Free Radical Mechanisms in Enzymology

Perry A. Frey,* Adrian D. Hegeman, and George H. Reed

Department of Biochemistry, University of Wisconsin—Madison, 1710 University Avenue, Madison, Wisconsin 53726

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Contents

1. Introduction

The past 15 years have seen an explosive growth in the number of enzymatic reactions found to proceed by mechanisms involving free radicals as intermediates. One of us learned as a graduate student in the 1960s that "... a nonpolar reaction mechanism may be invoked for an enzymatic reaction when no reasonable polar mechanism is available".[1] While this rule remains in force, a large number of enzymatic processes proceed by radical mechanisms and not by polar mechanisms. Radical reactions in enzymology are most often associated with redox-active cofactors, usually flavin, pterin, quinone, or metallo-cofactors. However, a few enzymatic reactions involving molecular oxygen as a substrate proceed without cofactor involvement when the cosubstrate possesses appropriate chemical properties.[2] Nonetheless, most radical-based reactions in enzymology may be ordered according to cofactor involvement as in Table 1. They might be further classified according to the mechanism of substrate radical formation—hydrogen atom abstraction or electron transfer. However, this would not be enzymologically helpful because some enzymes, such as ribonucleotide reductases, employing different cofactors in variant species, initiate radical formation by either or both mechanisms.

Flavin semiquinones were the first enzymatic radical species to be recognized in electron transfer reactions of flavoproteins and reactions of dihydroflavins with molecular oxygen. Flavin semiquinones function in terminal electron transport complexes by facilitating electron transfer between obligatory two-electron-reducing agents, such as succinate and NADH, and one-electron acceptors such as iron-sulfide centers and the cytochromes, and these processes are further mediated by quinone cofactors such as coenzyme Q. Flavin semiquinones and the semiquinone forms of quinone electron transfer mediators are highly stabilized free radicals, owing to extensive delocalization of the unpaired electrons. For many years they were thought to be the only practical radical intermediates in enzymology. Flavin semiquinones are frequently observed spectroscopically, for example in the enzymatic reactions listed with flavoproteins in Table 1. In reactions of flavoprotein oxidases, superoxide and flavin semiquinones are intermediates in the oxidation of dihydroflavin coenzymes by molecular oxygen. Flavin semiquinones mediate electron transfer in the reactions of the other flavoproteins listed.

The discovery of superoxide and superoxide dismutase in biological systems focused attention on how electron transfer involving molecular oxygen is managed in the biosphere.[3,4] Many enzymes engaged in oxygenation function through the intermediate formation of superoxide ($O_2^{\bullet-}$), which can react as a radical intermediate in oxygenation reactions. Superoxide is a transient intermediate in reactions of molecular oxygen with dihydroflavins and related cofactors, as well as with metallo-cofactors such as mononuclear iron and heme. Superoxide is an intermediate in a number of the reactions of flavins and metallo-cofactors listed in Table 1. Superoxide dismutase catalyzes the transformation of superoxide into H_2O_2 and O_2, which is itself a radical reaction. The role of superoxide dismutase in controlling superoxide adventitiously released in the metabolism of molecular oxygen in no way diminishes the significance of superoxide as an intermediate in oxygenation reactions catalyzed by flavoproteins and metallo-oxygenases. Moreover, the detoxification of superoxide is an important topic in the literature on reactive oxygen species. Because this review is focused on free radicals as intermediates in enzymatic catalysis, detoxification of reactive oxygen species will not be discussed here.

Quinoproteins have quinone cofactors such as pyrroloquinoline quinone, topaquinone, tryptophan tryptophylquinone, lysyltopaquinone, or cysteinyltopaquinone. They carry out dehydrogenation or oxidation of alcohols or amines. In general, the dehydrogenations of cosubstrates to two-electron-oxidized products by quinoptroteins take place by polar mechanisms leading to the two-electron-reduced quinol forms of the cofactors, which are then reoxidized by one-electron-oxidizing agents, either molecular oxygen or a metallo-cofactor such as a cytochrome. The reoxidations of the quinol cofactors are thought to proceed through semiquinone radicals as intermediates. The radicals detected have not been thoroughly characterized.[5]

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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 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.

Adrian D. Hegeman was born in San Francisco, California, in 1970. He obtained his B.A. degree in Biochemistry from Oberlin College in 1992, after which he worked as a technician at the University of Michigan medical campus. In 1995 he began his doctoral training with Prof. Perry A. Frey at the University of Wisconsin—Madison. His thesis, which was successfully defended in 2001, focused on the catalytic mechanism of an isoform of dTDP-glucose 4,6-dehydratase. This work included pre-steady-state and steady-state kinetic analysis of enzyme catalysis and stable isotope exchange reactions using mass spectrometry. His postdoctoral work under Prof. Michael R. Sussman began in 2002 at the University of Wisconsin Biotechnology Center, where he continues to explore quantitative mass spectrometry methodologies.

George H. Reed was born and raised in Indiana. He received a B.S. in Chemistry from Purdue University in 1964 and a Ph.D. in Chemistry from the University of Wisconsin in 1968. He did postdoctoral work in Biophysics and Physical Biochemistry at the University of Pennsylvania with Prof. Mildred Cohn. During his postdoctoral years, he was introduced to electron paramagnetic resonance (EPR) and developed applications of EPR to studies of manganous complexes with enzymes and other proteins. He joined the faculty of the Department of Biophysics and Physical Biochemistry at the University of Pennsylvania as an Assistant Professor in 1971. In 1985 he moved to the Enzyme Institute and Department of Biochemistry at the University of Wisconsin—Madison. He is currently a professor in the Department of Biochemistry. His research interests include metal ion complexes with enzymes, radical intermediates in enzymatic reactions, and other aspects of mechanistic enzymology. His outside interests include angling, birding, amateur astronomy, bluegrass music, and wilderness canoe trips.

The entries in Table 1 are representative of enzymes engaged in radical reactions. While Table 1 is not comprehensive, the enzymes listed are too numerous to discuss in a single review. Moreover, many have been subjects of recent reviews cited in Table 1. This article focuses primarily on a representative collection of radical-based enzymatic reactions for which significant new information has recently become available. Examples are chosen from the lists of enzymes dependent on mononuclear iron, adenosylcobalamin, *S*-adenosylmethionine (SAM), and thiamine pyrophosphate (TPP).

2. Mononuclear Iron

Enzymes employing mononuclear iron include dioxygenases and monooxygenases, also known as hydroxylases and epoxidases, and incorporate atoms from molecular oxygen into the reaction products. They also include synthases such as isopenicillin N synthase and deacetoxycephalosporin C synthase. The synthases also use molecular oxygen as an oxidizing agent but do not necessarily incorporate oxygen atoms. Many, but not all, mononuclear iron enzymes bind iron in a 2-His, 1-carboxylate motif, meaning that the iron is ligated by two histidine ligands plus either aspartate or glutamate. This is true of phenylalanine and tyrosine hydroxylases, listed under tetrahydrobiopterin-dependent enzymes in Table 1, as well as of most of the enzymes listed under mononuclear iron.

The reactions of O_2 with iron cofactors, such as heme in cytochrome P450s and di-iron complexes such as in soluble methanemonoxygenase, generally begin with the Fe^{III} form of the cofactor and proceed through reduction to the ferrous form, the binding of O_2, electron transfer to generate the ferric peroxide, and acid-catalyzed dehydration to a ferryl-oxy species that initiates the oxygenation process. The series of transformations relevant to cytochrome P450 is illustrated in Figure 1. An analogous process occurs in the actions of di-iron complexes, in which a different sequence of electron transfer steps occurs. In the action of a mononuclear iron monooxygenase, molecular oxygen reacts with iron to form a ferryl-oxy complex, which carries out the oxidation or oxygenation of the substrate. The sources of reducing electrons for iron-dependent monooxygenases are external reducing agents or cosubstrates, such as NADPH, tetrahy-

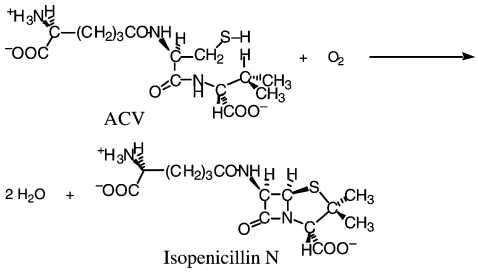
Table 1. Cofactors Associated with Enzymatic Free Radical Mechanisms

