

# Biological Radical Sulfur Insertion Reactions

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## I. Introduction

Sulfur-containing biochemicals are present in all living organisms. The molecules shown in Figure 1 are primary cell metabolites which are essential for life. Their formation depends on the introduction of sulfur atoms into metabolic precursors and the selective formation of C–S bonds. In cells, cysteine is the common source of the sulfur atom in these reactions. During biosynthesis of coenzyme A, no new C–S bond is formed since one is already present in cysteine. However, in the other cases the biosynthetic pathway involves the formation of new C–S bonds. A number of secondary (nonessential) metabolites also contain C–S bonds which are formed during biosynthesis. Some examples of these antibiotics and natural products are shown in Figure 2. Biological macromolecules such as proteins and transfer RNAs also contain a large amount of sulfur atoms (Figures 3 and 4). In the case of proteins, these are mainly contained within cysteines and methionines. In the case of a few enzymes, cross-linking reactions between the sulfur atom of cysteine and the side chains of tyrosine, tryptophan, histidine, aspartate, or glutamate are responsible for the generation of important cofactors (Figure 3). Finally, a number of thionucleosides have been characterized in tRNAs from both prokaryotes and eukaryotes. In Figure 4

are shown those present in *Escherichia coli*: 4-thiouridine ( $s^4U$ ), 2-thiocytidine ( $s^2C$ ), 5-methylamino-methyl-2-thiouridine ( $mnm^5s^2U$ ), and  $N^6$ -(4-isopentenyl)-2-methylthioadenosine ( $ms^2i^6A$ ).

The current understanding of sulfur compound biosynthesis is surprisingly quite limited, and when mechanisms have been proposed, they are mostly requiring further investigation.<sup>1</sup> The known biochemical mechanisms of C–S bond formation can be divided into two classes. The first class contains reactions with ionic chemical mechanisms that will be briefly discussed. The second one contains reactions that presumably proceed through radical mechanisms. In this review article, we will mainly focus on the progress made over the past few years in the understanding of radical sulfur insertion and the C–S bond-forming reactions during synthesis of biotin, lipoic acid, protein-bound cofactors, various antibiotics, and transfer RNAs. A special discussion on the chemical mechanism of biotin synthase is provided.

## II. C–S Bond Formation: Ionic Mechanisms

The first class of reactions involves the simple attack of an electrophilic precursor by a nucleophilic sulfur atom. This is the case for the synthesis of S-adenosylmethionine (SAM or AdoMet), which is the major methylating biological agent and a source of 5'-deoxyadenosyl radicals.<sup>2,3</sup> SAM is a product of a reaction between methionine and ATP, in which a S–C(5'-deoxyadenosyl) bond is created and phosphate and pyrophosphate are released (Figure 5). Among archaeobacteria, coenzyme M (2-mercaptoethanesulfonic acid) and coenzyme B (7-mercaptoheptanoylthreonine phosphate) play central roles in the anaerobic production of methane and are associated with energy conversion by methanogens.<sup>4,5</sup> Coenzyme M is derived from the reaction of cysteine with sulfoacetaldehyde. It occurs through a thiazolidine intermediate which then is cleaved, allowing the transfer of the sulfur atom from cysteine to the sulfonic acid moiety (Figure 5). The biosynthesis of coenzyme B is not well studied. However, it is likely that it occurs through similar mechanisms, once the precursor  $\alpha$ -ketosuberate has been decarboxylated to heptan-7-ol-oic acid (Figure 5).

The newest concept in ionic sulfur insertions derives from the discovery of two novel key intermediate sulfur donors, persulfides (R–S–SH) and thio-carboxylates (R–CO–SH). These compounds have a

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Etienne Mulliez was born in 1949 near Paris. He began his graduate study at Université René Descartes, Paris V, under the direction of Professor J. C. Chottard and received his Ph.D. in 1979. He then joined the group of Professor G. A. Hamilton at Penn State University, where he was introduced to the field of mechanistic enzymology. Back in Paris, he set up a research program on mechanistic studies on lipoxygenase, a non-heme iron protein using radical intermediates, and developed chemical models of their active sites. In 1991, he joined the group of Professor M. Fontecave in Grenoble to work on the anaerobic ribonucleotide reductase, a prototypical free-radical protein. In the recent years, his interests have leaned toward the chemistry and biochemistry of iron–sulfur clusters. He has been a Research Assistant at the CNRS since 1976.



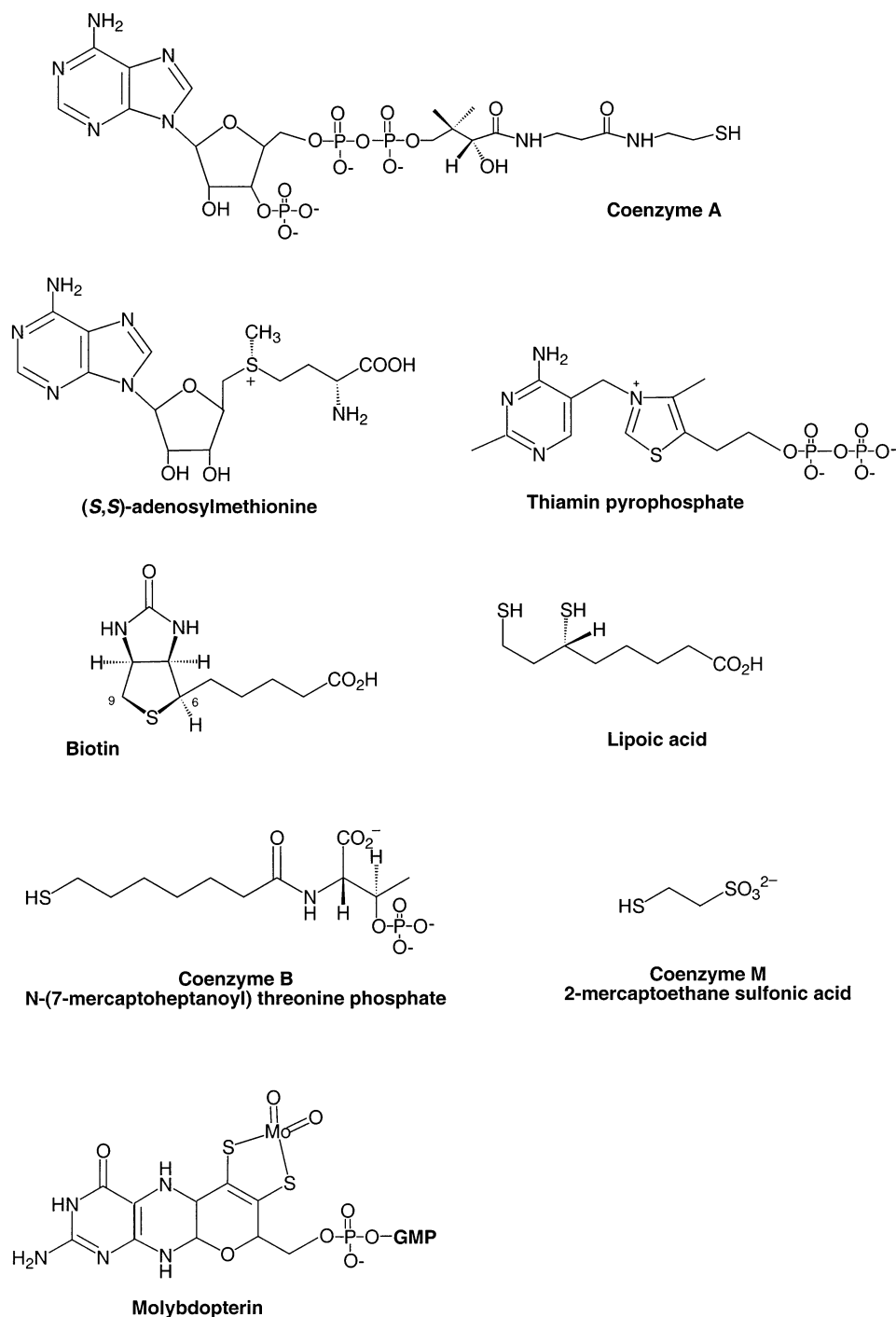
Sandrine Ollagnier-de Choudens was born August 19, 1971, in Gap, France, spent her younger years in Alpes, and received her Ph.D. from Grenoble University in 1997. She was a postdoctoral fellow at the Karlsruhe University, Germany, with Pr J. Rétey and then at Grenoble CEA, France, with Pr. M. Fontecave. She is mainly interested in the reactivity and biosynthesis of the iron–sulfur clusters and the mechanisms of metalloenzymes (biotin synthase, lipoate synthase).

reactive terminal sulfur atom which can be readily transferred to electrophilic precursors.<sup>6</sup> The simplest persulfide is thiocysteine produced by C-DES, a cystine lyase that cleaves cystine into cysteine persulfide (thiocysteine), yielding pyruvate and ammonia.<sup>7</sup> The protein-bound persulfide groups are located on protein cysteine side chains, whereas the thiocarboxylate groups specifically derive from modification of protein C-terminal carboxylates (Figures 6 and 7). The key enzymes in the biosynthesis and the utilization of persulfides are cysteine desulfurases. The various mechanistic and physiological properties of this class of enzymes and the general properties of persulfides are discussed in recent review articles.<sup>8,9</sup> In general the same organism contains several cysteine desulfurases. For example, *E. coli* has three such enzymes, named IscS (iron sulfur cluster), CSD (cysteine sulfinate desulfinase), and SufS (initially named CsdB).<sup>10,11</sup> These are pyridoxal-5'-phosphate (PLP)-dependent homodimer-

ic enzymes, present in all living organisms, that catalyze the conversion of L-cysteine to L-alanine and sulfane sulfur. The reaction proceeds via the formation of a protein-bound cysteine persulfide intermediate on a conserved cysteine residue (Figure 6).<sup>12,13</sup> A mechanism for this reaction has been established by elegant studies from D. Dean on NifS, a cysteine desulfurase from *Azotobacter vinelandii*.<sup>14,15</sup> The role of cysteine desulfurases in the biosynthesis of iron–sulfur clusters is well documented,<sup>16,17</sup> and there are increased evidences for their involvement in thiamin, thionucleosides and molybdopterin biosynthesis, as briefly discussed in the following.

