^{-1} was consistent with an incomplete cysteinyl coordination. Incubation with dithionite in 60% ethylene glycol resulted in a nearly stoichiometric conversion to an $S = 0$, $[4Fe\text{-}4S]^{2+}$ cluster, whereas prolonged incubation with excess dithionite produced a paramagnetic $[4Fe\text{-}4S]^{+}$ cluster. The coordination environment of the $[4Fe\text{-}4S]^{2+}$ cluster was ambiguous, displaying a breathing mode at 338 cm^{-1}, which lies between typical values for a 4S coordination (∼335 cm^{-1}) and 3S, 1O/N coordination (∼340−342 cm^{-1}).[137,138] Characterization of the 1+ oxidation state by EPR spectroscopy showed that the Fe−S cluster displayed mixed spin states, with $S = 1/2$ ($g = 2.044, 1.944, 1.914$) and $S = 3/2$ ($g = 5.6$) ground states.[137] These results prompted the investigators to conclude that the [4Fe-4S] cluster is formed by reductive dimerization at the interface of subunits, akin to what had been proposed for ARR-activase,[40] and that cluster interconversion could serve a regulatory role in response to oxygen.

More recent work has provided evidence against the bridging of clusters at the dimer interface. Jarrett and co-workers demonstrated the formation of two $[4Fe\text{-}4S]^{2+}$ clusters per mole of BioB upon incubation with iron, sulfide, and DTT and complete reduction of these clusters by dithionite.[139] Interestingly, upon excluding 60% ethylene glycol from the samples, reduction by dithionite resulted exclusively in the formation of $[4Fe\text{-}4S]^{+}$ clusters, and reversibility between the 1+/2+ redox pair was established, suggesting that this form is the redox-active form of BioB under strict anaerobic conditions. Fontecave and co-workers confirmed the presence of one [4Fe-4S] cluster per monomer of anaerobically prepared samples of ^{57}Fe-reconstituted BioB by Mössbauer spectroscopy.[140] Both oxidation states were stable under anaerobic conditions, and quantitative conversion to the $[2Fe\text{-}2S]^{2+}$ cluster was seen upon exposing the samples to air, an observation that had, in fact, been noted in a previous Mössbauer study of BioB.[141]

Like other members of the radical SAM superfamily, biotin synthase contains a conserved Cys motif ($C{-}X_3{-}C{-}X_2{-}C$) which is thought to provide ligands for the [4Fe-4S] cluster of the protein. In BioB, these residues are Cys53, Cys57, and Cys60. The role of these and other less conserved cysteines have been probed by site-directed mutagenesis studies.[142−145] In the most comprehensive study, single Cys→Ala mutations of all eight cysteine residues in BioB were prepared, and the variants were assayed for activity and characterized spectroscopically.[144] Interestingly, only two mutations (C276A, C288A) displayed behavior similar to that of the wild-type protein, whereas the other six Cys→Ala mutants rendered the enzyme inactive, even though these proteins were still able to assemble a [4Fe-4S] cluster under highly reducing conditions. If one assumes that the [4Fe-4S] cluster of BioB has only three coordinated cysteine residues (Cys53, Cys57, Cys60), in accordance with work on PFL-AE, ARR-AE, and lysine 2,3-aminomutase, the role of the other three cysteines (Cys97, Cys128, Cys188) remains unknown (see below).