coenzyme or cofactor	enzyme	ref
flavoproteins	electron transfer	6−8
	DNA photolyase	7, 9−11
	flavoprotein oxidases	12−14
	nitric oxide synthase	8, 15−18
tetrahydropterins	aromatic amino acid hydroxylases	19, 20
	nitric oxide synthase	15
Cu, Zn, Mn, Ni, Fe	superoxide dimutases	3, 4
quinoproteins	methanol dehydrogenase	21−24
	glucose dehydrogenase	21, 23
	copper amine oxidases	25−29
	methylamine dehydrogenase	22, 24
	lysyl oxidase	27, 30
heme proteins	cytochrome P450s	31, 32, 35
	peroxidases	33, 34
	catalase	36, 37
	prostaglandin synthase	38−42
di-iron complexes	methane monooxygenase	43−47
	fatty acyl-ACP desaturase	48, 49
	ribonucleotide reductase (class I)	50−54
	toluene monooxygenase	55
mononuclear iron	lipoxygenase	56, 57
	isopenicillin N synthase	58−60
	catechol dioxygenases	60, 61
	taurine dioxygenase	62
	hydroxypropyl phosphonate epoxidase	63, 64
	prolyl-4-hydroxylase	65
	lysyl-5-hydroxylase	66, 67
	clavaminate synthase	68
	γ-butyrobetaine hydroxylase	69
	thymine hydroxylase	70, 71
	deacetoxycephalosporin C synthase	72
mononuclear copper	galactose oxidase	73, 74
multi-copper	laccase	75
	ascorbate oxidase	76
adenosylcobalamin	glutamate mutase	77−82
	methyl-malonyl-CoA mutase	83−87
	dioldehydrase	89−91
	ribonucleotide reductase (class II)	88, 89, 92
	lysine 5,6-aminomutase	93, 94
	methyleneglutarate mutase	95−97
	ethanolamine-ammonia lyase	89, 98−101
	isobutyryl CoA mutase	102−104
radical SAM	lysine 2,3-aminomutase	105−107
	pyruvate-formate lyase and activase	108, 109
	anaerobic ribonucleotide reductase (class III)	110−112
	biotin synthase	113−116
	lipoyl-ACP synthase	117−119
	benzylsuccinate synthase and activase	120−122
	spore photoproduct lyase	123−125
	coproporphyrinogen III oxidase (HemN)	126−129
	molybdopterin synthesis and MoaB	130
	glycerol dehydratase and activase	131, 132
thiamine pyrophosphate	pyruvate:ferredoxin oxidoreductase	133−135
	pyruvate oxidase (FAD)	136−137
no cofactor	urate oxidase	2

drobiopterin, or α-ketoglutarate. Iron-dependent monooxygenases incorporate one atom of oxygen from O_2 into the product and the other into water. Dioxygenases do not use a reducing substrate and incorporate both oxygen atoms into the product. Isopenicillin N synthase, a mononuclear iron enzyme, carries out an oxidase reaction that does not involve an external reducing agent or the incorporation of oxygen into the product.

2.1. Isopenicillin N Synthase (IPNS)

The biosynthesis of penicillin begins with the assembly of a tripeptide, L-α-aminoadipoyl-L-cysteinyl-L-valine (ACV).[138] In a remarkable reaction, IPNS catalyzes the O_2-dependent cyclization of ACV into the bicyclic β-lactam isopenicillin N according to eq 1. Any cyclic organic



(1)

compound may be regarded as derived from a corresponding acyclic precursor by the removal of the elements of molecular hydrogen from the joined atoms. The two rings in isopenicillin N are in fact derived by the removal of the elements of two hydrogen molecules. Removal of a C3(H) and the peptide-N(H) from the cysteinyl residue leads to the β-lactam ring, and removal of the cysteinyl-S(H) and valyl-C3(H) leads to the five-membered ring. The resulting reducing equivalents are used to reduce molecular oxygen to two molecules of water. The mechanism by which this process takes place is a fascinating chapter in enzymology.

It has been pointed out that "any enzymatic reaction in which C−H cleavage occurs at an unactivated and unfunctionalized carbon will be found to require the action of a metal-containing cofactor that provides a paramagnetic hydrogen-abstracting species".[1] IPNS follows this rule: the valyl-C3(H) is unactivated and must be removed, and IPNS is a mononuclear iron enzyme. Figure 2 shows the structure of the active site of IPNS with ACV bound.[129,140] The ligands to iron are His^{214}, Asp^{216}, His^{270}, and the sulfhydryl group of ACV. Thus, IPNS has a 2His and one carboxylate motif and binds the substrate through its thiolate group. The side chain of Arg^{87} binds the carboxylate group of the α-aminoadipoyl moiety.

The mechanism in Figure 3 allows the substrate ACV to serve as the internal reducing agent for O_2 in its own cyclization to isopenicillin N. Unlike many monooxygenases, the resting form of IPNS has ferrous iron, which can bind O_2 in the manner of heme, as well as the thiolate group of ACV. As in oxyhemoglobin, the Fe^{II}- -O_2 may be regarded as a ferric superoxide ($Fe^{III}-O_2^{\bullet}$), the dominant resonance form. Abstraction of C3(H) as a hydrogen atom from the cysteinyl side chain of ACV by superoxide leads through a six-membered cyclic transition state to a transient thioaldehyde ligated to $Fe^{II}-O-OH$. Dehydration of the peroxide in concert with abstraction of the cysteinyl-NH leads to cyclization to the β-lactam ring, elimination of a water molecule, and generation of the ferryl-oxy species. The nearby valyl isopropyl group presents the valyl-C3(H) for abstraction by the ferryl-oxy species to form the tertiary

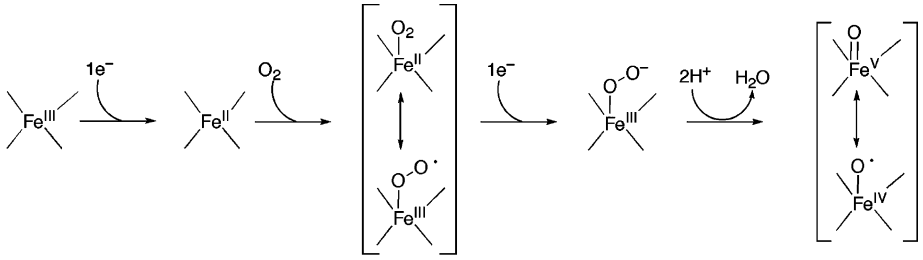


Figure 1. Steps to generate a ferryl-oxy intermediate in a typical reaction of a ferric cofactor with molecular oxygen.

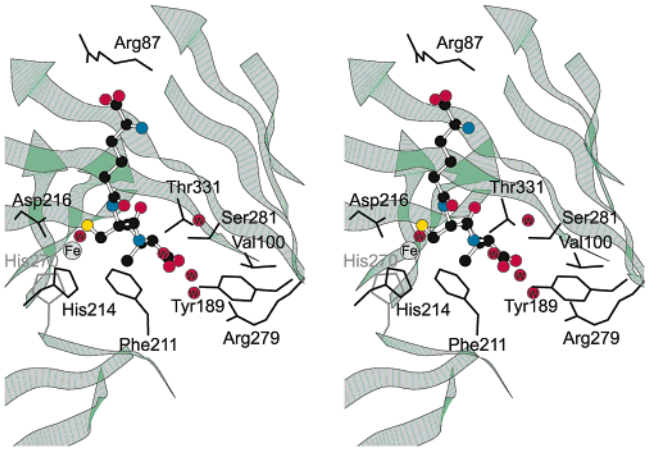


Figure 2. Active site of isopenicillin N synthase. The *Emericella nidulans* IPNS is shown with δ-(L-α-aminoadipoyl)-L-cysteinyl-D-valine (ACV) bound in the active site. The structural model (PDB 1BK0) was refined to 1.30 Å resolution.[145] The cysteine of the tripeptide substrate forms one of five ligands to the octahedrally coordinated ferrous iron atom, leaving one open valence for molecular oxygen to bind. This binding mode for dioxygen is supported by similar structural models with ACV and NO bound to the active site iron.[145]

isopropyl radical. Bonding between the tertiary radical and the thiolate ligand on Fe^{III}−OH closes the five-membered ring and reduces iron to Fe^{II}−OH in preparation for the next catalytic cycle after dissociation of the product.

Abstraction of the cysteinyl-C3(H) by the ferric superoxide radical in Figure 3 may appear questionable. Hydrogen atom abstraction by iron cofactors is normally ascribed to ferryl-oxy species. However, in the case of ACV, the β-carbon of cysteine is bonded to sulfur and the γ-thiolate is ligated to iron. Sulfur bonded to carbon in dialkyl sulfides dramatically stabilizes carbon-centered radicals owing to the spin-delocalizing effect of the nonbonding electrons on sulfur.[142] This effect has been exploited to stabilize a secondary radical in an enzymatic reaction sufficiently to allow for its observation and characterization by EPR spectroscopy.[143,144] In the case of ACV, the thiolate bonded to the cysteinyl β-carbon should be even more stabilizing than sulfur in a sulfide. This effect should weaken the bond to C3(H) and facilitate hydrogen abstraction by ferric superoxide. Whether a discrete, carbon-centered radical is an intermediate or the reaction is concerted, as shown in Figure 3, is not known. In either case, the thiolate ligand would promote hydrogen abstraction.

Evidence for the ferryl-oxy radical intermediate has been obtained. Crystals were grown anaerobically with a substrate analogue in which *S*-methyl-D-cysteine replaced the D-valyl moiety.[141] The structure showed the cysteinyl thiolate group ligated to Fe^{II}. Upon exposure of these crystals to a high pressure of O_2, a yellow chromophore appeared transiently, and crystallographic analysis of the resulting crystals revealed the product structure. The five-membered ring was absent, the β-lactam ring was present, and the C-terminus was *S*-methyl-D-cysteine sulfoxide. Apparently, the yellow chromophore was the ferryl-oxy species, and the *S*-methyl group presented a sulfoxidation target. Ferryl-oxy species generally oxygenate thioethers when they are present in substrate analogues. This reaction apparently revealed the ferryl-oxy species but circumvented abstraction of the hydrogen atom from the isopropyl group of D-valine.