It has been shown that the sulfur source in the biosynthesis of 4-thiouridine (s<sup>4</sup>U) in tRNAs is a persulfide group bound to a specific cysteine of a protein named ThiI (Figure 6). The reaction proceeds in the presence of ATP-Mg by nucleophilic attack on the C-4 of an activated uridine (Figure 6).<sup>18</sup> This persulfide is the result of a trans-persulfuration reaction in which the sulfur atom from a persulfide group on the IscS protein is transferred to ThiI (Figure 6).<sup>19,20</sup> Indeed, deletion of the *iscS* gene in *E. coli* results in a mutant strain that lacks 4-thiouridine in its tRNAs.<sup>21</sup>

It is likely that synthesis of another thionucleoside mnm<sup>5</sup>s<sup>2</sup>U (Figure 4) also proceeds by similar mechanisms. First it requires MnmA, an enzyme with sequence similarities to ThiI.<sup>22</sup> Second, a recent study using *E. coli* deletion mutants of each of the genes encoding cysteine desulfurases as well as double mutants showed that IscS is specifically required for the biosynthesis of mnm<sup>5</sup>s<sup>2</sup>U and s<sup>4</sup>U and, furthermore, accounts for more than 95% of the sulfur content in tRNA.<sup>19,23</sup> A similar study in *Salmonella enterica* reached the same conclusions.<sup>24</sup> The importance of IscS in this biosynthetic pathway is clearly demonstrated from the observations that overexpres-

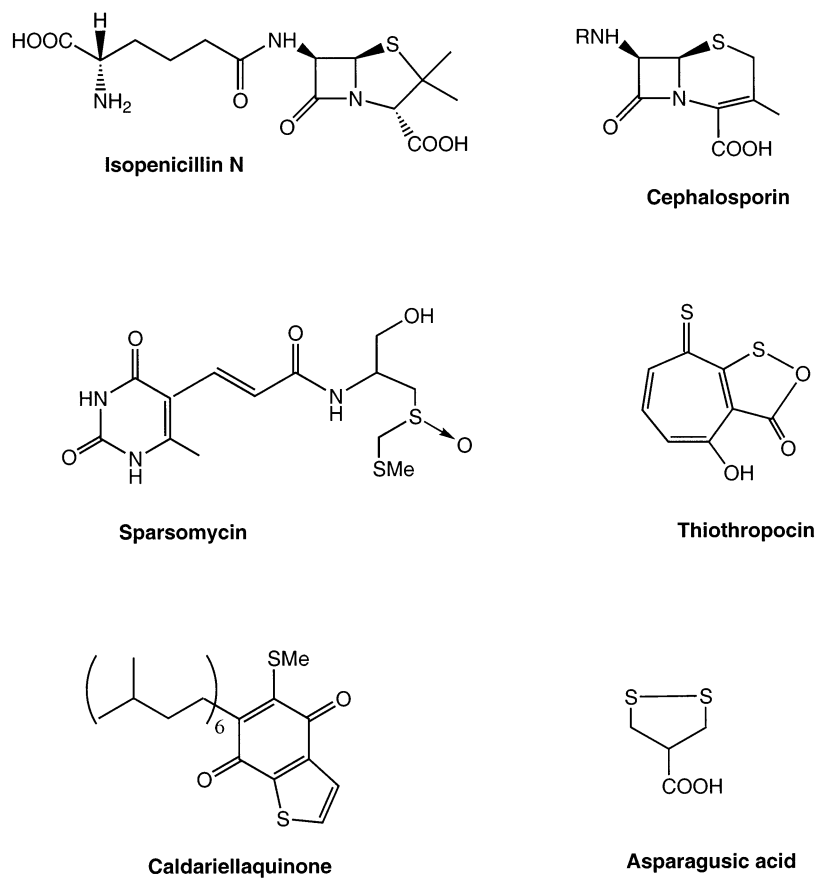


**Figure 1.** Structure of sulfur-containing cofactors (GMP, guanosine monophosphate).

sion of CSD and SufS did not complement any of the phenotypic characteristics of the *iscS* mutant and that the modified nucleoside profiles of a double *csd-sufS* mutant are superimposable to that of the parent strain. The cases of *s<sup>2</sup>C* and *ms<sup>2</sup>i<sup>6</sup>A* (Figure 4) are less clear since a poorly efficient IscS-independent pathway could account for the presence of small amounts of these nucleosides in logarithmically growing *E. coli* cells.<sup>23,24</sup> The case of *ms<sup>2</sup>i<sup>6</sup>A* will be addressed in the last section, as recent studies from our laboratory strongly suggested that in this case sulfur incorporation proceeds by radical mechanisms.

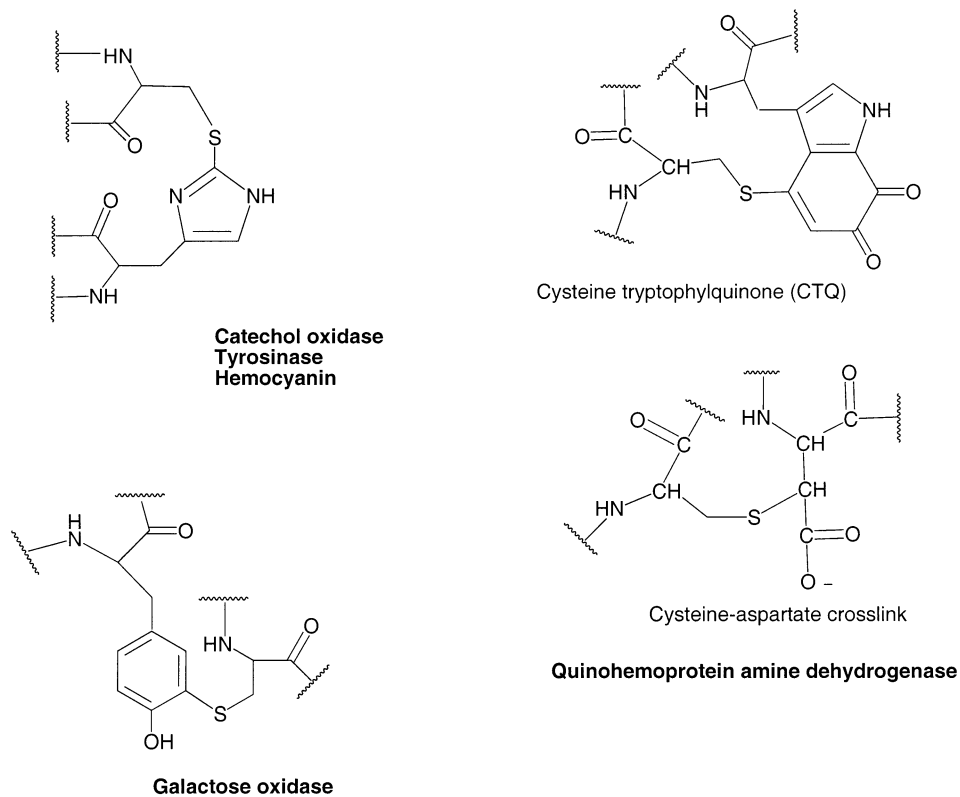
During biosynthesis of molybdopterin, which is a pyranopterin containing a unique dithiolene group

coordinating molybdenum or tungsten in all Mo- and W-enzymes except nitrogenase,<sup>25</sup> both sulfur atoms are provided by a thiocarboxylate at the C-terminus of MoaD, the small subunit of the molybdopterin synthase.<sup>26</sup> This thiocarboxylate is derived from the reaction of the adenylated carboxylate with a persulfide moiety provided by a cysteine desulfurase, either IscS, CSD, or SufS (Figure 7). CSD is the most efficient system for molybdopterin synthesis *in vitro*, but IscS and SufS can work as well.<sup>27</sup> The molybdopterin cofactor can be produced in an *iscS*<sup>-</sup> *E. coli* strain, indicating that *iscS* is not absolutely required for the *in vivo* activation of molybdopterin synthase.<sup>27</sup>

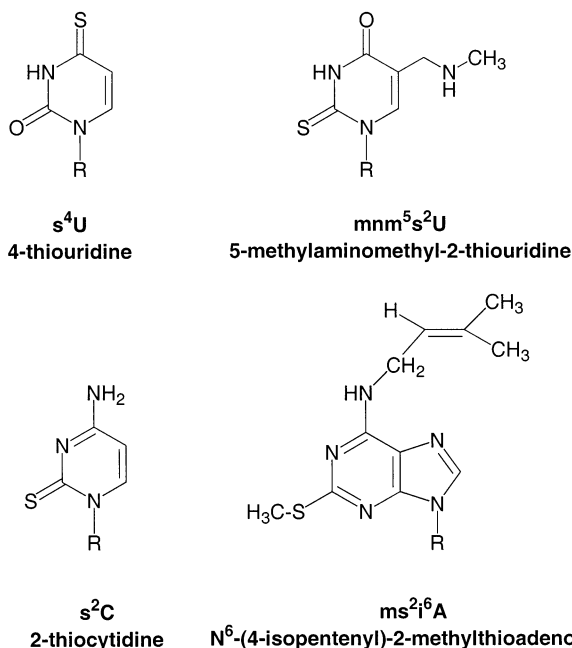


**Figure 2.** Structure of selected sulfur-containing antibiotics and natural products.

**Protein bound cofactors**



**Figure 3.** Structure of modified amino acids in proteins.



**Figure 4.** Structure of thionucleosides in tRNAs (R, ribose).

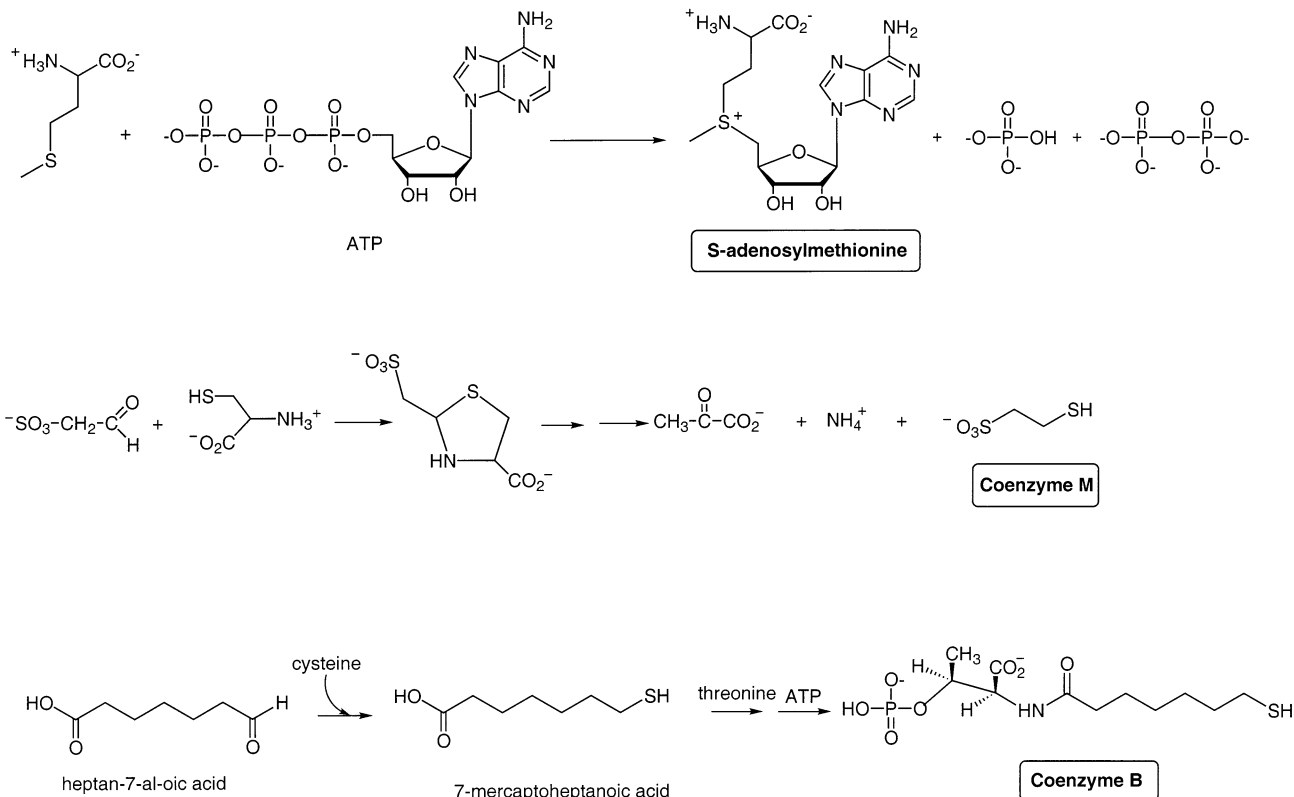
A thiocarboxylate on the ThiS protein has also been proposed to be a key intermediate during biosynthesis of the thiazole moiety of thiamin.<sup>6</sup> In addition to ThiS, several other proteins, ThiG, ThiH, and ThiF, participate in the conversion of tyrosine and deoxy-D-xylulose-5-phosphate to the thiazole moiety of thiamin (Figure 7).<sup>6</sup> However, the mechanism of the reaction is complex and far from being understood. An alternative sulfur donor for this reaction has been proposed to be a  $-CO-S-S-$  acyl disulfide interme-

diolate, bridging the carboxyl terminus of ThiS to a cysteine of ThiF.<sup>28</sup> Finally, it seems that ThiS does not receive a sulfur atom directly from the persulfide of IscS but from a ThiI-bound persulfide, which is generated through the action of IscS/cysteine (trans-persulfuration).<sup>29</sup> The *iscS*<sup>-</sup> *E. coli* strain requires thiamin and nicotinic acid for growth in minimal media, which implies that the IscS protein is critical for thiamin synthesis.<sup>21</sup>