The interplay between SAM and the [4Fe-4S] cluster of BioB was studied by Fontecave and co-workers.[145] They showed that reductive cleavage of SAM accompanies oxidation of the $[4Fe\text{-}4S]^{+}$ cluster. Using EPR and Mössbauer spectroscopies in conjunction with methionine quantitation, a direct correlation was obtained with the rate and amount of 1+/2+ cluster conversion and formation of methionine. These results are similar to those observed for other enzymes in the radical SAM superfamily and provide evidence for the role of SAM as a source of a 5′-deoxyadenoyl radical in catalysis. Mutations of Cys53, Cys57, and Cys60 abolished the reductive cleavage of SAM, providing further evidence for their role as ligands to the cluster. An interesting observation in this study was that the substrate (DTB) was not required for the cleavage reaction—a surprising result, given the substrate requirement for cleavage of SAM in lysine 2,3-aminomutase, PFL-activase, and ARR-activase. It appears that reductive cleavage of SAM is more thermodynamically favorable for BioB than the aforementioned enzymes, because the requirement for substrate may, in general, provide a driving force for this highly unfavorable reaction via coupling to the formation of more stable substrate-derived radicals. The fate of the 5′-deoxyadenosyl radical formed upon reductive cleavage of SAM was not addressed in this study.

The origin of sulfur in biotin has long been a matter of some controversy. Cysteine has been described as the most likely source of sulfur in vivo[146] and in crude cell extracts.[127,136] However, incorporation of sulfur from cysteine does not occur with the purified enzyme.[130,147] The Marquet group has shown that reconstitution of apoprotein from either *E. coli* or *B. sphaericus* with $FeCl_3$, $Na_2[^{34}S]$, and DTT yields ^{34}S-labeled biotin with ∼65% incorporation.[148] These results were corroborated by another study where biosynthetic incorporation of sulfur into BioB by growth in the presence of [^{35}S]cysteine and [^{35}S]-sulfide showed significant transfer of radioactivity into biotin.[149] These experiments suggest that the sulfur donor in biotin synthase is the Fe−S cluster of the protein itself, which implies that BioB is acting as a reagent in the reaction but not as an actual catalyst. This is certainly consistent with the fact that no preparations of biotin synthase have ever been shown to undergo more than a single turnover.

The idea that the [4Fe-4S] clusters of BioB would serve both as a redox catalyst, providing an electron for the cleavage of SAM, and as a source of sulfur in biotin is certainly puzzling. Recent experiments by Jarrett and co-workers have provided an elegant solution to this dilemma. By doing careful iron, sulfide, and spectrophotometric analysis, they showed

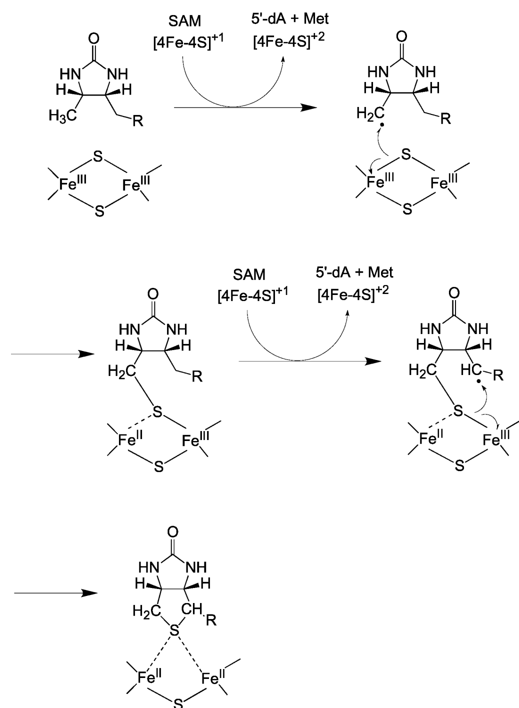


Figure 17. Mechanism of sulfur insertion into dethiobiotin, as proposed by Jarrett and co-workers.