3. Adenosylcobalamin

Adenosylcobalamin (AdoCbl) is a complex organometallic cofactor (see Figure 4) that serves as a radical initiator in the catalytic cycles of the AdoCbl-dependent enzymes. Many aspects of the AdoCbl cofactor and of the AdoCbl-dependent enzymes have been recently reviewed,[77,89,90,106,146,147−149] so only a brief summary and update are necessary here. In the actions of AdoCbl-dependent enzymes (Scheme 1), homolytic cleavage of the cobalt−carbon bond of AdoCbl (BDE ∼ 30 kcal M^{-1}) provides a facile route to an energetic primary alkyl radical, 5′-deoxyadenosine-5′-yl (5′-deoxyadenosyl radical). The cofactor-derived organic radical initiates radical chemistry by abstracting a hydrogen atom from the substrate or, in the specific case of class II ribonucleotide

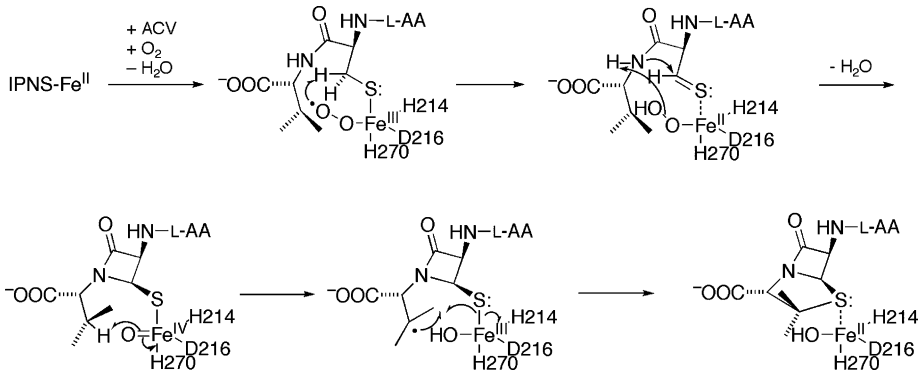


Figure 3. Steps in a mechanism for the action of isopenicillin N synthase.

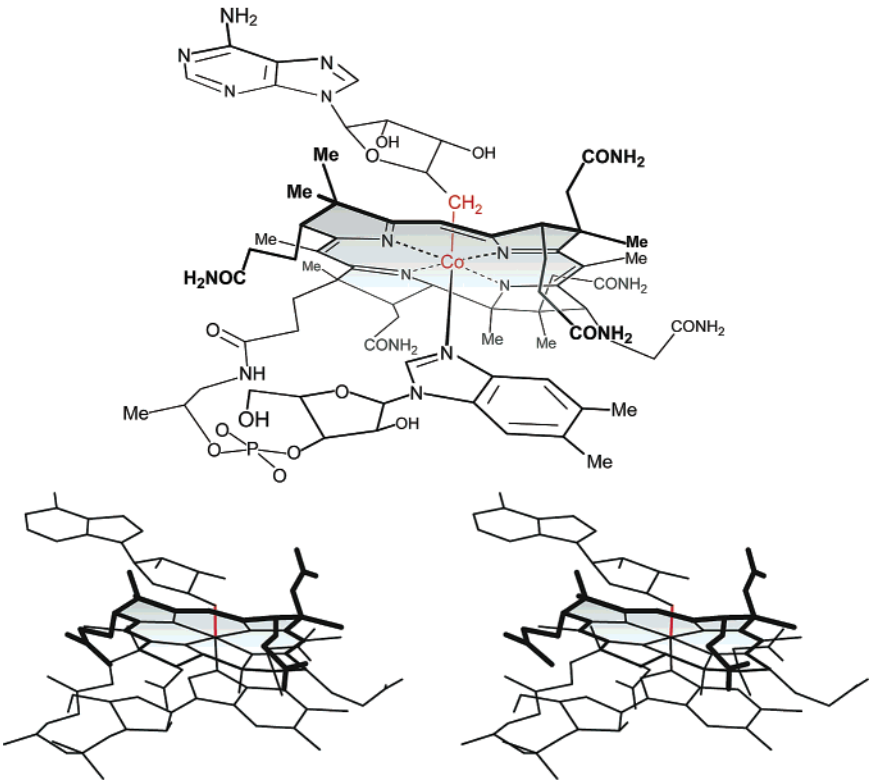
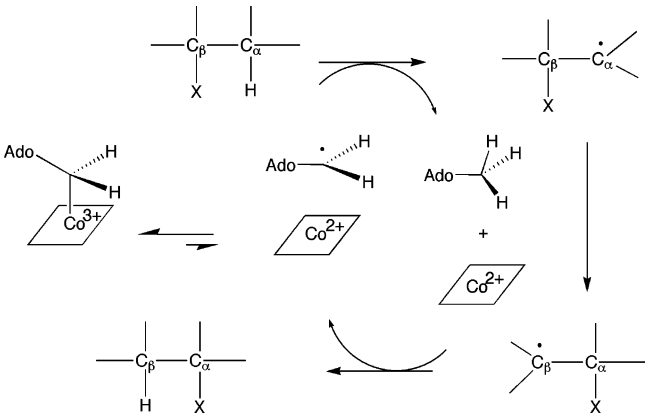


Figure 4. Structure of adenosylcobalamin.

Scheme 1



reducatases, a thiol group of the protein. Following a radical-based substrate rearrangement, a product-derived radical gains back a hydrogen atom from the 5′-CH_3 of the 5′-deoxyadenosine intermediate. The resulting 5′-deoxyadenosyl radical recombines with the Cob(II)alamin to reform the AdoCbl to complete the catalytic cycle. The half-life of the cobalt−carbon bond of AdoCbl in the dark at 37 °C is close to 6 years. The AdoCbl-dependent enzymes shorten this bond lifetime by 10^{12} or greater.[77] The mechanisms for this rate acceleration have not yet been clearly established and remain active areas of research. The mechanisms of the 1,2 rearrangements (Scheme 1) catalyzed by the enzymes are, as yet, poorly understood. The rearrangement mechanisms have been explored by computational approaches.[100,150−153]

Although the 5′-deoxyadenosyl radical is a transient species that has, thus far, escaped direct detection spectroscopically, there is ample indirect evidence for its intermediacy in AdoCbl-dependent reactions.[89,154] In contrast, radicals based on the carbon skeleton of the substrates or products have been observed by EPR in the steady-state of the reactions by freeze-trapping.[156] The EPR spectra of these radical intermediates are typically dominated by electron spin interactions with the low-spin Co^{2+} ($S = {}^1/_2$) of Cob(II)-alamin, which is also present in the active site. The organic radical ($S = {}^1/_2$) and low-spin Co^{2+} ($S = {}^1/_2$) couple to form a spin triplet ($S = 1$). The zero-field splitting of the triplet depends on the distance between the radical and the low-spin Co^{2+} in the active site. The isotropic exchange interaction of the two unpaired electrons also plays a role in the appearance of the EPR spectra. Advances in computational capabilities have made analysis of these complicated spectra tractable.[88,155] The spectra are also influenced by the presence of nuclear spins (e.g., ^1H, ^{13}C, $^{14,15}N$) in the substrate such that isotopic substitutions are a useful method for assigning the structure of the radical components. Rapid mix/freeze quench methods can provide time resolution on the order of a few milliseconds.[156] Several forms of optical spectroscopy and computational approaches have also been applied in these systems.[87,157−159]

3.1. Methylmalonyl-CoA Mutase

Methylmalonyl-CoA mutase (MCM) catalyzes the interconversion of methylmalonyl-CoA and succinyl-CoA. MCM is the sole AdoCbl-dependent enzyme found in mammals. Many aspects of MCM and its catalytic mechanism have been reviewed recently.[77] MCM is a member of the "base off/His-on" AdoCbl-dependent enzymes. The dimethylbenzimidazole lower axial ligand of the free cofactor is displaced by a His residue from the protein upon binding. Thinking about the reaction as it steps through the catalytic cycle has been guided, of late, by elegant three-dimensional structures determined by X-ray crystallography (Figure 5).[160] The mechanism of the rearrangement is thought to involve a cyclopropyl intermediate radical as shown in Scheme 2. A radical intermediate can be freeze-trapped by manually

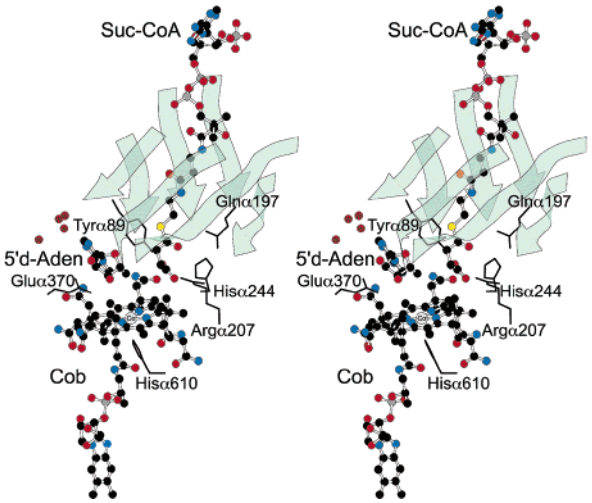


Figure 5. Active site of methylmalonyl-CoA mutase. Succinyl-CoA (Suc-CoA), cobalamin (Cob), and 5′-deoxyadenosine (5′d-Aden) are shown bound in the active site of methylmalonyl-CoA mutase. The image is of the enzyme from *Propionibacterium freudenreichii* subsp. shermanii and was prepared using PDB coordinate file 4REQ for the X-ray crystallographic model refined to 2.20 Å resolution.[84]

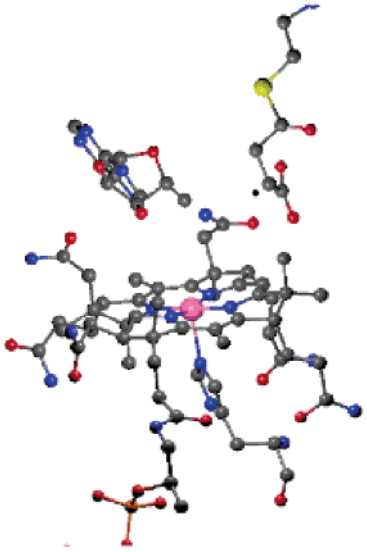


Figure 6. Position of the succinyl-CoA radical in the active site of MCM. Reprinted with permission from ref 162. Copyright 2005 American Chemical Society.