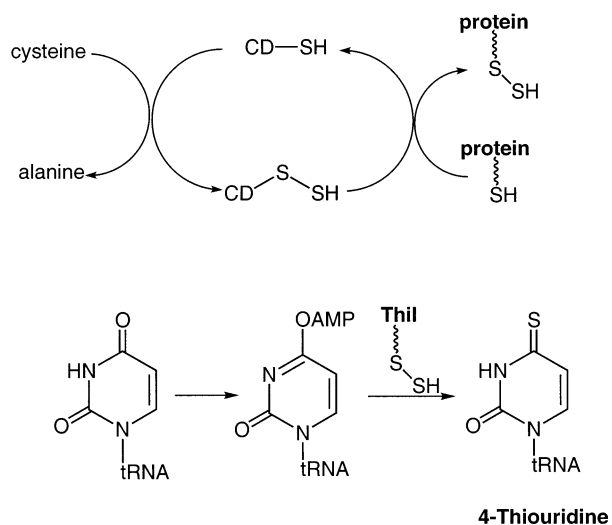
### III. C–S Bond Formation: Radical Mechanisms

#### A. Biosynthesis of Penicillins: The Isopenicillin Synthase

The final step of the isopenicillin biosynthetic pathway is a good example of a C–S bond-forming reaction through a radical mechanism.<sup>30,31</sup> Studies by J. Baldwin's group offer a beautiful and rare example of how a thorough combination of biochemical, synthetic, and structural approaches can delineate the mechanism of an enzymatic C–S bond formation with great detail. The precursor of isopenicillin is the linear tripeptide L- $\alpha$ -aminoacyl-cysteiny-D-valine (ACV) (Figure 8). Isopenicillin is created through an oxidative bicyclization reaction catalyzed by isopenicillin synthase (IPNS) (Figure 8). This reaction has no precedent in synthetic organic chemistry. The oxidant is provided by a single molecule of oxygen which is fully converted to two water molecules during the reaction. IPNS is a non-heme iron protein containing a single ferrous iron atom at the active site.<sup>32</sup> This active site can now be described with some accuracy on the basis of the recently determined three-dimensional structures of the protein under



**Figure 5.** Biosynthetic pathways for S-adenosylmethionine, coenzyme M, and coenzyme B.

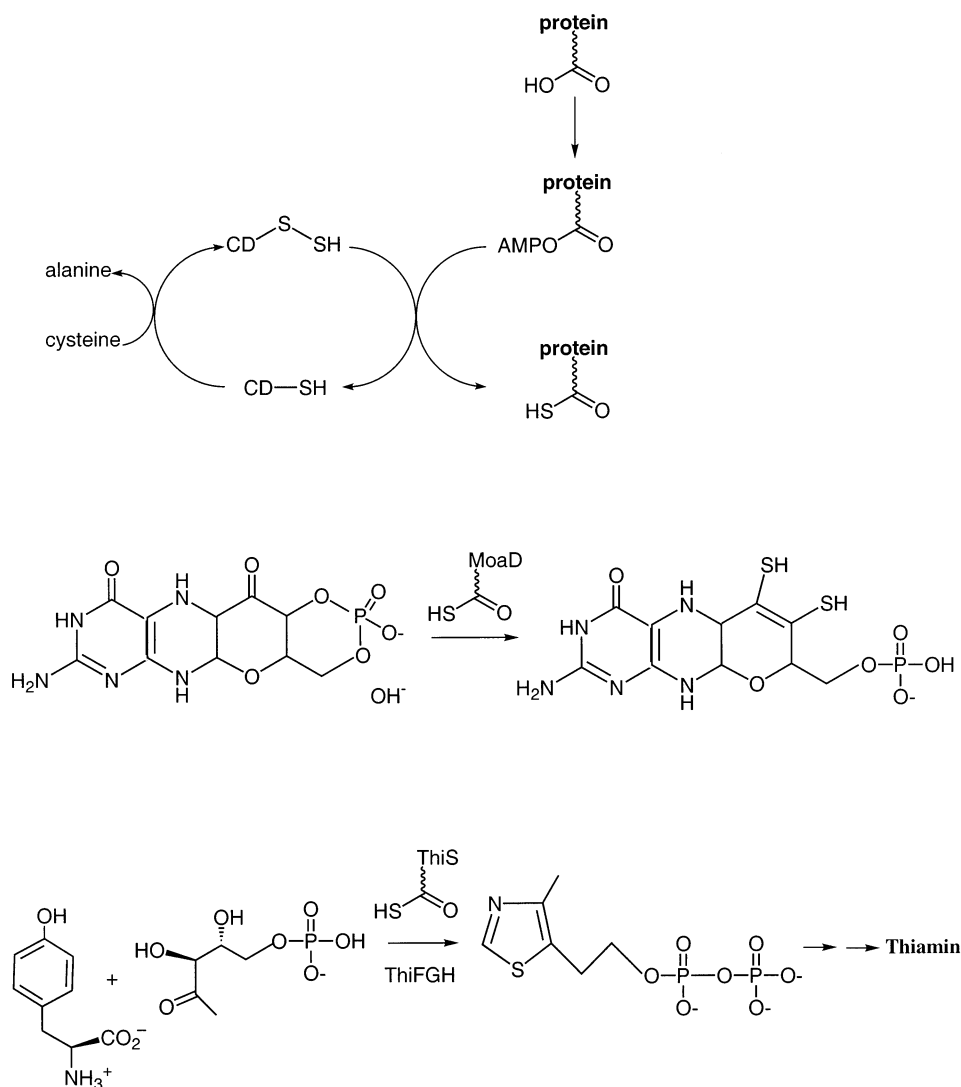


**Figure 6.** Protein-bound persulfides in 4-thiouridine biosynthesis (AMP, adenosine monophosphate; CD-SH, cysteine desulfurase).

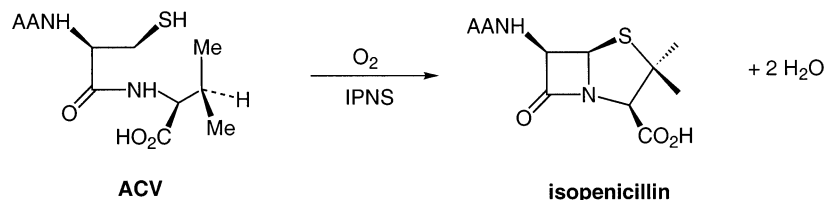
different forms.<sup>32–34</sup> The iron center is chelated by two histidines and one aspartate from the polypeptide chain and offers enough coordination sites for

binding molecular oxygen and the ACV substrate, through the deprotonated thiol group from its cysteine moiety (Figure 9). The reaction occurs in two steps. The first one consists of the formation of the  $\beta$ -lactam ring and is partially rate-limiting. Iron binds oxygen, and the resulting  $\text{Fe}(\text{O}_2)$  complex is first converted into an intermediate peroxide  $\text{Fe}-\text{O}-\text{OH}$  species during a reaction which involves a two-electron oxidation of the thiol of the substrate to give a thioaldehyde while  $\text{O}_2$  is reduced to the peroxide (Figure 9). Formation of the  $\beta$ -lactam ring proceeds, with concomitant formation of a ferryl  $\text{Fe}(\text{IV})=\text{O}$  species. The closure of the thiazolidine ring follows, during a two-electron oxidation of the ligand by the ferryl moiety, which is converted back to the initial ferrous state (Figure 9). The participation of the Fe-oxo species is suggested from the reaction with an allylglycyl analogue, which gives products arising from the nucleophilic attack of the thiolate on an intermediate epoxide (Figure 10). The epoxide is generated during the oxo transfer from the ferryl center to the double bond.<sup>35</sup>

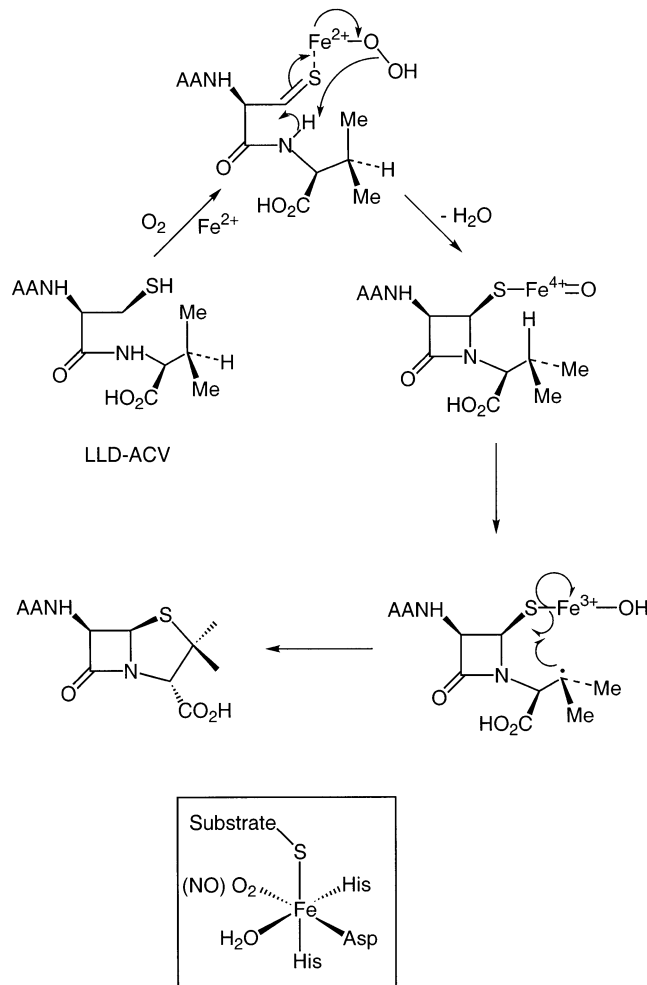
Even though no free intermediate radicals could be spectroscopically detected during this second step,



**Figure 7.** Protein-bound thiocarboxylates in biosynthesis of the molybdopterin cofactor and thiamin (AMP, adenosine monophosphate; CD-SH, cysteine desulfurase).