that aerobically purified BioB contains two apparently distinct $[2Fe-2S]^{2+}$ clusters per monomer.[150] These clusters can both be converted to the [4Fe-4S] form in the presence of excess iron, sulfide, and a strong reducing agent, but most importantly the redox potential of these Fe−S centers is different. One $[2Fe-2S]^{2+}$ cluster is converted to a $[4Fe-4S]^{2+}$ cluster with a midpoint potential of −140 mV. Further reduction to the 1+ oxidation state occurs at −450 mV. This cluster is proposed to serve as the reducing agent for SAM. The other cluster has a much lower potential and is converted directly to the $[4Fe-4S]^{+}$ form at −505 mV. This cluster, in its $[2Fe-2S]^{2+}$ form, is proposed to serve as the sulfur donor for biotin formation. Enzyme incubated under anaerobic assay conditions, where the electrochemical potential is −330 ±30 mV, an environment that is similar to that of aerobically grown *E. coli*, yields protein that has one [4Fe-4S] center and one [2Fe-2S] center per monomer, as expected.[150] This newly identified [2Fe-2S] center is presumably ligated by Cys97, Cys128, and Cys188, residues that are important for catalysis.[144] By following changes in the iron and sulfide content of BioB and changes in the UV−vis and EPR spectra of the protein, Jarrett's group was able to demonstrate that the $[2Fe-2S]^{2+}$ center undergoes selective degradation under turnover conditions, while the [4Fe-4S] center remains intact.[151] A mechanism for the oxidative insertion of sulfur into DTB based on these results is presented in Figure 17.

Further evidence for the existence of two distinct Fe−S clusters in BioB was provided in a very recent Mössbauer study,[152] and the results are shown in Figure 18. ^{57}Fe-labeled BioB reconstituted with ^{57}Fe in the presence of Na_2S and $FeCl_3$, followed by gel filtration, yields a ∼1:1 mixture of $[2Fe-2S]^{2+}$ and $[4Fe-4S]^{2+}$ clusters in addition to mononuclear iron

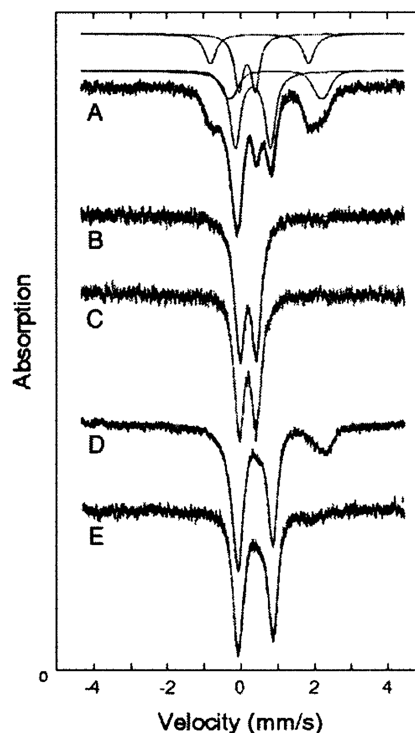


Figure 18. Mössbauer spectra of biotin synthase (BioB): (A) ^{57}Fe-BioB reconstituted with $^{57}FeCl_3$, Na_2S and DTT; (B) as-isolated ^{57}Fe-BioB containing only $[2Fe-2S]^{2+}$ clusters; (C) ^{57}Fe-BioB reconstituted with unlabeled $FeCl_3$, Na_2S and DTT; (D) ^{57}Fe-BioB containing primarily $[4Fe-4S]^{2+}$ clusters generated by reduction with $Na_2S_2O_4$ in 60% ethylene glycol; (E) unlabeled BioB reconstituted with $^{57}FeCl_3$, Na_2S, and DTT. Simulated spectra used to fit spectrum A are shown on top. The Mössbauer parameters for the different states of iron are as follows: $[2Fe-2S]^{2+}$, $\delta = 0.27$ mm/s, $\Delta E_Q = 0.49$ mm/s; $[4Fe-4S]^{2+}$, $\delta = 0.42$ mm/s, $\Delta E_Q = 1.00$ mm/s; $Fe^{2+}S_4$, $\delta = 0.63$ mm/s, $\Delta E_Q = 2.80$ mm/s; $Fe^{2+}(O/N)_{5/6}$, $\delta = 1.08$ mm/s, $\Delta E_Q = 2.60$ mm/s. Reprinted with permission from ref 152. Copyright 2002 American Chemical Society.