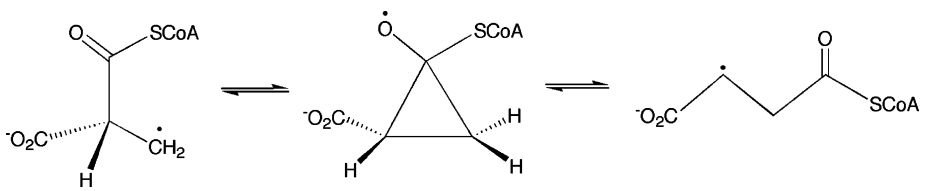
mixing substrate or product with the MCM−AdoCbl complex and freezing the solution in liquid N_2. The EPR spectrum of the intermediate has a *g*-tensor and a ^{59}Co ($I = {}^7/_2$) hyperfine tensor that are consistent with those expected of a strongly coupled, hybrid triplet spin system.[161] The triplet signal was subjected to a detailed analysis that provided estimates of the electron−electron spin coupling parameters, *D* and *J*.[162] The magnitude of *D* and the positions of the ^{59}Co hyperfine signals, in turn, provided an estimate of the position of the organic radical component in the *g*-axis system of cob(II)alamin. Isotopic substitutions,^{2}H and ^{13}C, in the methylmalonyl portion of the initial substrate lead to narrowing and broadening, respectively, of the EPR signals, and they thereby confirm that the organic radical component of the triplet is derived from the substrate and not a group on the protein. Furthermore, the pattern of nuclear hyperfine splitting was consistent with that expected for the succinyl-CoA radical (Scheme 2). Of the three radicals shown in Scheme 2, the succinyl-CoA radical is expected to have the highest stability, owing to delocalization of the unpaired spin onto the adjacent carboxylate group. These EPR results were used to model the radical intermediate into the active site of the enzyme (see Figure 6).[162] Isotope-edited EPR methods have also been used to characterize cob(II)alamin−organic radical pairs in ribonucleotide reductase, glutamate mutase, and ethanolamine-ammonia lyase.[80,88,98,101]

4. Radical SAM

A rapidly growing superfamily of enzymes catalyze radical-based reactions in which *S*-adenosylmethionine (SAM) and a [4Fe-4S] cluster initiate radical formation.[163] In the actions of those members so far studied, SAM is ligated to the iron−sulfur cluster through the α-amino and α-carboxylate groups of the methionyl moiety and undergoes transient 1-electron reduction by the $[4Fe\text{-}4S]^{1+}$ cluster, with concomitant fragmentation to the 5′-deoxyadenosyl radical and methionine ligated to the $[4Fe\text{-}4S]^{2+}$ cluster.[105] The 5′-deoxyadenosyl radical then initiates radical formation by abstracting a hydrogen atom from a substrate to form 5′-deoxyadenosine and a substrate-related free radical.

Two subclasses of radical SAM enzymes employ the 5′-deoxyadenosyl radical in different ways. In one subclass, the SAM-dependent enzyme has a [4Fe-4S] cluster and catalyzes a reaction in which the 5′-deoxyadenosyl radical derived from SAM abstracts a hydrogen atom from the substrate to generate the substrate-related radical and 5′-deoxyadenosine. The substrate radical undergoes further reaction to form the product-related radical, which abstracts a hydrogen atom from the methyl group of 5′-deoxyadenosine to generate the product and reconstitute the 5′-deoxyadenosyl radical. Examples of this subclass in Table 1 include lysine 2,3-aminomutase and spore photoproduct lyase. In another subclass, the 5′-deoxyadenosyl radical abstracts a hydrogen atom from the substrate to produce 5′-deoxyadenosine, methionine, and a substrate-related radical, which undergoes sulfuration to form the substrate-derived product. Examples include biotin synthase and lipoyl synthase. In the third subclass, a SAM-dependent activase has the [4Fe-4S] cluster and employs the 5′deoxyadenosyl radical to activate an enzyme. The 5′-deoxyadenosyl radical generated at the active site of the activase abstracts a hydrogen atom from a specific glycyl residue in the other protein, producing 5′-deoxyadenosine and the corresponding glycyl radical in the

Scheme 2



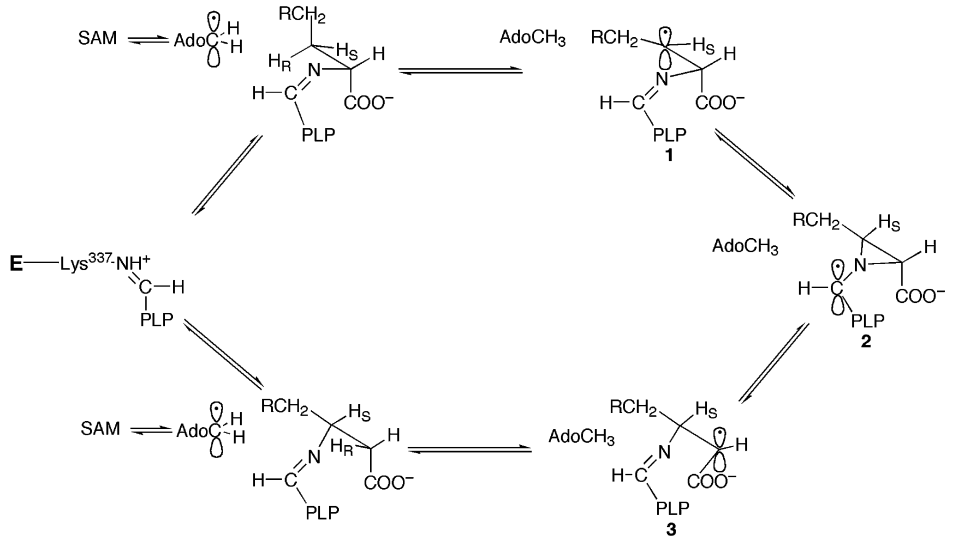


Figure 7. Mechanism for the reaction of lysine 2,3-aminomutase.

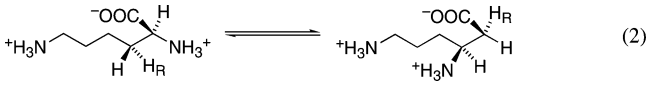
second enzyme, which is thereby activated. Examples in this class include pyruvate formate lyase and its activase, anaerobic ribonucleotide reductase and its activase subunit, benzylsuccinate synthase and its activase, and glycerol dehydratase and its activase.

While numerous examples of radical SAM enzymes have been identified, only a few have been studied in depth. Many other members of the superfamily have not been studied at all, and the chemistry in which they are engaged is not known with certainty. Examples from two subclasses will be discussed briefly in the following sections.

The [4Fe-4S] clusters in radical SAM enzymes so far investigated are constructed within the unique cysteine motif characteristic of the superfamily. The generic motif Cxxx-CxxC provides three cysteinyl ligands for the iron−sulfur cluster, and this leaves a unique iron lacking a cysteinyl ligand.[163,164]

4.1. Lysine 2,3-Aminomutase (LAM)

LAM catalyzes the interconversion of L-α-lysine (lysine) and L-*β*-lysine (*β*-lysine): a reaction in which the α-amino group migrates from C2 of lysine to C3 of *β*-lysine, and the 3-*pro-R* hydrogen of lysine migrates to the 2-*pro-R* position of *β*-lysine, as illustrated in eq 2. As such, the reaction is



(2)

analogous to adenosylcobalamin-dependent rearrangements but does not require a vitamin B_{12} coenzyme.[105−107]

LAM requires SAM and a [4Fe-4S] cluster to initiate radical formation and mediate hydrogen transfer, which is analogous to the role of adenosylcobalamin in the B_{12}-dependent rearrangements. Figure 7 shows the function of the 5′-deoxyadenosyl radical derived from SAM and the mechanism of the isomerization of lysine at the active site of LAM. In the resting enzyme, pyridoxal 5′-phosphate (PLP) is bound to Lys337 as the internal aldimine, and SAM is ligated to the [4Fe-4S] cluster. The reaction begins with the binding of lysine to the internal aldimine of PLP by transaldimination to give the external aldimine of PLP in an imine linkage to the α-amino group of lysine. The [4Fe-4S]-dependent fragmentation of SAM to the 5′-deoxyadenosyl radical initiates the radical mechanism. Abstraction of the 3-*pro-R* hydrogen from the lysyl side chain produces 5′-deoxyadenosine and generates lysyl radical **1** at the active site. Isomerization of radical **1** to the *β*-lysyl radical **3** occurs in two steps by way of the azacyclopropylcarbinyl radical **2**. *β*-Lysyl radical **3** is the product-related radical, and hydrogen transfer from the methyl group of 5′-deoxyadenosine quenches this radical to the external aldimine of *β*-lysine with PLP and regenerates the 5′-deoxyadenosyl radical. Transaldimination with Lys337 releases *β*-lysine and restores the internal aldimine for the next cycle. EPR spectroscopic and kinetic characterizations of the intermediate **3** and closely related analogues of the intermediates **1** and the 5′-deoxyadenosyl radical verify the mechanism in Figure 7.[105−107]

The focus now shifts to the mechanism by which a reaction of SAM with the[4Fe-4S] cluster leads to the 5′-deoxyadenosyl radical. The process may be described as in eq 3,

$$\mathbf{E}-[4\text{Fe-}4\text{S}]^{1+}\cdot\text{SAM} \rightleftharpoons \mathbf{E}-[4\text{Fe-}4\text{S}]^{2+}\cdot\text{Met}\cdot 5'\text{-dAdo}^{\bullet} \quad (3)$$

in which electron transfer from the iron−sulfur cluster to SAM leads to the reversible fragmentation of SAM into the 5′-deoxyadenosyl radical and methionine.[165] *Se*-adenosyl-L-selenomethionine (*Se*SAM) is nearly as efficient as SAM in activating LAM. A selenium XAS study of LAM activated with *Se*SAM implicated a direct ligation of selenium with iron (2.7 Å) in the [4Fe-4S] cluster under conditions in which the C5′−Se bond of *Se*SAM was cleaved, that is, in radical complexes such as that on the right side of eq 3.[166] Close interactions of selenium and iron were not observed before cleavage of *Se*SAM. Because SAM and *Se*SAM should function similarly, the sulfur of methionine should be ligated to iron in complexes in which SAM is cleaved to 5′-deoxyadenosine and methionine. These results suggest that the sulfur of SAM is poised to ligate the unique iron in the [4Fe-4S] cluster in the precleavage structure, and direct ligation takes place upon electron transfer and cleavage to the 5′-deoxyadenosyl radical.