**Figure 8.** The reaction catalyzed by isopenicillin synthase (AA, L- $\alpha$ -aminoadipoyl).



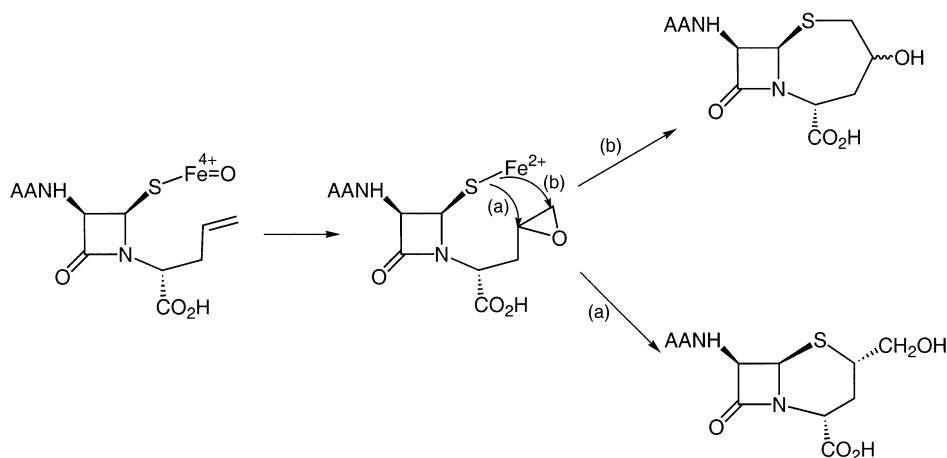
**Figure 9.** The iron center and the proposed reaction mechanism of isopenicillin synthase (AA, L- $\alpha$ -aminoadipoyl).

studies with substrate analogues, labeled substrates, and inhibitors support a radical mechanism.<sup>30</sup> The ferryl intermediate is proposed to abstract a hydrogen atom from the isopropyl group, and the resulting isopropyl radical combines with the iron-bound thiolate sulfur atom, thus closing the thiazolidine ring (Figure 9). With ACV analogues in which the valinyl residue was replaced by amino acids containing cyclopropylmethyl groups, radical clock products derived from the rearrangement of a cyclopropyl-carbinyl radical are observed.<sup>36</sup> This is in agreement with the proposed radical mechanism. Furthermore, with substrates containing selectively deuterated cyclopropyl groups, the reaction produced 3-exo-methylene homocephans with the single deuterium at C-5, present as a 1:1 mixture of epimers, in agreement with the mechanism shown in Figure 11.<sup>36</sup>

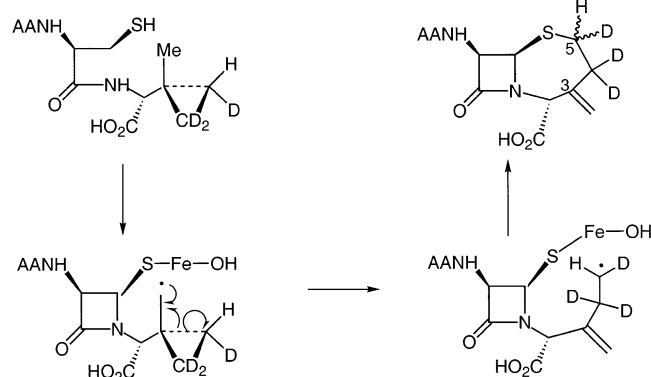
## B. Biosynthesis of the Cofactor of Galactose Oxidase

Galactose oxidase is a monomeric copper-containing enzyme that catalyzes the stereospecific oxidation of primary alcohols to their corresponding aldehydes.<sup>37</sup> The crystal structure of the enzyme from *Fusarium* sp. shows a thioether bond resulting from the covalent cross-link between S $\gamma$  of a cysteine residue and C $\epsilon$  of an adjacent tyrosine residue.<sup>38</sup> This modified tyrosine serves as a ligand to the catalytically essential copper atom and is in the form of a phenoxyl radical when the enzyme is in an active state.<sup>39</sup> The proposed catalytic cycle is shown in Figure 12.<sup>40</sup> The reaction is initiated by the abstraction of a hydrogen atom from the substrate by the Cu(II)-radical center, followed by electron transfer to copper. In this first step a two-electron oxidation of the alcohol substrate to the corresponding aldehyde occurs and the Cu(II)-radical center is converted into the Cu(I)-tyrosine complex. In the second step the active Cu(II)-radical center is regenerated through the reaction of the Cu(I) center with molecular oxygen, with the concomitant production of one equivalent of hydrogen peroxide (Figure 12). The role of the tyrosine-cysteine cross-link for the enzymatic reaction is still unclear: it might contribute to lowering the one-electron redox potential of the tyrosyl radical from 1 to 0.4 V, thus facilitating the formation and stabilization of the radical but a structural role cannot be excluded.<sup>41</sup>

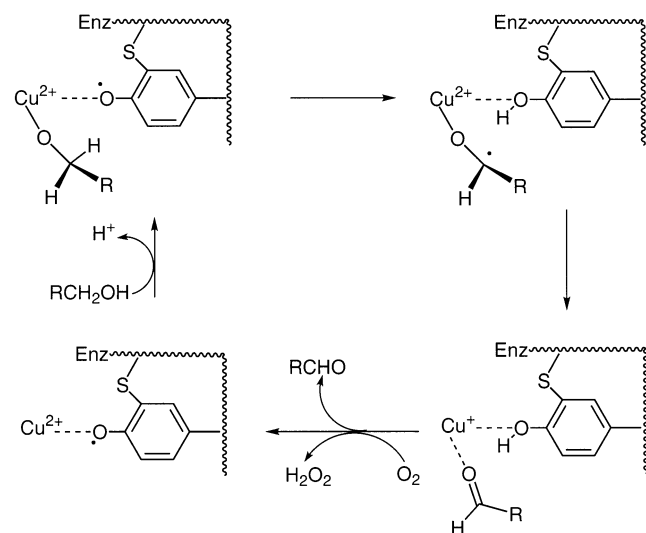
Very little is known about the formation of the thioether bond of the cofactor and the details of the chemical mechanism are only speculative. Nevertheless, *in vitro* experiments have shown that generation of the thioether bond is not an enzymatic reaction but simply occurs during incubation of the apoprotein form of galactose oxidase with Cu(II) and oxygen.<sup>42</sup> This suggests that an oxidative self-processing reaction occurs *in vivo* as well, with no requirement for other proteins or enzymes during the assembly of the catalytically active enzyme. A speculative radical mechanism leading to the cofactor is shown in Figure 13.<sup>43</sup> During the first step of the reaction Cu(II) binds to the unmodified tyrosine. Indeed, on aerobic exposure of the apoprotein to Cu(II) sulfate broad transitions at 410 and 750 nm appear as an indication of metal binding. With time, the band at 410 nm shifts to 445 nm, whereas that at 750 nm shifts to 800 nm and the intensity increases. These changes reflect the generation of the tyrosyl radical from the initial Cu(II)-protein complex. In this complex, the tyrosine ligand is activated as the result of electron delocalization to Cu(II) and appearance of a radical density on the carbon at the ortho position with regard to the hydroxyl group. Then, the electron-enriched Cu



**Figure 10.** Evidence for an intermediate ferryl species in isopenicillin synthase (AA. L- $\alpha$ -aminoadipoyl).



**Figure 11.** Evidence for an intermediate free radical in isopenicillin synthase (AA. L- $\alpha$ -aminoadipoyl).



**Figure 12.** The reaction catalyzed by galactose oxidase.

atom binds and reduces molecular oxygen. Formally in the superoxide oxidation state, oxygen is proposed to couple to the aromatic ring of the tyrosyl radical generating a labile peroxide which can be attacked by the nucleophilic thiolate group of an adjacent cysteine (Figure 13, pathway A). The observation that peroxides are the major products during reactions between radiolytically generated phenoxyl radicals and superoxide radicals<sup>44</sup> supports this mechanism. Alternatively, Cu-bound activated oxygen may serve to generate a thiyl radical which then couples to the

tyrosyl radical (Figure 13, pathway B). Superoxide radicals have been indeed shown to have the capacity to oxidize thiols.<sup>45</sup> These hypotheses can explain the formation of the thioether bond but not that of the final radical. Whether this occurs subsequently during a reaction with a second molecule of oxygen remains to be established. Model studies of cofactor synthesis, still not available, might be useful for understanding this complex mechanism.

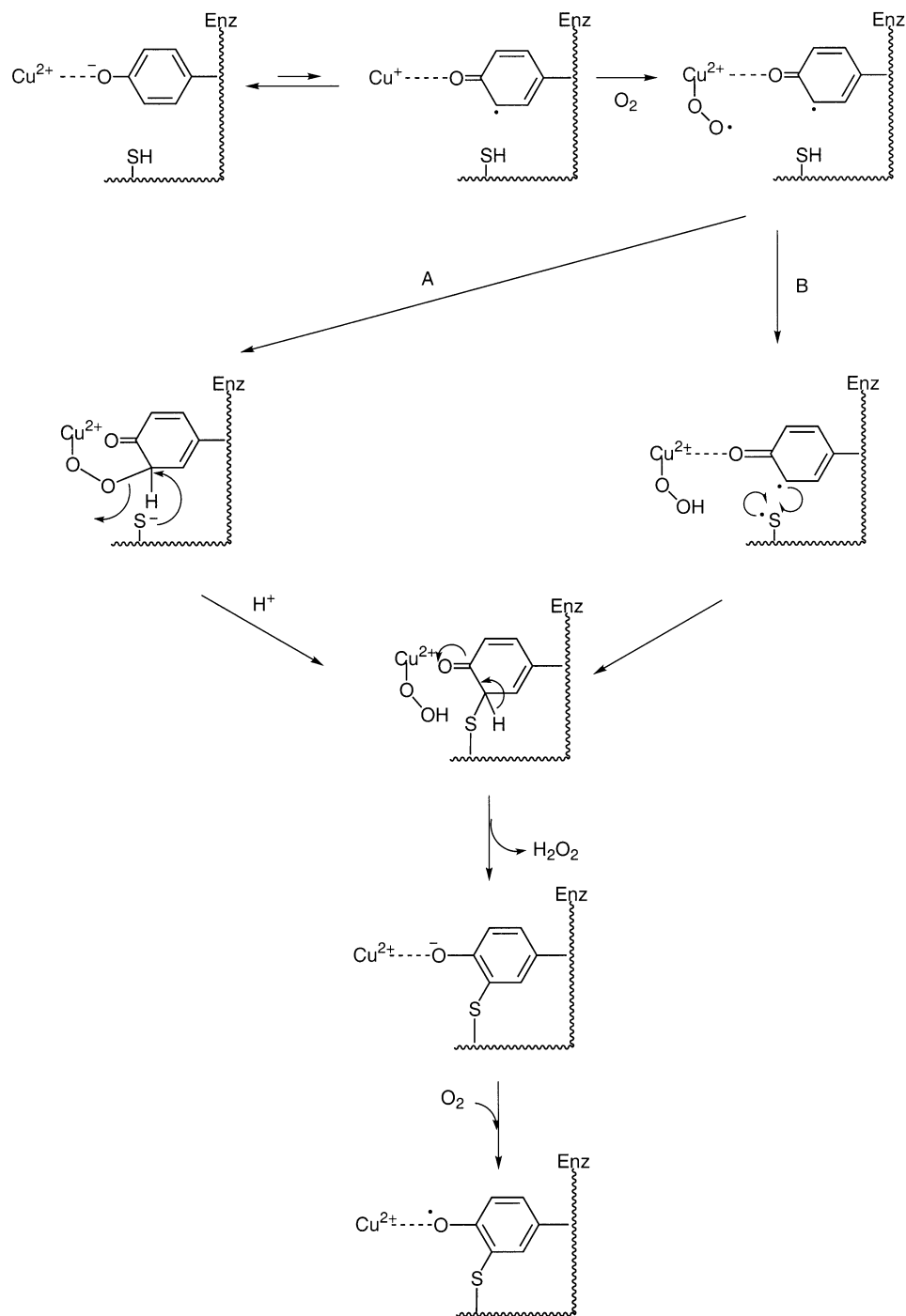
Another example of an enzyme carrying a similar copper-radical center in which the radical is provided also by a modified cysteinyl-tyrosine residue is glyoxal oxidase.<sup>46,47</sup> Glyoxal oxidase is produced by the wood-rot fungus *Phanerochaete chrysosporium* and is an essential component of its extracellular lignin degradation pathway. It catalyzes the oxidation of glyoxal OHCCHO and other aldehydes to the corresponding carboxylic acids coupled to reduction of dioxygen to hydrogen peroxide. Biochemical and spectroscopic characterization of wild-type and site-directed mutant enzymes supports structural correlations between galactose oxidase and glyoxal oxidase.<sup>46,47</sup> No information regarding the cysteine-tyrosine coupling reaction in this enzyme is available so far.