(Figure 18A). Most importantly, identical reconstitution with ^{56}Fe yields only a $[2Fe-2S]^{2+}$ cluster (Figure 18C) or a very similar result, as seen for the as-purified enzyme (Figure 18B). This means that the added ^{56}Fe, which is invisible by Mössbauer spectroscopy, must be used exclusively to form the [4Fe-4S] cluster. In a complementary experiment, ^{56}Fe-BioB was reconstituted with ^{57}Fe, and the Mössbauer spectrum showed only the presence of a [4Fe-4S] center (Figure 18E). In other words, the [2Fe-2S] cluster in the as-isolated protein remains intact during the reconstitution process, which is performed under mild reducing conditions.[152]

The major challenge facing investigators studying the biotin synthase reaction is to demonstrate multiple turnovers of the enzyme. If, in fact, the sulfur is derived from a [2Fe-2S] center, a reconstitution process must be in place in vivo that would render the system catalytic. Currently, these additional factors are unknown. A valuable insight into this problem was provided by the Marquet group, which showed that apo-biotin synthase could be reconstituted by NifS from *Azetobacter vinelandii* in the presence of iron and [^{35}S]cysteine, resulting in the formation of ^{35}S-labeled biotin.[153] NifS is known to catalyze sulfide incorporation into Fe−S clusters,[154]

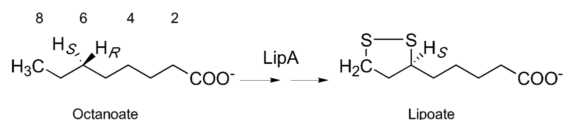


Figure 19. Reaction catalyzed by lipoate synthase (LipA).

presumably via its activity as a cysteine desulfurase, which produces S^{2-} and alanine.[155] Even though reconstitution of apoBioB into holoBioB was accomplished, sustained turnovers could not be established. An *E. coli* homologue of NifS called IscS, in combination with IscU, which is an Fe–S cluster scaffold protein, is involved in Fe–S cluster assembly.[156] Perhaps these proteins, in combination with other unidentified factors, hold the key for biotin synthase catalysis? An interesting twist to this problem was recently presented by Fontecave and coworkers, who showed that BioB itself displays cysteine desulfurase activity.[157] This activity is dependent on the presence of PLP, suggesting that this cofactor, which is also commonly used by other cysteine desulfurases, is also important for BioB. Although changes in the absorption spectrum of BioB were apparent upon sodium borohydride reduction of the Schiff base form of the cofactor, covalent attachment of the cofactor to BioB was not directly established in this study. In addition, two cysteine residues (Cys97, Cys128) were shown to be critical for the desulfurase activity. How this exciting discovery fits into the current thinking about the mechanism of biotin synthase is unclear at the moment, but biotin synthase is undoubtedly going to reveal more surprises.

B. Lipoate Synthase

The formation of lipoic acid involves the insertion of two sulfur atoms into carbons 6 and 8 of octanoic acid, respectively (Figure 19). Early in vivo labeling studies in *E. coli* showed that sulfur insertion at C8 is likely to precede that at C6, because [8-$^2H_2$]-thiooctanoic acid could serve as a good precursor for lipoic acid, whereas [6-$^2H_2$]-thiooctanoic acid was much poorer.[124] Hydroxylated intermediates could also be ruled out on the basis of the inability of labeled forms of hydroxyoctanoic acid(s) to yield lipoic acid. The stereochemistry at C6 has been elucidated using radiolabeled substrate. The results showed that the 6-*pro-R* hydrogen is removed upon sulfur insertion.[124]