In an ENDOR study patterned after a similar study of the pyruvate formate lyase activase,[167] the ligands to the unique iron in the [4Fe-4S] cluster in LAM were assigned as the

Figure 8. Mechanism for the reversible homolytic cleavage of SAM at the active site of lysine 2,3-aminomutase to form the 5′-deoxyadenosyl radical.

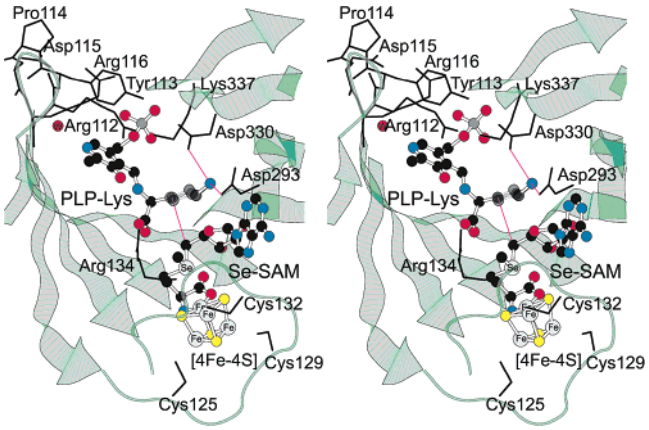


Figure 9. Active site of lysine 2,3-aminomutase. The active site of lysine 2,3-aminomutase from *Clostridium subterminale* is shown with the lysyl-PLP external aldimine (PLP-Lys), seleno-S-adenosylmethionine (Se-SAM), and [4Fe-4S] iron sulfur cluster bound. The red line shows the potential contact between C5′ of the deoxyadenosyl moiety of the SAM and C3(H) of the lysyl side chain. The structural model (PDB 2A5H) was refined to 2.10 Å resolution.[169]

α-amino and α-carboxylate groups of SAM.[168] Using the $[4Fe\text{-}4S]^{1+}$ form of the cluster as the paramagnetic probe, and ^{17}O in [methionyl-1-^{17}O]SAM or ^{15}N in [methionyl-2-^{15}N]SAM as the NMR nuclei, the ENDOR experiments definitively identified the α-amino and α-carboxylate groups as iron ligands. Correlating the XAS and ENDOR results led to the mechanism in Figure 8 for the reversible fragmentation of SAM at the active site of LAM. In this mechanism, the sulfonium group of SAM is poised near the unique iron of the $[4Fe\text{-}4S]^{1+}$ cluster in the resting enzyme, with the α-amino and α-carboxylate groups directly ligated to iron. Upon electron transfer from the cluster to its ligand SAM, the cluster is oxidized to $[4Fe\text{-}4S]^{2+}$, the C5′−S bond in SAM breaks, the methionyl-sulfur becomes ligated to the [4Fe-4S] cluster, and the 5′-deoxyadenosyl radical is formed. In an alternative mechanism, based on the ENDOR studies of the irreversible cleavage of SAM by the action of pyruvate formate lyase activase, the sulfur of SAM becomes ligated to a sulfide in the $[4Fe\text{-}4S]^{2+}$ cluster upon electron transfer and cleavage of SAM.[168]

The structure of a complex of LAM with PLP, *Se*SAM, lysine, and the $[4Fe\text{-}4S]^{2+}$ cluster at 2.1 Å resolution supports the mechanism for reversible cleavage of SAM in Figure 8.[169] This complex is closely analogous to the Michaelis complex, except for the oxidation state of the [4Fe-4S] cluster, which is (2+) instead of (1+). Spectroscopic evidence by ENDOR does not reveal a difference in the structures of the two oxidation states.[168] The structure at the active site in Figure 9 shows lysine in an imine linkage to PLP, with the α-carboxyl group bound to conserved Arg134 and the e-amino group bound to the conserved Asp293 and Asp330. *Se*SAM ligates the unique iron in the [4Fe-4S] cluster through its α-amino and α-carboxylate groups. Carbon-3 of the lysyl side chain is within 3.8 Å of C5′ in SAM, in excellent range for hydrogen abstraction upon formation of the 5′-deoxyadenosyl radical.

The structure shows the selenium of *Se*SAM within 3.2 Å of the unique iron in the [4Fe-4S] cluster and poised in perfect position to form a coordination bond upon cleavage of the C5′−Se bond. Upon cleavage and ligation, the Fe and Se would close to the 2.7 Å distance observed in the XAS studies. The unique iron is the nearest noncovalently bonded atom to selenium. The proximity of selenium and iron, together with the conformation of the selenomethionyl side chain, definitely supports the mechanism in Figure 8.

The current and future focus on this mechanism concerns the reduction potential of the [4Fe-4S] cluster in LAM with reference to the required potential for electron transfer to the sulfonium group of SAM. The midpoint reduction potentials of biological redox cofactors are not compatible with the requirements for electron transfer to a sulfonium group. Some means for modulating the midpoint reduction potential of the [4Fe-4S] cluster must exist in the radical SAM superfamily. (Recent redox titrations show that the reduction potential for the $[4Fe\text{-}4S]^{2+}$ cluster in LAM in its complex with SAM is −430 mV [Hinckley, G. T.; Frey, P. A. *Biochemistry* **2006**, *45*, 3219.] Further measurements show that, upon binding L-lysine to this complex, the reduction potential is lowered to −600 mV [Wang, S.; Frey, P. A. Unpublished results]. Independent data indicate that the equilibrium constant for the reductive cleavage of SAM at the active site is about 10^{-5}. On this basis, the reduction potential for the reductive cleavage of SAM bound to the [4Fe-4S] cluster is about −870 mV. Thus, the binding of SAM to the iron−sulfur cluster elevates the reduction potential by about 730 mV, and the binding of L-lysine depresses the reduction potential of the $[4Fe\text{-}4S]^{2+}$ cluster by 170 mV, so that the two are separated by about 270 mV.)

4.2. Pyruvate Formate Lyase and Activase

Pyruvate formate lyase (PFL) catalyzes the conversion of pyruvate and CoA to acetyl-CoA and formate.[170] The enzymatically active form of PFL from *E. coli* harbors a radical equivalent on a specific glycyl residue (Gly734).[171,172] PFL is not itself a radical SAM enzyme. The glycyl-734 radical in PFL, as in other glycyl radical-containing enzymes, is produced post-translationally by PFL activase, a SAM-dependent and [4Fe-4S]-containing radical SAM enzyme.[172,173] In the absence of O_2, the glycyl-734 radical in PFL is persistent owing to the resonance stabilization provided by the adjacent amide nitrogen and glycyl carbonyl groups. Under aerobic conditions, the enzyme is protected from O_2-mediated peptide cleavage by a deactivase enzyme.[174] Characterization of PFL, including aspects of its catalytic mechanism, has been reviewed,[106,175] so a brief update is presented here. Spectroscopic characterization of the PFL activase has been recently reviewed.[173]

All evidence indicates that the glycyl-734 radical in PFL does not interact directly with pyruvate or CoA. Rather, the radical center is first transferred to nearby residues Cys418 and Cys419, the thiyl radical forms of which are responsible for the radical chemistry involving the substrates.[106,176] The structural arrangement of Gly734, Cys418, and Cys419 at the active center is shown in Figure 10. Cys419 is responsible for the exchange of the C-2(H) of the glycyl-374 radical with solvent protons.[176,177] Although ping pong kinetics has not been established in the action of PFL, the overall reaction is

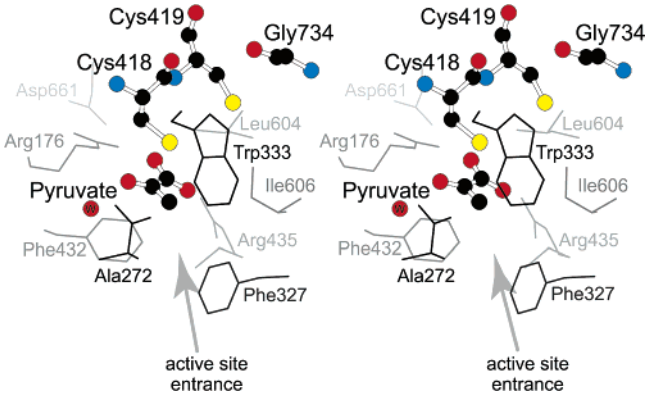


Figure 10. Active site of pyruvate formate lyase. Pyruvate is bound in the active site of *Escherichia coli* PFL. The structural model (PDB 1H16) was refined to 1.53 Å resolution.[178]

discussed in terms of two half-reactions.[178] The first half-reaction is the conversion of pyruvate to formate and an acetyl enzyme intermediate as shown in Figure 11.