Other thio-cross-links between cysteines and histidine, tryptophane, aspartate, and glutamate (Figure 3) have been recently observed but nothing is known with regard to the mechanism of their generation.<sup>48–51</sup>

### C. Biosynthesis of Biotin: The Biotin Synthase

In bacteria, plants, and mammals, biotin synthase catalyzes the last step of the biotin biosynthetic pathway leading from dethiobiotin to biotin. An *E. coli* strain lacking an active biotin synthase is unable to grow in minimal medium unless it is complemented with biotin. The reaction involves activation/cleavage of C–H bonds of dethiobiotin and insertion of a sulfur atom (Figure 14). The enzyme from *E. coli*, the product of the *bioB* gene, has been the most extensively studied biotin synthase and is named BioB in the following.<sup>52,53</sup> It is a homodimer of 77 kDa, with each polypeptide chain carrying an oxygen-sensitive (4Fe–4S) cluster, probably ligated by three cysteines of a CXXXCXXC box conserved among all BioB known sequences and a fourth still not identi-

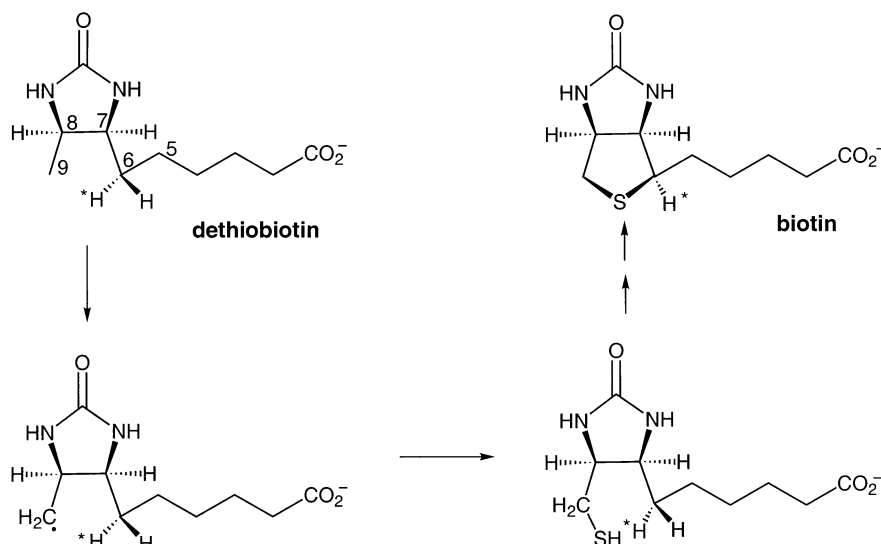




**Figure 13.** Proposed mechanisms for the generation of the cofactor in galactose oxidase.

fied ligand.<sup>54–60</sup> In the presence of oxygen, the iron center is unstable and loses iron and sulfide, being converted into a (2Fe–2S) cluster. However, when the enzyme returns to reductive anaerobic conditions, iron and sulfide are mobilized to regenerate a (4Fe–4S) cluster.<sup>54–56,61,62</sup> The active enzyme form is the one containing the (4Fe–4S) cluster. Site-directed mutants of BioB in which the cysteines of the conserved sequence (Cys53, Cys57, Cys60) have been changed into alanine are inactive.<sup>57–60</sup> It has been reported that under certain reconstitution conditions each polypeptide can carry two clusters, one (4Fe–4S) cluster chelated by the cysteine triad and a (2Fe–2S) cluster presumably bound to three other con-

served cysteines, Cys97, Cys128, and Cys188.<sup>63–65</sup> The presence of two different clusters in different sites in this type of enzyme preparation has been recently demonstrated by Mössbauer spectroscopy using differently substituted <sup>57</sup>Fe protein.<sup>65</sup> The cluster composition of BioB is, however, not yet firmly established. Actually, similar activities, have been reported for preparations containing either two clusters, one (4Fe–4S) cluster and one (2Fe–2S)<sup>63,64</sup> or one (4Fe–4S) cluster.<sup>66</sup> In the latter case, the presence of cysteine and pyridoxal-phosphate are required.<sup>66</sup> Thus, different mechanisms have been proposed for each type of BioB preparation that are discussed in detail below.



**Figure 14.** The reaction catalyzed by biotin synthase: free radicals and 9-mercaptodethiobiotin as postulated intermediates.

In an attempt to determine whether the biologically active relevant BioB form contains one or two clusters, Mössbauer studies of whole *E. coli* cells overexpressing BioB and aerobically grown in media enriched with  $^{57}\text{FeCl}_3$  have been undertaken by two groups independently.<sup>67,68</sup> In the first study only (2Fe–2S) clusters could be detected<sup>68</sup> whereas in the second one a mixture of (4Fe–4S) and (2Fe–2S) clusters was observed with the latter being the major species.<sup>67</sup> During purification of BioB from these cells the proportion of (4Fe–4S) clusters declined progressively and the pure enzyme only contained (2Fe–2S) clusters.<sup>67</sup> These *in vivo* experiments thus did not allow a firm conclusion regarding the cluster composition in BioB within cells.

Whereas this enzyme has been the subject of a large number of studies in the last 20 years, there are still a number of other unresolved questions, especially regarding the mechanism. There is compelling evidence that free radical intermediates are involved during the reaction.<sup>52,53</sup> On the other hand, the various proposed mechanistic scenarios for sulfur insertion have not received much experimental support so far. Even though whole-cells experiments have shown that cysteine is the source of sulfur for biotin biosynthesis,<sup>69</sup> the nature of the activated sulfur species that is directly incorporated into dethiobiotin is unknown. This topic will be discussed in a following section. Finally, the reported activities *in vitro* with well-defined assay mixtures rarely exceed 1 nmol of biotin per nmol of monomer after several hours of reaction. This may reflect one of three things. Either unidentified important cofactors, necessary for catalytic turnover, are missing (this would be in agreement with the observation that moderately increased activities are obtained when assay mixtures are complemented with bacterial soluble extracts), or rapid inactivation, whose mechanism has not been unravelled yet, occurs during the reaction in test tubes. Finally biotin synthase may not be an enzyme but instead a reactant.<sup>70</sup>

In fact, the following observations better suggest that biotin synthase is a true enzyme. First, using extracts of an *E. coli* strain overexpressing the biotin

synthase from *Arabidopsis thaliana* and a complex assay mixture, it was reported an activity of 7 mol biotin/mol monomer over a 6 h incubation period.<sup>71</sup> Multiple turnovers were also observed when a purified preparation of the enzyme, which alone had no activity, was complemented with extracts from wild-type *E. coli* or with mitochondrial fractions from plant (pea leaf cells or potato tubers) extracts.<sup>71</sup> This suggests not only that biotin synthase, at least from *A. thaliana*, is capable of multiple turnovers but also that cell extracts contain unknown stimulating factors. Second, BioB from *E. coli* was shown to be able to achieve a second catalytic cycle provided that, after a first cycle, the protein is filtered and then incubated with a fresh assay mixture.<sup>72</sup> Thus, the lack of multiple turnovers in the case of the *E. coli* enzyme is not due to an irreversible inactivation but rather to an inhibition by a product of the reaction. It was indeed shown that 5'-deoxyadenosine, a byproduct of AdoMet cleavage, is a very potent inhibitor of the reaction.<sup>72</sup> It is thus believed that biotin synthase is a true catalytic enzyme, and it becomes crucial to understand how within cells the enzyme manages to avoid the inhibition/inactivation process that is observed *in vitro*.

### 1. Evidence for a Radical Mechanism

There are several lines of evidence supporting a radical mechanism for dethiobiotin to biotin conversion, even though a free radical has yet to be detected during the reaction. A strong indication for the involvement of free radicals in biotin synthase came from the observation that this enzymatic machinery shares a number of properties with radical enzymes such as lysine 2,3-aminomutase, the activating components of ribonucleotide reductase and pyruvate-formate lyase, all members of the "radical-SAM" superfamily.<sup>3,73–75</sup> The second indirect evidence is based on results obtained with substrate analogues and regio- and stereospecifically labeled substrates.<sup>52,53</sup>

**a. Studies with Substrate Analogues.** It was first shown, using tritiated and deuterated forms of dethiobiotin, that the hydrogen atoms at C-5, C-7, and C-8 were not involved in the reaction, thus

excluding unsaturated intermediates.<sup>76</sup> Only two hydrogen atoms were found to be removed from dethiobiotin, one from C-6 and one from C-9.<sup>77</sup> Furthermore, only the pro-(S) hydrogen at C-6 (unlabeled H in Figure 14) is eliminated, and sulfur insertion at C-6 occurs with overall retention of configuration (Figure 14).<sup>78</sup> Stereochemical information with respect to the introduction of sulfur at C-9 of dethiobiotin was obtained through experiments with a substrate bearing a chiral methyl group at C-9.<sup>79</sup> It was shown that sulfur insertion at this position occurs with complete racemization, suggesting a radical mechanism and the involvement of H atom abstraction from the C-9 methyl group (Figure 14). This first free radical intermediate might then generate 9-mercaptodethiobiotin proposed to be the precursor of biotin (Figure 14). In fact, 9-mercaptodethiobiotin has been shown to be converted to biotin to a significant extent during incubation with resting *Bacillus sphaericus* cells and can support the growth of an auxotrophic *E. coli* mutant, with an inactivated *bioA* gene, whose biotin biosynthetic pathway is blocked before dethiobiotin.<sup>80,81</sup> It is also able to support the growth of an *E. coli* mutant strain lacking the biotin operon but containing the *bioB* gene encoded on a plasmid.<sup>80</sup> This compound has been labeled either with <sup>35</sup>S or <sup>34</sup>S. In both cases, during incubation with *B. sphaericus* cells, the label was recovered into biotin with high yield (80% of biotin contained <sup>34</sup>S).<sup>70</sup> Finally 9-(9-<sup>2</sup>H<sub>2</sub>)mercaptodethiobiotin was transformed by resting cells of *B. sphaericus* into (9-<sup>2</sup>H<sub>2</sub>)biotin, with no loss of deuterium.<sup>81</sup> Resting cells were necessary for these experiments since in growing bacterial cells an active and complex desulfurization of the substrate is occurring. On the contrary, 6-mercaptodethiobiotin could not be converted to biotin by resting bacterial cells and is probably not a reaction intermediate. 9-Hydroxydethiobiotin and both 6(*R*)- and 6(*S*)-hydroxydethiobiotin as well as 6(*R*),9-dihydroxydethiobiotin were unable to support the growth of an auxotrophic *bioA* *E. coli* mutant strain and were excluded as intermediates during biotin synthesis.<sup>82</sup>

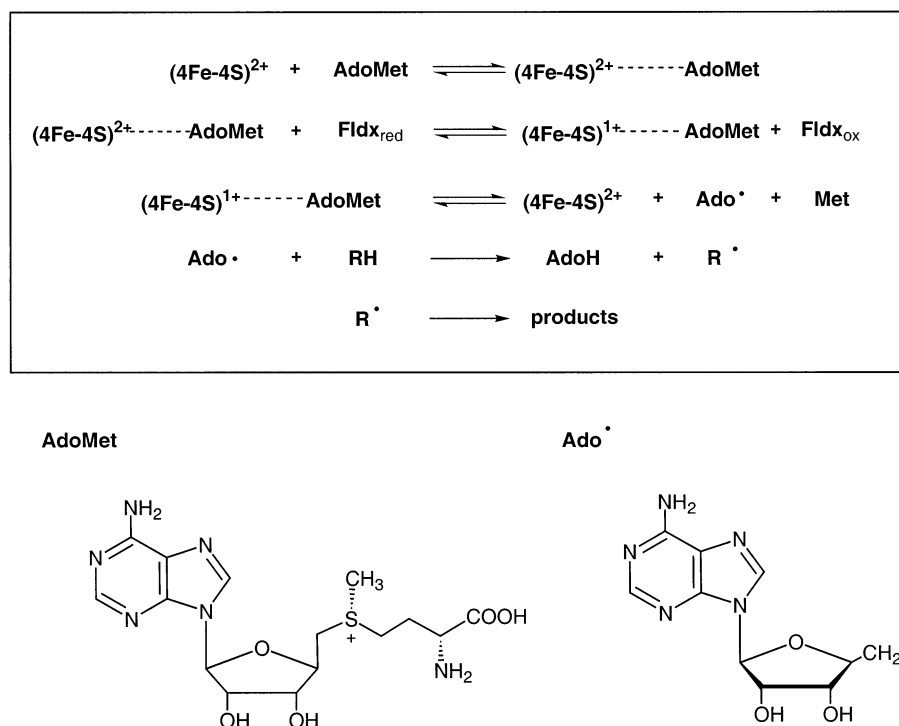
Another result supporting a radical mechanism came from preliminary studies with the substrate analogue 5,6-dehydrodethiobiotin.<sup>83</sup> When <sup>14</sup>C-labeled versions of this compound were incubated with S-adenosylmethionine (AdoMet) and pure preparations of biotin synthase illuminated in the presence of deazaflavin (DAF) as a source of electrons, a small amount of covalent binding of the substrate to the protein was observed.<sup>83</sup> No binding could be detected if DAF was omitted or if dethiobiotin was added in excess. As discussed below, AdoMet and a strong reducing agent are required in vitro for the production of biotin from dethiobiotin. It is tempting to suggest that, with such a substrate analogue, H atom abstraction at C-6 results in an allylic radical in which spin delocalization of the radical center makes it reactive with respect to the protein itself and thus results in covalent binding.