Genetic and biochemical work indicated that insertion of both sulfur atoms into octanoate might be catalyzed by the product of the *lipA* gene.[158–160] The *lipA* gene encodes for a 321-amino-acid protein with an estimated molecular mass of 36 kDa.[159,161] Significant sequence similarities with BioB were also noted, which is not surprising, given the striking resemblance between the BioB and LipA reactions. In particular, a $C-X_3-C-X_2-C$ motif was found (Figure 10), which led investigators to propose that LipA would contain an Fe–S cluster.[159,161] Purification of LipA by the groups of Marletta[162] and Fontecave[163] indeed demonstrated that LipA is an iron–sulfur protein. The protein is purified mainly in a dimeric form, and characterization by UV–vis and resonance Raman spectroscopies showed that it contains mainly a $[4Fe\text{-}4S]^{2+}$ cluster.[162] Purification of the enzyme from insoluble inclusion bodies, followed by reconstitution under strict anaerobic conditions in the presence of iron, sulfide, and DTT, shows that each subunit binds one $[4Fe\text{-}4S]^{2+}$ cluster, as determined by iron and sulfide analysis and UV–vis and Mössbauer spectroscopies.[163] Reduction by dithionite or 5-deazaflavin leads to partial reduction to the $[4Fe\text{-}4S]^{+}$ form ($g = 2.04$, 1.93), and exposure of these clusters to O_2 results in degradation to the $[2Fe\text{-}2S]^{2+}$ form. These characteristics are, in fact, reminiscent of the spectroscopic properties of biotin synthase. Whether the sulfur donor of LipA is a [2Fe-2S] cluster, as has been proposed for BioB,[151] is currently unclear.

Studies on LipA have been hampered by the inability to demonstrate enzyme activity. Experiments by Jordan and Cronan provided evidence that the true substrate in the LipA reaction might be the acylated form of octanoic acid covalently attached to acyl-carrier protein (ACP).[164] This hypothesis was tested by Marletta and co-workers, who devised an assay for LipA and showed for the first time that this protein is a lipoate synthase.[165] The assay uses octanoyl-ACP as substrate and relies on the transfer of lipoyl-ACP to apo-pyruvate dehydrogenase complex (apo-PDC) via the action of LipB. Spectrophotometric detection of the product is accomplished through the reduction of 3-acetylpyridine adenine dinucleotide by PDC. Due to the nature of this assay, a considerable amplification response is obtained, which allows for the detection of minute quantities of product. However, only 0.032 mol of lipoyl-ACP per mole of LipA could be generated in the assay, which shows that additional components are lacking that are needed to yield a sustainable catalytic system. The researchers were also able to show that the reaction was dependent on the addition of SAM, which led to ~10-fold increase in activity, and that cluster reduction to the $[4Fe\text{-}4S]^{+}$ state was required.[165]

V. Concluding Remarks

The commonly held opinion that the major role of SAM in biology is to serve as a methylating agent has now been seriously challenged. One can argue, on the basis of the wide variety of reactions facilitated by this coenzyme, that the major role of SAM is to serve as an initiator of various enzymatic radical reactions. This exciting property of SAM has now ushered in a new field in “radical enzymology” and will undoubtedly provide new and previously unknown avenues for researchers in the field. Even though a lot has been learned over the past two decades about the radical role of SAM, there still remain many unanswered questions, especially with regard to the interplay between the Fe–S center(s) and SAM. Some of these questions will perhaps be answered when X-ray crystal structure(s) of radical SAM enzyme(s) become available.

Although SAM is not as structurally elegant as B_{12}, this relatively simple molecule provides enough functional diversity to be used in the same fashion as different forms of B_{12}. In fact, by the dual role of

SAM in Nature, this coenzyme is able to function in the same manner as both adenosylcobalamin and methylcobalamin, that is, to initiate radical catalysis via the 5′-deoxyadenosyl radical and as a methyl group donor, respectively. This intriguing property of SAM adds further credential to our metaphor for this coenzyme as a "rich man's adenosylcobalamin".

VI. Note Added after ASAP Posting

Two reference citations were changed after the original version of this paper was posted ASAP on 4/17/2003: in section III.A.2, paragraph 5, line 13, and in section IV.A.1, third line from the end. Additionally the reference citations in section III.C, paragraph 2, line 2, were removed. The correct version was posted 4/21/2003.

VII. References

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