In the second half-reaction, CoA reacts with the acetyl enzyme to form the acetyl-CoA and regenerate the enzyme-based radical. A radical mechanism of transacetylation is shown in Figure 12. However, a conventional polar transacetylation mechanism between CoASH and the acetyl-enzyme (complex 7 in Figures 11 and 12) would be simpler; in this case, the radical center at Gly734 would be a spectator.

The reaction sequence is intriguing because of the movement of the radical center among groups on the protein and onto substrates and products. The timing of formation of the adduct between pyruvate and Cys418 is of interest because an earlier mechanistic proposal assumed formation of a thiohemiketal between pyruvate and one of the active site cysteinyl residues.[179] Crystallographic data at 1.6 Å resolution[178] for the complex of the unactivated enzyme (i.e., enzyme lacking the radical at Gly734) with pyruvate and CoA show pyruvate bound between Arg176 and Arg435 and in intimate contact with the side chain SH of Cys418. Carbon-2 of pyruvate appears to be in an sp^2 configuration, suggesting that a thiol hemiketal adduct is not present. The ribosyl-pantetheine moiety of CoA is positioned away from the active site. However, rotation about the glycosidic bond (*syn* to *anti*) would bring the pantethiene group into position to acquire the acetyl group from Cys418.

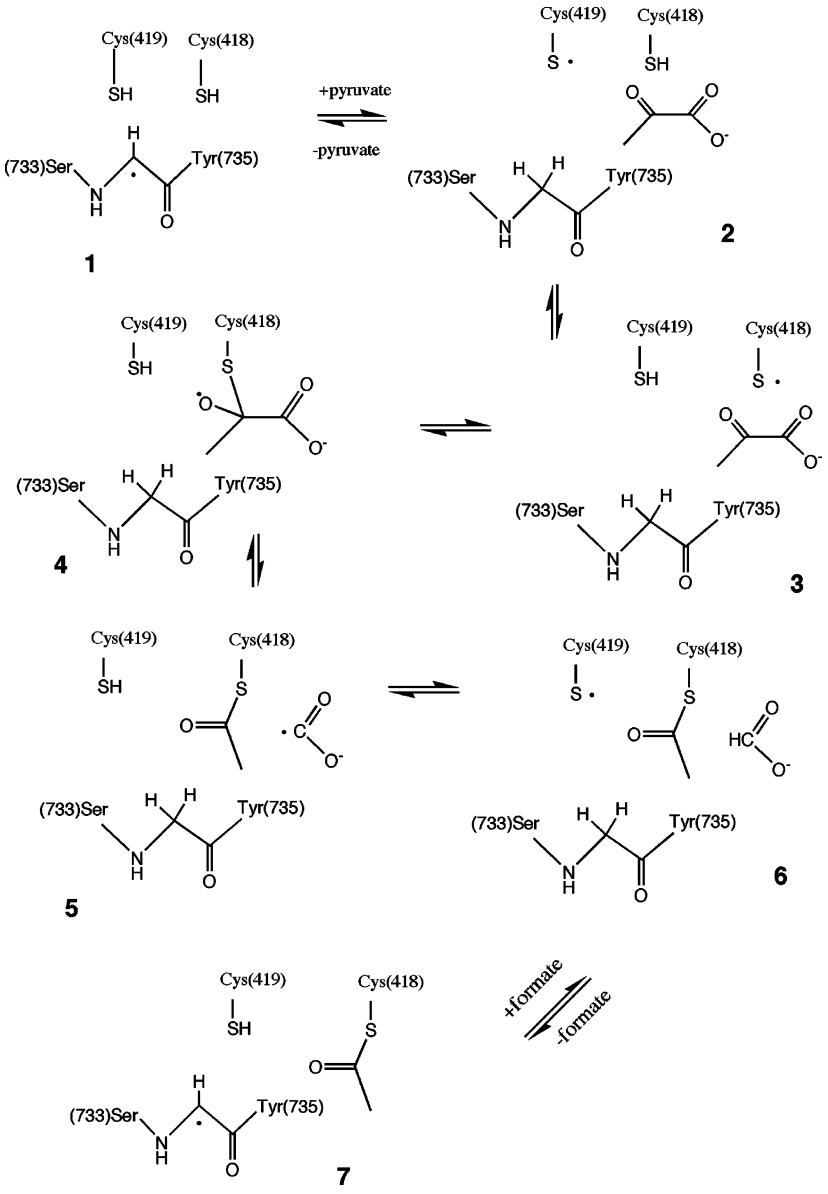


Figure 11. Mechanism for the first half-reaction in the action of PFL.

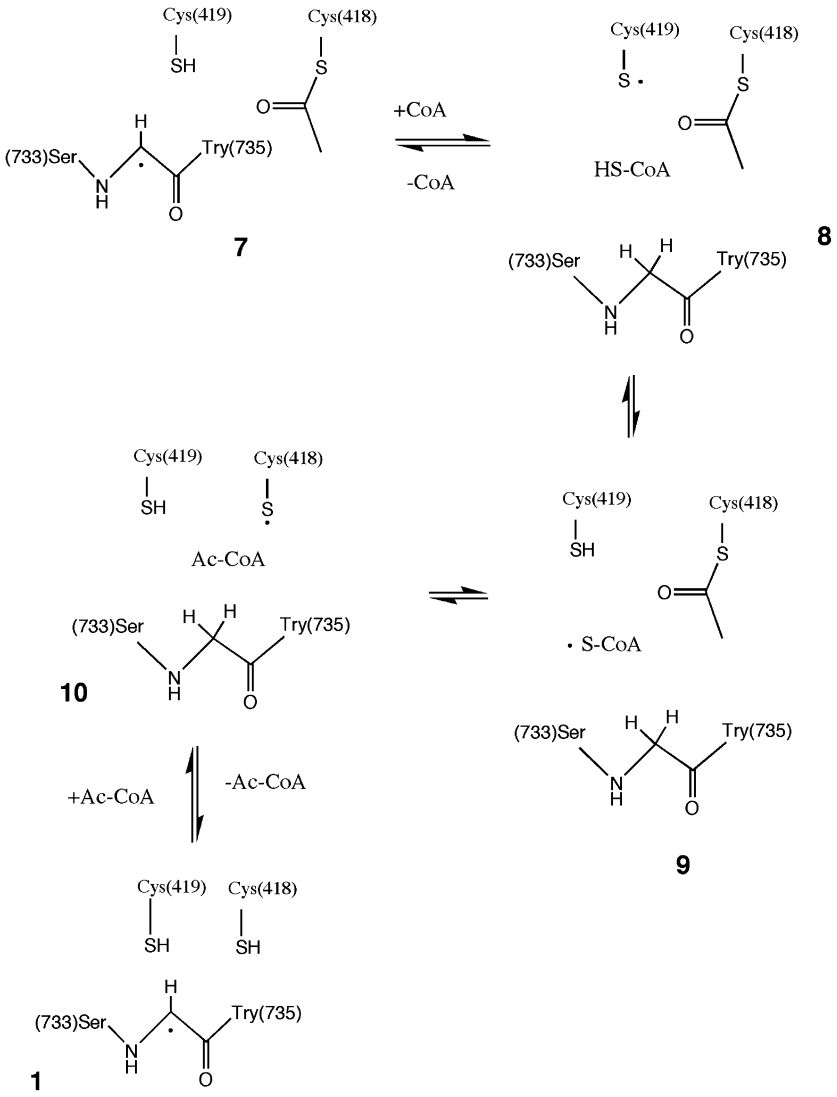


Figure 12. Mechanism for the second half-reaction in the action of PFL.

5. Hydroxyethylidene-thiamin Pyrophosphate Radicals

While many enzymatic radical mechanisms are initiated by abstraction of hydrogen atoms, radical mechanisms in enzymology occur in a more broadly based context that includes electron transfer as a mechanism for radical formation. Well-known examples include (i) the oxygen-activating enzymes that transiently form superoxide by inner sphere electron transfer to molecular oxygen, either from a dihydroflavin or a transition metal, and (ii) the class I ribonucleotide reductases that generate enzyme-thiyl radicals by long-range electron transfer.[52−54] The FAD-dependent pyruvate oxidase (POX) and the [4Fe-4S]-dependent pyruvate ferredoxin oxidoreductase (PFOR) are thiamine pyrophosphate (TPP)-dependent enzymes that catalyze radical reactions in which electron transfer from the intermediate 2-(α-hydroxyethylidene)-TPP initiates radical formation. The initial electron acceptor in POX is FAD, and in PFOR it is a [4Fe-4S] cluster.

TPP functions as an essential coenzyme in enzymatic reactions involving carbon−carbon cleavage/formation of α-hydroxycarbonyl compounds or the decarboxylation of α-ketoacids. Most of these reactions proceed by polar mechanisms. However, the fact that all TPP-dependent enzymatic reactions can be assayed by the ferricyanide reduction method, in which ferricyanide is reduced to ferrocyanide, suggests the ability of TPP to participate in radical reactions.[180,181] The ferricyanide assay is based on the oxidation of essential intermediates in TPP-dependent reactions, either 2-(α-hydroxyethylidene)-TPP or 2-(α,β-dihydroxyethylidene)-TPP, the structures of which are shown in Figure 13, to 2-acetyl-TPP or 2-glycolyl-TPP. Hydrolysis of the 2-acyl-TPP regenerates TPP for the next catalytic cycle.