**b. Biotin Synthase is a Member of the Radical-SAM Family.** Biotin synthase has been proposed to belong to the radical-SAM enzyme family for the

following reasons.<sup>75</sup> First it contains a (4Fe-4S)<sup>2+/1+</sup> cluster required for catalysis<sup>3,73,75</sup> and is coordinated by the three cysteines of the CXXXCXXC motif, which is a unique sequence common to members of that enzyme family. In addition, S-adenosylmethionine (AdoMet or SAM) and an electron-donating system consisting of NADPH, flavodoxin reductase, and flavodoxin were shown to be required for biotin synthase activity in vitro.<sup>84-87</sup> Photoreduced deazaflavin could serve as the reducing agent as well.<sup>88,89</sup> The requirement for these components is a signature for enzymes of the radical-SAM superfamily.<sup>3,73,75</sup>

The AdoMet/FeS/e<sup>-</sup> combination has been proposed, in the case of prototypic enzymes such as the anaerobic ribonucleotide reductase, pyruvate formate lyase, and lysine 2,3-aminomutase, to function as a source of methionine and a 5'-deoxyadenosyl radical, noted Ado<sup>o</sup> in Figure 15, resulting from reductive cleavage of AdoMet.<sup>3,73-75</sup> The dissociation energy of the S-C(5'-deoxyadenosyl) bond of AdoMet is about 60 kcal/mol, and homolytic cleavage of that bond is possible only after a one-electron reduction. It has been demonstrated that the electron is provided by the reduced (4Fe-4S)<sup>+</sup> and that the flavodoxin reductase/flavodoxin system serves to convert the cluster into this reduced active state (Figure 15). The highly energetic 5'-deoxyadenosyl radical, which has never been directly observed, is proposed to initiate the reaction by selectively abstracting a H atom from the substrate RH (Figure 15). With the first two enzymes mentioned above, the substrate is a glycine residue of the polypeptide chain and H atom abstraction leads to an oxygen-sensitive glycy radical that is essential for enzyme activity.<sup>90,91</sup> Experiments with proteins containing deuterated glycines have shown in both cases that the label is transferred to the 5'-deoxyadenosyl group, strongly supporting the hypothesis of an intermediate 5'-deoxyadenosyl radical.<sup>90,92</sup> Furthermore, a 5'-deoxyadenosyl group was trapped by a peptide analogue of the pyruvate formate lyase radical site.<sup>93</sup> In the case of lysine 2,3-aminomutase, the substrate is lysine, and the reaction involves the intermediate sequential formation of substrate- and product-derived radicals.<sup>74</sup> Using a variety of substrate analogues, P. Frey et al. could observe several of these species by EPR spectroscopy, thus providing a strong support to the hypothesis of a radical mechanism in this radical-SAM enzyme family.<sup>74</sup> Finally, using 3',4'-anhydroadenosylmethionine as well as <sup>2</sup>H- and <sup>13</sup>C-labeled versions of that compound as an analogue of AdoMet, they could observe the stabilized allylic 5'-deoxy-3',4'-anhydroadenosin-5'-yl radical, further supporting the mechanism proposed in Figure 15.<sup>94,95</sup>

The ability of reduced clusters to reduce sulfonium ions and cleave them in a reaction affording the most stable radical has been demonstrated with Fe<sub>4</sub>S<sub>4</sub>-(SEt)<sub>4</sub><sup>3-</sup> and Fe<sub>4</sub>S<sub>4</sub>(SPh)<sub>4</sub><sup>3-</sup> model complexes and AdoMet analogues.<sup>96</sup> In these experiments the selection of sulfonium salts was made on the basis of their irreversible reduction potentials so that the clusters were thermodynamically competent for reduction. However, with these model systems, several other processes occurred which have not been observed



**Figure 15.** The reductive cleavage of *S*-adenosylmethionine to the 5'-deoxyadenosyl radical, catalyzed by an iron–sulfur center (Fldx<sub>ox</sub>, oxidized flavodoxin; Fldx<sub>red</sub>, reduced flavodoxin; RH, substrate; Met, methionine).

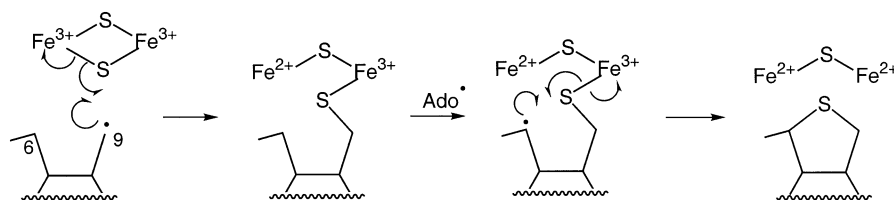
with the enzymes: (i) one-electron reduction of the radical followed by protonation (in the enzymes the Ado<sup>•</sup> radical further reacts by H atom abstraction); (ii) electrophilic attack of the sulfonium on coordinated thiolate as a minor pathway. Finally there is no evidence for the formation of C–S bonds from reaction of the intermediate radical with sulfur atoms of the cluster.

As a member of this class of enzymes, the biotin synthase reaction might also involve, as in Figure 15, binding of AdoMet to the (4Fe–4S) cluster, electron transfer from the reduced cluster to AdoMet, and cleavage of AdoMet to methionine and the 5'-deoxyadenosyl radical. The latter is supposed to serve for the activation of dethiobiotin, through hydrogen atom abstraction at the C-9 and/or C-6 carbons. The resulting carbon radicals have the potential then to couple to a sulfur atom.

First, binding of AdoMet to the (4Fe–4S)<sup>2+</sup> cluster has been demonstrated by Raman resonance and Mössbauer spectroscopy from changes, upon addition of AdoMet, of the spectroscopic features characteristic of the cluster.<sup>97</sup> AdoMet is likely to bind to the fourth unique site of the (4Fe–4S) cluster, each of the three other irons being coordinated by a cysteine of the CXXXCXXC peptide. As in the case of the activase of the pyruvate formate lyase, AdoMet coordination might occur via the amino and carboxylate groups of the methionine moiety.<sup>98</sup> Second, our laboratory recently showed that the reduced (4Fe–4S)<sup>1+</sup> form of the cluster is competent for a one-electron reduction and cleavage of AdoMet, resulting in the production of methionine.<sup>99</sup> Using tritiated AdoMet, Marquet et al. showed that 5'-deoxyadenosine and methionine were formed in equimolar amounts.<sup>100</sup> The methionine (or 5'-deoxyadenosine)/biotin ratio has been addressed in a few studies. Using well-defined as-

says, Marquet et al. reported that almost 3 times more 5'-deoxyadenosine was formed with respect to biotin.<sup>100,101</sup> Shaw et al., with assay mixtures containing cell extracts, reported similar results.<sup>102</sup> Both concluded that their results were most consistent with a ratio of 2, since this is the maximal number that can be obtained if AdoMet needs to be cleaved, one time for each C–H bond to be abstracted. However ratio values larger than 2 indicate the occurrence of unspecific reactions leading to 5'-deoxyadenosine and do not allow firm conclusions. Furthermore, these numbers are fraught with weaknesses: the assays either have very low activities<sup>100,101</sup> or are done using crude extracts.<sup>102</sup> Finally, these results were recently challenged by the numbers we obtained using the cysteine/PLP-dependent system and BioB preparations containing only the (4Fe–4S) cluster. As discussed below, we determined a methionine/biotin ratio of 1 and a mechanism fitting with this result has been proposed.<sup>72</sup>

Marquet et al., in 1999, also provided the strongest evidence for the involvement of an intermediate 5'-deoxyadenosyl radical and its role in the direct abstraction of H atoms in dethiobiotin.<sup>101</sup> They synthesized dethiobiotin samples deuterated at the C-9 or C-6 positions and observed that during in vitro conversion of these substrates to biotin, deuterium was recovered into 5'-deoxyadenosine. Even though this experiment is crucial for the understanding of the reaction mechanism, it is not possible to unambiguously conclude whether deuterium in 5'-deoxyadenosine was originating from both C-9 and C-6. Indeed, the substrates were mixtures of dethiobiotin molecules, with various degrees of deuteration at both C-9 and C-6 positions, and the amount of deuterium incorporated into biotin was about half of the expected value.



**Figure 16.** An iron–sulfur center as the source of sulfur during biotin synthesis: an hypothetical mechanism.

## 2. The Incorporation of Sulfur

### a. An Iron–Sulfur center as the Sulfur Donor?

When the *bioB* gene was cloned at the beginning of the 90's, recombinant *E. coli* and *B. sphaericus* strains became available allowing preparation of enriched solutions of the protein (in the form of soluble extracts from these bacteria). The first preparation of a pure enzyme was achieved in 1994.<sup>103</sup> It then became possible to define the minimal requirements for biotin formation activity *in vitro*, even though the activity in most cases did not exceed 1 mol of biotin per mol of enzyme monomer. *AdoMet* and a source of electrons<sup>85,88,89</sup> are absolutely required very likely for production of 5'-deoxyadenosyl radicals as discussed above. *AdoMet* is not the source of sulfur since no label from (<sup>35</sup>S)*AdoMet* could be incorporated into biotin.<sup>84,104</sup> *Dithiothreitol* is in some cases required but its function is still unidentified.<sup>89,105,106</sup> Finally, in a number of assays, addition of  $\text{Fe}^{2+}$  and  $\text{S}^{2-}$  has a significant stimulating effect.<sup>64,70,89,106</sup> A common interpretation is that purified biotin synthase preparations are cluster-deficient, and iron and sulfide serve to replenish the iron center.

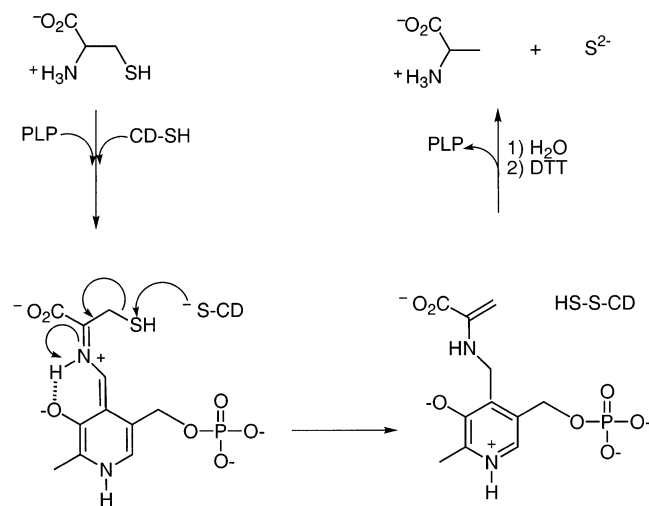
The involvement of cysteine in the reaction deserves a special discussion since its importance during *in vitro* biotin synthesis has been a matter of controversy. Cysteine has been shown to be the sulfur donor *in vivo* and thus was introduced early in *in vitro* assay mixtures. In experiments using crude bacterial extracts, sulfur from (<sup>35</sup>S)cysteine was recovered into biotin, however, with a significantly decreased specific activity, indicating that other unlabeled sources of sulfur present in the extracts could be used by the enzyme.<sup>102,104</sup> Instead, working with pure enzyme and well-defined assays, several authors reported no transfer of <sup>35</sup>S from (<sup>35</sup>S)cysteine to biotin unless a cysteine desulfurase such as NifS or IscS, producing sulfide and alanine from free cysteine, was present in the reaction mixture.<sup>84,106,107</sup> In agreement with this observation, <sup>34</sup>S or <sup>35</sup>S was found to be transferred from labeled  $\text{Na}_2\text{S}$  to biotin.<sup>70</sup> This indicated that, at least *in vitro*,  $\text{S}^{2-}$  could serve as the sulfur donor. Finally, with biotin synthase preparations reconstituted by treatment of the apoenzyme with either  $\text{Fe}^{2+}/^{34}\text{S}^{2-}$  or with  $\text{Fe}^{2+}/(^{35}\text{S})\text{-cysteine/NifS}$ , a significant amount of label was recovered into biotin.<sup>70,106</sup> A disturbing observation in these experiments, using BioB preparations presumably containing exclusively <sup>35</sup>S or <sup>34</sup>S sulfur, was that <sup>32</sup>S sulfur was also recovered into the isolated biotin. Starting with a pure apoenzyme reconstituted with  $\text{Fe}^{2+}/^{35}\text{S}\text{-cysteine/NifS}$  and assay mixtures without sulfur-containing compounds except DTT, the level of incorporation of <sup>35</sup>S to cysteine was 56%, whereas that of <sup>34</sup>S was 62% using the enzyme

reconstituted with  $\text{Fe}^{2+}/^{34}\text{S}^{2-}$ . Thus, in fact, a large amount of <sup>32</sup>S was retained on the protein in a mobilizable form, either in remaining clusters in the initial "apoprotein" or in sulfur species covalently bound to the protein, of the sulfane type such as persulfides or others. That biotin synthase has the potential to carry and deliver sulfur in the absence of a sulfur donor was shown in an experiment using a purified enzyme from *E. coli* cells grown in the presence of (<sup>35</sup>S)cysteine.<sup>108</sup> In an assay lacking additional S-containing compounds, <sup>35</sup>S was found in biotin. However, this last experiment does not tell whether the sulfur is coming from the cluster, a cysteine of the polypeptide chain or some other species such as a protein-bound persulfide.

These labeling experiments allowed A. Marquet to put forward the hypothesis first proposed by Flint,<sup>103</sup> that sulfur could be provided by the iron–sulfur cluster itself.<sup>70</sup> In this view the added <sup>34</sup>S<sup>2-</sup> or <sup>35</sup>S<sup>2-</sup> is supposed to provide the sulfur bridges of the cluster from which the label would be transferred to biotin. However, other sulfur donors such as sulfane species, which could have been formed during treatment with <sup>34</sup>S or <sup>35</sup>S sulfur sources, cannot be excluded. If Marquet's hypothesis were correct, a mechanism providing S atoms for reconstitution of the cluster should be required for multiple turnovers. However, addition in the assay of cysteine desulfurases or  $\text{Na}_2\text{S}$ , known to participate to cluster reconstitution, did not allow multiple turnovers.

Later, this hypothesis was improved by J. Jarrett who reported that biotin synthase was able to chelate two clusters, one (4Fe–4S) cluster for *AdoMet* reductive activation and one (2Fe–2S) cluster proposed to serve as the S donor specifically.<sup>63–65</sup> It was reported that only a preparation containing both clusters was active, but it is still not able to undergo multiple turnovers. Furthermore EPR and UV–vis spectroscopy studies showed that, during a single turnover, the (4Fe–4S) cluster was preserved while the (2Fe–2S) one was destroyed. This observation made it possible to propose a mechanism for biotin formation in which 2 molecules of *AdoMet* are used up and the sulfur atom is provided by an iron sulfur cluster (Figure 16).<sup>64</sup> After abstraction of the H atom at C-9 of dethio-biotin, the intermediate substrate radical couples to one sulfur atom of the cluster thus generating Fe-bound deprotonated 9-mercaptodethio-biotin. Ring closure occurs after abstraction of the H atom at C-6 by a second 5'-deoxyadenosyl radical. In the overall process, the cluster has received two electrons and lost one sulfur bridge.

**b. A Persulfide as the Sulfur Donor?** An alternative sulfur donor has been recently proposed on the basis of the observation that preparations of

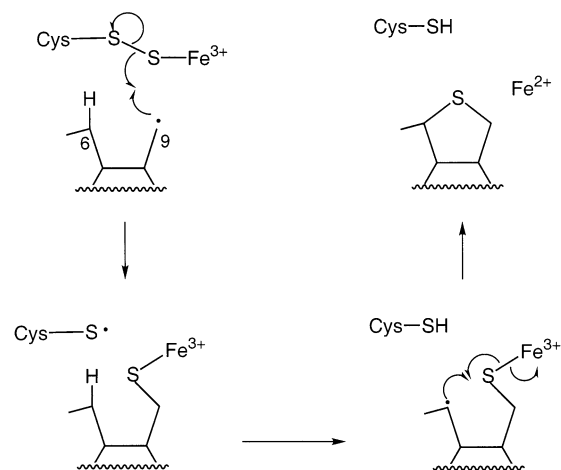


**Figure 17.** Proposed mechanism for the enzymatic cysteine desulfuration reaction (CD-SH: cysteine desulfurase).

biotin synthase containing only the (4Fe–4S) center displays a cysteine desulfurase activity.<sup>66</sup> These preparations catalyze the mobilization of the sulfur atom from L-cysteine, but not D-cysteine, in the presence of DTT producing alanine and sulfide.<sup>66</sup> As for all cysteine desulfurases reported so far, this activity critically depends on the presence of pyridoxal phosphate (PLP) and thus certainly proceeds by similar mechanisms (Figure 17). The simplest scenario implies the intermediate formation of a substrate cysteine–pyridoxal phosphate ketimine adduct and the subsequent nucleophilic attack by the thiolate anion of an active protein-bound cysteine.<sup>14,15</sup> These events result in the formation of a PLP-bound enamine which is ultimately released as alanine and a protein-bound persulfide. Sulfide then derives from the persulfide by reaction with DTT. Accordingly, biotin synthase binds one equivalent of PLP, even though there is in BioB no obvious sequence corresponding to PLP-binding motifs, and is able to stabilize a persulfide during reaction with cysteine.<sup>66</sup> The importance of this cysteine desulfuration activity for biotin synthesis is shown from the acceleration of biotin formation by addition of PLP and cysteine and the rather good correlation between the two activities.<sup>66</sup> However, again these conditions do not allow multiple turnovers.

An active cysteine desulfurase depends on a critical cysteine residue which provides the persulfide site. Accordingly, in addition to the three cysteines, namely, Cys53, Cys57, and Cys60 of the CXXXCXXC sequence providing the ligands to the (4Fe–4S) center, BioB from *Escherichia coli* contains three more cysteines Cys97, Cys 128, and Cys188 required for biotin synthesis activity.<sup>59,60</sup> However, these cysteines belong to sequences with no homology to those found in others desulfurases. Nevertheless, Cys97 and Cys128 were shown to be critical, but not essential, to the cysteine desulfurase activity.<sup>66</sup> Even though the function of these cysteines is still unclear, it is tempting to suggest that one may be the site for an intermediate persulfide. The function of Cys188, which is fully conserved, also remains to be identified.

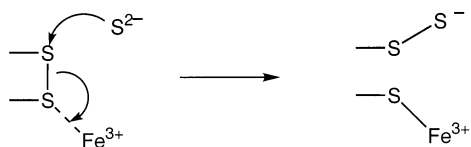
The discovery of a PLP-dependent cysteine desulfurase activity for biotin synthase has led us to



**Figure 18.** A persulfide as the source of sulfur during biotin synthesis: an hypothetical mechanism.

propose a new mechanism shown in Figure 18, which applies to active preparations containing only one (4Fe–4S) cluster and thus does not involve a second cluster. This mechanism features a protein-bound persulfide as the direct sulfur donor to the intermediate substrate radical. Whether ancillary cysteine desulfurases such as IscS, CSD, and SufS participate to the generation of the persulfide in BioB is a possibility that, however, has not been studied yet. In this regard, it is important to note that the three *iscS*<sup>-</sup>, *CSD*<sup>-</sup>, and *sufS*<sup>-</sup> *E. coli* mutant strains grow perfectly well in minimal medium in the absence of biotin (Mihara, Esaki, personal communication). The mechanism also accounts for the observation that, in this system, only one molecule of AdoMet is consumed for the production of one molecule of biotin.<sup>72</sup> Monitoring the reaction from the formation of biotin, methionine (as a probe for AdoMet cleavage) and alanine (as a probe for cysteine desulfuration) in parallel, we indeed showed that both methionine and alanine were formed in equimolar amounts with regard to biotin. This result nicely fits with the requirement for only one sulfur atom per biotin molecule and further supports the importance of the cysteine desulfurase activity of BioB. On the other hand, it is in disagreement with previous studies which found 2.8–2.9 5'-deoxyadenosine or 2.6–3.1 methionine per biotin.<sup>100–102</sup> The reasons for this inconsistency are not clear, but differences in BioB preparations and assay conditions could account for it.

In the mechanism of Figure 18, the persulfide is proposed to bind to the cluster. There are precedents for such persulfido-metal complexes in other metalloproteins. One example is the “putative prismane” protein of unknown function from *Desulfovibrio vulgaris* whose determined three-dimensional structure revealed the presence of a hybrid (4Fe–2S–3O) cluster ligated by seven protein residues, three cysteines, one persulfido-cysteine, two glutamates, and one histidine.<sup>109</sup> In the first step of the biotin synthase reaction, the (4Fe–4S) cluster catalyzes the one-electron reduction of AdoMet by reduced flavodoxin. The resulting cleavage of AdoMet gives rise to methionine and the 5'-deoxyadenosyl radical which subsequently abstracts a hydrogen atom from the



**Figure 19.** Persulfides as products of the reaction of sulfide with a disulfide bridge.

carbon at position 9 in dethiobiotin. In parallel, sulfur from free cysteine is transferred to the protein, generating a reactive protein-bound persulfide species. As shown in Figure 18, the substrate C-9 radical couples to the terminal sulfur of the persulfide giving rise to a thiyl radical and the Fe-bound deprotonated 9-mercaptodethiobiotin. The thiyl radical abstracts a H atom at C-6, and during a reaction similar to the ring closure in isopenicillin synthase (Figure 9), formation of biotin occurs with a one-electron reduction of the cluster. Even though abstraction of the C-6 hydrogen atom by a cysteinyl radical is thermodynamically unfavorable, the reaction is likely to be driven by coupling to the following favorable one. One should also consider a mechanistic scenario, comparable to that shown in Figure 18, in which the persulfide is not bound to an iron atom but is instead protonated for direct sulfur transfer to the intermediate substrate radical.<sup>72</sup> It is important to note that formation of biotin requires that an electron is released at the end of the reaction. In both Figures 16 and 18 the electron acceptor is a ferric iron. This mechanistic hypothesis is speculative and has no chemical precedents. Nevertheless, it is not inconsistent with reported labeling experiments. As indicated above, sulfur from sulfide, added in excess as a salt or derived from cysteine desulfuration by exogenous cysteine desulfurases such as NifS or IscS, and in the presence of iron, is incorporated into biotin. It is likely that under these reaction conditions a persulfide is generated, similar to that formed during BioB-bound PLP-dependent cysteine desulfuration. This could occur for example through a nucleophilic attack by the sulfide anion of a protein-bound disulfide bridge (Cys97–Cys128?), which is a reaction previously documented<sup>110</sup> and is shown in Figure 19.

It is quite amazing that the same enzyme can function similarly, at least *in vitro*, with different cofactors, either two iron–sulfur clusters or one cluster and PLP. How this is possible and which combination is biologically relevant remain to be understood. Furthermore, a truly catalytic system is absolutely required for deeper mechanistic studies as well as how inhibition by the 5'-deoxyadenosine product might be removed has to be investigated.