Because ferricyanide is an obligatory one-electron acceptor, it must oxidize a hydroxyethylidene-TPP in two steps, so that an hydroxyethylidene-TPP radical must be an intermediate. Two 2-(α-hydroxyethylidene)-TPP radicals at different protonation levels are shown in Figure 13. While hydroxyethylidene-TPP radicals play no role in many TPP-reactions, POX and PFOR catalyze electron transfer from 2-(α-hydroxyethylidene)-TPP, to FAD in the reaction of POX or to a [4Fe-4S] cluster in the case of PFOR, to form 2-(α-hydroxyethylidene)-TPP radicals as intermediates.

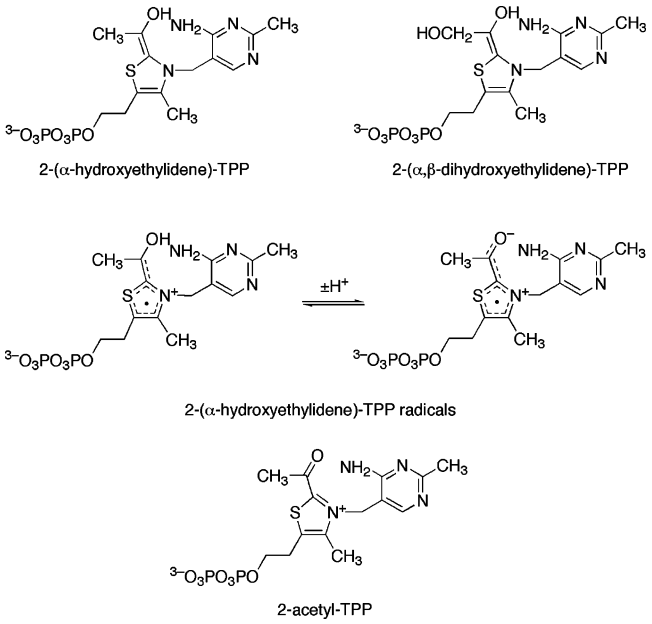


Figure 13. Structures of TPP intermediates in enzymatic reactions. 2-(α-Hydroxyethylidene)-TPP and 2-(α,*β*-dihydroxyethylidene)-TPP are intermediates in most TPP-dependent reactions. In a few cases, 2-(α-hydroxyethylidene)-TPP undergoes electron transfer to form a species of the 2-(α-hydroxyethylidene)-TPP radical as the successive intermediate. This radical exists at the two protonation levels shown in the figure. Upon further one-electron oxidation, the radical can be transformed into the successive intermediate 2-acetyl-TPP.

5.1. Pyruvate Oxidase (POX) from *Lactobacillus Plantarum*

POX from *L. plantarum* or *Aerococcus viridans* catalyzes the reaction of pyruvate with phosphate and molecular oxygen to form acetyl phosphate, CO_2, and hydrogen peroxide.[6,136] POX from *Lactobacilli* or *Aerococci* is a TPP-dependent flavoprotein. The reaction proceeds by the sequence of steps in eqs 4a−4d. The first two steps (eqs 4a and 4b) take place by the polar mechanisms typical of TPP. The last three steps (eqs 4c−4e) involve free radicals. Reaction 4c is a multistep process involving two electron transfers leading to $FADH_2$ and an acetyl group transfer leading to acetyl phosphate. The oxidation of $FADH_2$ by molecular oxygen likely proceeds by the conventional two-step mechanism outlined by eqs 4d and 4e, in which electron transfer from $FADH_2$ leads to the transient formation of superoxide and a FAD-semiquinone, and further electron and proton transfers in eq 4e produce hydrogen peroxide and FAD.

$$CH_3COCOO^- + \mathbf{E}\cdot FAD\cdot TPP + H^+ \rightleftharpoons \mathbf{E}\cdot FAD\cdot \text{lactyl-TPP} \quad (4a)$$

$$\mathbf{E}\cdot FAD\cdot \text{lactyl-TPP} \rightarrow \mathbf{E}\cdot FAD\cdot \text{2-(α-hydroxyethylidene)-TPP} + CO_2 \quad (4b)$$

$$\mathbf{E}\cdot FAD\cdot \text{2-(α-hydroxyethylidene)-TPP} + P_i \rightarrow \mathbf{E}\cdot FADH_2\cdot TPP + \text{acetyl-P} \quad (4c)$$

$$\mathbf{E}\cdot FADH_2\cdot TPP + O_2 \rightleftharpoons \mathbf{E}\cdot FADH\cdot(O_2^{\bullet -})\cdot TPP + H^+ \quad (4d)$$

$$\mathbf{E}\cdot FADH\cdot(O_2^{\bullet -})\cdot TPP + H^+ \rightarrow \mathbf{E}\cdot FAD\cdot TPP + H_2O_2 \quad (4e)$$

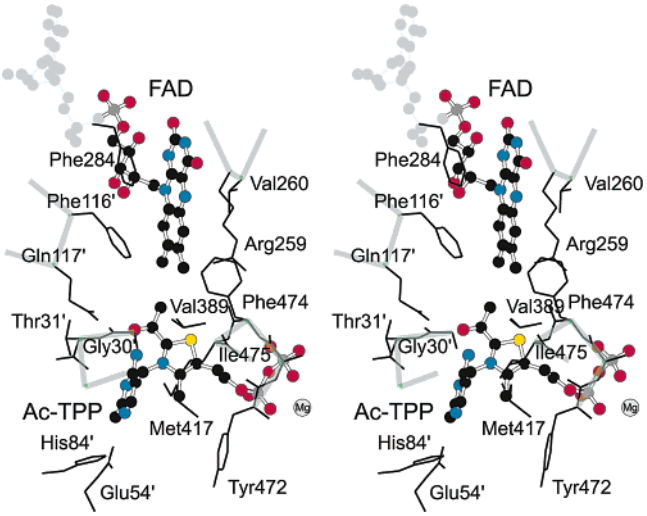


Figure 14. Active site of pyruvate oxidase. The active site of POX from *Aerococcus viridans* is shown with flavin adenine dinucleotide (FAD) and 2-acetyl thiamin pyrophosphate (Ac-TPP) bound. The structural model (PDB 1V5G) was refined to 1.96 Å resolution (Hossain et al., 2003, unpublished PDB deposition 1V5G).

Structures of POX from *L. plantarum* and *Aerococcus viridans* are available, and all show the proximity of the FAD and TPP binding sites. Figure 14 shows the active site of POX from *A. viridans* with $FADH_2$ and 2-acetyl-TPP bound at the active site. $FADH_2$ lies within 7 Å of 2-acetyl-TPP, and this is most likely essentially the same in the intermediate with FAD and 2-(α-hydroxyethylidene)-TPP in the site. The two coenzymes are easily within range for electron transfer on the time scale of enzymatic catalysis.

The mechanism of the multistep process in eq 4c is of particular interest in the context of radical enzymology. The transformation of FAD to $FADH_2$ can be monitored in transient kinetic studies by spectrophotometry. When the reaction is carried out in the presence of phosphate, no intermediate species of the flavin coenzyme can be detected spectrophotometrically, nor can acetyl-TPP be detected by NMR spectroscopy, either in the steady state or in transient kinetic experiments. However, when phosphate is excluded, and the transient reactions of FAD are observed spectrophotometrically, there are two distinct kinetic phases in the transformation of FAD into $FADH_2$, and 2-acetyl-TPP is an intermediate in the transient and steady states. The products in the absence of phosphate include acetate from the hydrolysis of 2-acetyl-TPP in place of acetyl phosphate.

The two kinetic phases in the reduction of FAD are of interest because the intermediate has been assigned as a FAD-semiquinone, based on the prominent, long-wavelength absorption band (550−650 nm) that transiently appears.[6] The first electron transfer step to form the semiquinone takes place independently of phosphate at the same rate as in the presence of saturating phosphate. The second electron transfer step is faster in the presence of phosphate, so that intermediates are not observed, but much slower by a factor of >100 in the absence of phosphate. Thus, intermediates accumulate in the absence of phosphate, specifically the FAD-semiquinone and presumably a hydroxyethylidene-TPP radical. The protonation state of the 2-(α-hydroxyethylidene)-TPP radical is not known, but the electronic spectra of the flavin intermediate are consistent with a mixture of the blue and red semiquinone forms. There is currently no spectroscopic evidence for the 2-(α-hydroxyethyl)-TPP radical, and

its existence is inferred from the transient formation of the FAD-semiquinone.

5.2. Pyruvate:Ferredoxin Oxidoreductase (PFOR)

PFOR from *Halobacterium halobium* catalyzes the TPP-dependent decarboxylation of pyruvate and the reduction of ferredoxin according to eq 5. The EPR spectrum of a 2-(α-

$$CH_3COCOO^- + 2Fd_{ox} + CoASH \rightarrow CO_2 + CH_3COSCoA + 2Fd_{red} \quad (5)$$

hydroxyethylidene)-TPP radical at the active site of PFOR has been known since 1981.[182] A structure of this form of PFOR is available and shows three iron−sulfur clusters situated appropriately for electron transfer to ferredoxin.[134] The active site and relative orientations of the three clusters are shown in Figure 15.

The active site binds the essential coenzyme TPP and catalyzes the initial polar steps of the mechanism, beginning with ionization of C2(H) from TPP to form the ylid-like TPP anion. Addition of this anion to the carbonyl group of pyruvate produces lactyl-TPP, which undergoes decarboxylation to 2-(α-hydroxyethylidene)-TPP at the active site, similar to the corresponding steps in many TPP-dependent reactions, including that of POX (eqs 4a and 4b). Oxidation of the latter takes place in two one-electron steps, with the electrons being relayed to ferredoxin through the participation of three [4Fe-4S] clusters, as illustrated in Figure 15. The clusters are aligned in an electron-transfer pathway spanning the distance from the active site to the surface of the protein. Clusters B and C are near the surface, and cluster A lies about midway between the active site and the surface. The first electron transfer to cluster A generates the 2-(α-hydroxyethylidene)-TPP radical independently of the presence of coenzyme A. The electron is channeled through

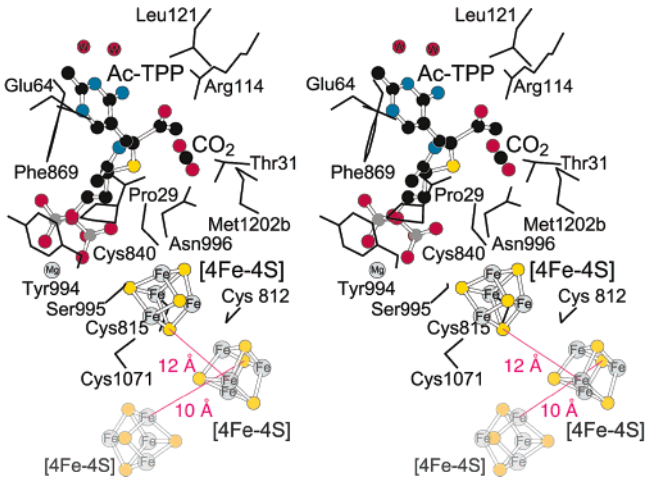


Figure 15. Active site and iron−sulfur clusters of pyruvate: ferredoxin oxidoreductase. The active site of PFOR from *Desulfovibrio africanus* is shown in a complex of acetyl thiamin pyrophosphate (Ac-TPP) and carbon dioxide that results from the reaction of pyruvate in the absence of CoA. Electron transfer to the active site from ferredoxin is mediated by three iron sulfur clusters [4Fe-4S] shown in the figure trailing off below and behind the active site. CoA is required for transfer of a second electron in the reaction of Ac-TPP to form CO_2 and AcCoA during catalysis. Accumulation of an acetyl thiamin pyrophosphate radical species in the PFOR active site has been demonstrated by EPR of the crystallized enzyme in the absence of CoA. The structural model (PDB 1KEK) was refined to 1.90 Å resolution.[134]

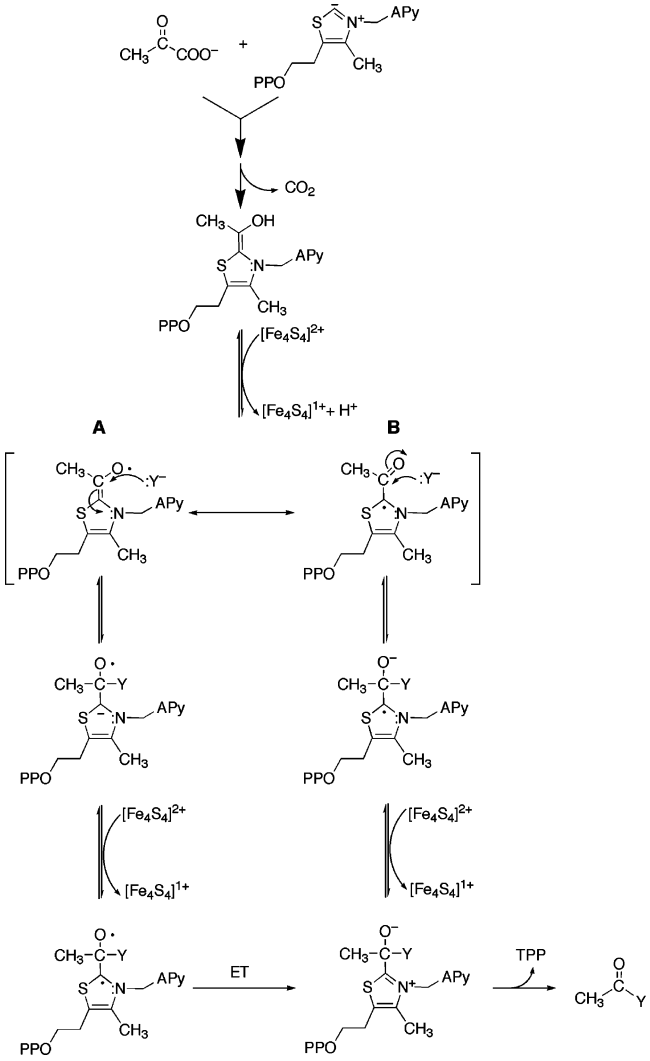


Figure 16. Two mechanisms for addition of nucleophiles to 2-[α-hydroxyethylidene]-TPP radicals. In the action of PFOR, the nucleophile $:Y^-$ is $CoAS^-$, and in the action of POX, it is $H_2PO_4^-$. In both mechanisms shown here, an electron is transferred from 2-[α-hydroxyethylidene]-TPP to an iron−sulfur cluster to generate the radical, which is shown in two resonance forms. In the actions of POX and PFOR, the second electron transfer has been suggested to proceed with the addition of the nucleophile to the 2-[α-hydroxyethylidene]-TPP radical to form an adduct.[6,183] In mechanism A shown at the left, the nucleophile $:Y^-$ undergoes addition to the 2-[α-hydroxyethylidene]-TPP radical to form a radical anion, the second electron transfer to an iron−sulfur cluster leads to a diradical, and internal electron transfer leads to the tetrahedral adduct of $:Y^-$ and 2-acetyl-TPP, which decomposes to TPP and either acetyl-phosphate or acetyl-CoA. In mechanism B, the 2-[α-hydroxyethylidene]-TPP radical is written in the resonance form shown at the right, which emphasizes the carbonyl nature of the C2′−O group, and nucleophilic addition to this carbonyl group leads to an alternative structure for the intermediate adduct. Electron transfer to an iron−sulfur cluster leads directly to the tetrahedral intermediate and then to TPP and acetyl-phosphate or acetyl-CoA. In mechanism B, the unpaired electron is highly delocalized in all intermediates, whereas, in mechanism A, the unpaired electron is localized after addition of the nucleophile.

cluster A to clusters B/C and then to the reduction of ferredoxin. In the overall reaction (eq 5), coenzyme A is the acetyl group acceptor, and it is also required for the second electron-transfer step. The second electron also reduces ferredoxin by the same electron-transfer pathway.

The initial, coenzyme A-independent electron-transfer allows the radical to be observed as a prominent signal in

the EPR spectrum obtained when the enzyme is incubated with pyruvate in the absence of coenzyme A.[182,183] In a recent study of PFOR from *M. thermoacetica*, analysis of isotope-edited EPR spectra and electronic structure calculations suggested that the radical was a π radical having unpaired spin density distributed between C2 of the substrate and the π system of the thiazolium ring of TPP.[184] In the complete system, with pyruvate and coenzyme A present, no intermediates are observed.

5.3. The Mechanism of Acceptor-Mediated Electron Transfer

In the actions of both POX and PFOR, the first electron-transfer step from the 2-(α-hydroxyethylidene)-TPP to an iron−sulfur cluster takes place in the absence of the acetyl group acceptor. However, the second electron transfer from the 2-(α-hydroxyethylidene)-TPP requires the acetyl group acceptor, phosphate in the case of POX or coenzyme A in the case of PFOR. Several mechanisms have been considered to account for acceptor-dependent electron transfer from the 2-(α-hydroxyethylidene)-TPP radical.[6,183] A mechanism proposed for both enzymes involves the transient formation of a radical adduct between the acceptor and the 2-(α-hydroxyethyl)-TPP radical, with the radical adduct serving as the source of the second electron. This would account for the requirement for the acceptor to drive the second electron transfer. The acceptor-dependent electron transfer is proposed to follow the course of mechanism A in Figure 16.[6,183] The structure of the adduct radical in mechanism A remains to be established. In an alternative formulation, the unprotonated 2-[α-hydroxyethylidene]-TPP radical can be written in the resonance form shown at the right in Figure 16 (mechanism B). This resonance form accentuates the resemblance of C2′ and C2′(O) to a carbonyl group that can undergo nucleophilic addition of either phosphate or CoASH to form a radical adduct, in which the unpaired electron is fully delocalized within the thiazole ring. Electron transfer and elimination of TPP generates acetyl-CoA or acetyl phosphate.

6. Conclusions

Free radical mechanisms have entered the mainstream of enzymological research. Free radicals are involved in amino acid, carbohydrate, and hydrocarbon metabolism; detoxification of xenobiotics; coenzyme and cofactor biosynthesis; hormone metabolism; DNA biosynthesis and repair; and antibiotic biosynthesis. Most free radical mechanisms require coenzymes or/and cofactors such as flavins, heme, pterins, quinone cofactors, di-iron complexes, mononuclear iron, copper, adenosylcobalamin, *S*-adenosylmethionine/[4Fe-4S], thiamine pyrophosphate, and pyridoxal-5-phosphate. Much has been learned about radical mechanisms in enzymology, but most are poorly understood from the standpoint of the energetics of radical initiation. The explosive growth in the number of enzymes employing *S*-adenosylmethionine/[4Fe-4S] or mononuclear iron chemistry has rekindled interest in free radical mechanisms in enzymology.

7. Acknowledgments

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