#### D. Biosynthesis of Lipoate: The Lipoate Synthase

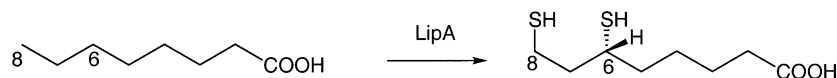
Lipoic acid synthase is a crucial cofactor in the pyruvate dehydrogenase, 2-oxoglutarate dehydrogenase, branched chain 2-oxoacid dehydrogenase and the glycine cleavage multienzyme complexes involved in oxidative metabolism in bacteria and in mitochondria of eukaryotic cells.<sup>111</sup> However, little is known about lipoate synthase, the product of the *lipA* gene, which catalyzes the insertion of sulfur atoms into

octanoic acid during the final step of lipoic acid synthesis (Figure 20). Genetic studies on *E. coli* showed that cells with mutations in *lipA* did not produce lipoic acid and phenotypes of these mutants suggest that LipA is responsible for the formation of both the C–S bonds.<sup>112,113</sup> Whole cells isotope transfer experiments suggest that octanoic acid is a precursor to lipoic acid.<sup>114</sup> Using specifically tritiated/deuterated forms of octanoic acid and analyzing their conversion to lipoic acid by *E. coli* cells, it was shown that sulfur atoms are introduced with loss of two hydrogen atoms, one from C-6 and one from C-8.<sup>115,116</sup> Sulfur is introduced at C-6 with overall inversion of configuration. It is introduced at C-8 with racemization, in agreement with the formation of an intermediate carbon radical at C-8.<sup>116–118</sup> 8-Thiooctanoic acid and 6-thiooctanoic acid can also be converted into lipoic acid, but to a lesser extent.<sup>119</sup>

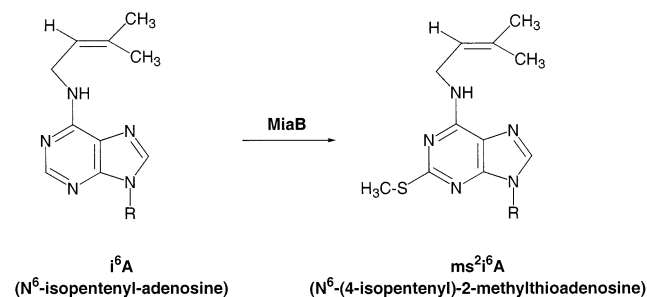
The LipA enzyme was first purified and characterized as an iron–sulfur protein, containing a (4Fe–4S)<sup>2+/1+</sup> cluster only in 1999 and a sensitive and quantitative *in vitro* assay became available in 2000.<sup>120–122</sup> Free lipoate and lipoyl-acyl carrier protein (lipoyl-ACP) were assayed indirectly using (i) the apo form of pyruvate dehydrogenase complex (apoPDC) as a lipoyl-accepting protein, (ii) purified LipA, and (iii) either purified lipoate-protein ligase A (LplA), ATP, octanoate as a substrate (for lipoate synthesis) or lipoyl-ACP-protein-N-lipoyl transferase (LipB) and octanoyl-ACP as a substrate (for lipoyl-ACP synthesis). Lipoylation of PDC was monitored spectrophotometrically.<sup>122</sup> Even though the activity did not exceed 0.03 mol of product per mol of polypeptide, thus very far from one turnover, the assay showed that octanoic acid is not the substrate of the enzyme. Lipoic acid formation has been observed using the octanoyl-acyl carrier protein (octanoyl-ACP) as the substrate, in a reaction strongly stimulated by a reductant and AdoMet<sup>122</sup>. All attempts to isolate a free lipoyl-ACP product in the assay were unsuccessful. Confirmation of formation of lipoyl groups from octanoyl-ACP was obtained by MALDI mass spectrometry of a recombinant PDC-lipoyl-binding domain that had been lipoylated in a LipA reaction. Whether this indicates that the product remains tightly associated with LipA remains to be established.

These results, together with the observation that the protein contains a CXXXCXXC sequence and is also able to assemble a (4Fe–4S) cluster required for activity, demonstrate that lipoic acid synthase is a member of the radical-SAM enzyme superfamily.<sup>54,73,75,120,121</sup> It thus strongly suggests that similar radical mechanisms for activation of the substrate and introduction of sulfur operate in biotin synthase and lipoic acid synthase. In the case of lipoate synthase, however, no evidence for either a cysteine desulfurase activity or the presence of two different clusters has been obtained so far but primary sequences show the presence of three conserved cysteines in addition to the CXXXCXXC motif.

In the *in vitro* assay described above lipoic acid was formed in the absence of exogenous sulfur-containing compounds.<sup>122</sup> This suggests, as in the case of biotin



**Figure 20.** The reaction catalyzed by lipoate synthase.



**Figure 21.** The reaction catalyzed by MiaB (R, ribose).

synthase, that the protein itself had some mobilizable sulfur atoms, either from the cluster, a persulfide or some other species, which can be introduced into the product. Marletta et al. favored a cluster as a source of sulfur, but the lousy enzyme activity, together with the observation that inclusion of iron and sulfide in the assay resulted in only small increases in activity, does not allow such a firm conclusion. Studies comparable to those carried out with biotin synthase are needed to evaluate the potential of Fe–S clusters and persulfides as sources for this “activated sulfur” in this system.

### E. Methylthiolation of tRNAs

Another iron–sulfur enzyme under investigation in our laboratory, the product of the *miaB* gene, is also likely to operate by related mechanisms.<sup>123</sup> The MiaB protein catalyzes the methylthiolation of tRNAs through the formation of 2-methylthio-N-6-isopentenyladenosine ( $ms^2i^6A$ ) from  $i^6A$  (Figure 21), at position 37 next to the anticodon on the 3'-position in almost all eukaryotic and bacterial tRNAs that read codons beginning with U except tRNA<sup>I,V</sup>Ser<sup>124</sup>.  $ms^2i^6A$  is one example of the numerous thionucleosides found in tRNAs from both prokaryotes and eukaryotes.<sup>125</sup> This modification is important for normal cell function and deficiency in methylthiolation results in decreased growth rate and cell yield.<sup>126</sup> We became interested in MiaB because the reaction, which consists of both sulfur insertion and methylation at position 2 of the adenine moiety, was earlier shown to require iron, cysteine, and AdoMet and because its amino acid sequence contains the CXXXCXXC sequence present in those of the enzymes of the radical-SAM family.<sup>127</sup> It is still unknown whether each step of the reaction is catalyzed by a specific or by the same enzyme. On the other hand, tRNAs from mutant strains lacking a functional *miaB* gene have been shown to contain only  $i^6A$ , lacking the methylthio group, suggesting that the MiaB protein is involved in C–S bond formation.<sup>127</sup> In vitro experiments using <sup>14</sup>C-labeled AdoMet and <sup>35</sup>S-labeled cysteine demonstrated that the sulfur atom of  $ms^2i^6A$  derives from cysteine and the methyl group of the methylthio moiety from AdoMet.<sup>128</sup>

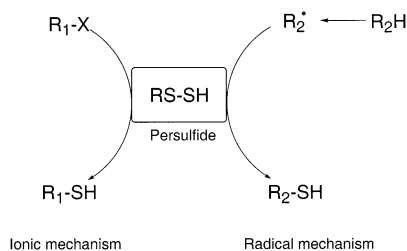
Purification and characterization of MiaB from *E. coli* and *Thermotoga maritima* demonstrated that it is an iron–sulfur protein.<sup>123,129</sup> It is the first and only tRNA-modifying enzyme known to contain an Fe–S cluster so far. The cluster is an oxygen sensitive (4Fe–4S) one with both accessible 2+ and 1+ redox states. UV–visible, EPR, and Raman resonance spectroscopic properties are comparable to those of biotin synthase and the anaerobic ribonucleotide reductase.

Since no in vitro enzyme assay is available yet, the functionality of MiaB was assayed in vivo using a *miaB*<sup>−</sup> *E. coli* strain lacking an active *miaB* gene.<sup>123</sup> tRNAs can be isolated and their modified nucleoside content analyzed by HPLC. The tRNAs from this mutant strain showed an accumulation of  $i^6A$  with no evidence of  $ms^2i^6A$ . Transformation with a MiaB-expressing plasmid converted this strain into one with nucleoside profiles similar to that of a wild-type *E. coli* strain, thus showing the formation of  $ms^2i^6A$ . Using this assay, we were able to show that Cys157, Cys161, and Cys164 from the CXXXCXXC sequence in *E. coli* were absolutely required for synthesis of  $ms^2i^6A$ : the plasmids expressing the corresponding site-directed mutated MiaB proteins were not able to complement the *miaB*<sup>−</sup> strain.<sup>123</sup> This suggested that these three cysteines, as in the case of biotin synthase, were involved in iron chelation.

Cysteine is the physiological source of sulfur for this reaction. An *E. coli* mutant strain lacking an active *iscS* gene, encoding one of the three cysteine desulfurases, showed a greatly decreased synthesis of  $ms^2i^6A$  demonstrating that cysteine desulfurases are involved in this process.<sup>22,23</sup> IscS plays a major role, but an IscS-independent pathway is also able to provide sulfur atoms. However, it is difficult to conclude that these cysteine desulfurases are important for methylthiolation of adenosine since MiaB is an iron–sulfur enzyme and *iscS* deficiency in *E. coli* has been clearly shown to result in greatly decreased activities of iron–sulfur enzymes.<sup>130</sup> On the other hand, IscS is absolutely required for supplying sulfur atoms during thiolation of other nucleosides for biosynthesis of s<sup>4</sup>U and mnm<sup>5</sup>s<sup>2</sup>U in tRNAs, which is not dependent on iron–sulfur enzymes.<sup>21–23</sup>

The MiaB reaction differs from that of biotin synthase and lipoate synthase in that the C–H bond to be converted into a C–S bond is not aliphatic but aromatic. Furthermore, the modified position is chemically rather unreactive when compared to position 8, which is the common target of free radicals.<sup>131</sup> This makes methylthiolation of carbon 2 a highly challenging reaction. Nevertheless considering the similarity of clusters in MiaB and BioB, the requirement for AdoMet and the involvement of cysteine desulfurases we can infer that the mechanism for  $ms^2i^6A$  synthesis is comparable to that of biotin synthesis and involves both persulfides and free radicals.





**Figure 22.** Persulfides as sulfur donors in ionic and radical C–S bond formation.

#### IV. Conclusion

Two recent discoveries gave new insights into the understanding of sulfur metabolism and mechanisms by which sulfur atoms are incorporated into biomolecules in biosynthetic reactions. First, it is established that cysteine is the common sulfur source from which sulfur atoms can be mobilized by cysteine desulfurases. These enzymes produce persulfides which are supposed to behave as excellent sulfur donors to electrophile or radical centers. This has been rather well shown in the case of ionic reactions such as the final steps of 4-thiouridine or molybdopterin synthesis.

The second discovery is that a number of enzymes such as BioB, LipA, and MiaB, catalyzing sulfur atom insertion reactions, are members of the radical-SAM enzyme superfamily. They are iron–sulfur proteins whose cluster is able to reductively cleave AdoMet to generate a 5'-deoxyadenosyl radical supposed to abstract hydrogen atoms on substrates and activate them. This has led to the novel hypothesis that conversion of C–H to C–S bonds might imply radical mechanisms, a notion not well appreciated before.

The most intriguing issue concerns the nature of the direct sulfur donor to the radical intermediates. The discovery of the involvement of cysteine desulfurase activities in biotin synthase leads us to make the suggestion that persulfides, or other sulfane species, play an important role as reactive intermediates also in radical sulfur insertion reactions. This unifying view with persulfides playing a central function in both ionic and radical reactions is summarized in Figure 22. It should be the basis for further important studies.

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