Importance of Organic Radicals in Enzymatic Cleavage of Unactivated C–H Bonds

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Contents

Perry Allen Frey was born in Plain City, OH, in 1935. He received his B.S. degree in chemistry from The Ohio State University in 1959 and his Ph.D. degree in biochemistry from Brandeis University in 1968. He was a Postdoctoral Fellow at Brandeis University and Harvard University. He worked from 1960 to 1964 as a Chemist with the U.S. Public Health Service, Cincinnati, OH. He then moved to the Department of Chemistry, The Ohio State University (1969–1981), where he attained Full Professor in 1979. Presently, he is Professor and Co-Director, Institute for Enzyme Research, and Professor of Biochemistry, University of Wisconsin. Research interests include mechanisms of enzyme and coenzyme action.

I. Introduction

In classical biochemistry the most important organic radicals were the flavin semiquinones and the semiquinone forms of coenzyme Q, which were known to be important in biological redox reactions. Most other enzymatic reactions were thought to proceed by polar reaction mechanisms. One of my teachers once observed that a nonpolar reaction mechanism may be invoked for an enzymatic reaction when no reasonable polar mechanism is available. Over the past 20 years a number of enzymatic reactions have been found to proceed by mechanisms involving organic radicals as intermediates. However, the rule, while it may be amended, has been durable, since most examples of nonpolar enzymatic mechanisms involve reactions for which no reasonable polar mechanism can be written. In many of these reactions a difficult step is the cleavage of unactivated carbon–hydrogen bonds in substrates, and the means by which unactivated C–H bonds are broken by enzymes is the principal subject of this paper.

This review will advance the thesis 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; and the mechanism will involve the formation of a substrate-derived organic radical as an intermediate. The most common and thoroughly studied of these reactions are the adenosylcobalamin (B_{12}) dependent rearrangements and the cytochrome P-450 dependent monooxygenations of hydrocarbons. These B_{12} and cytochrome P-450 systems have been extensively reviewed[1-3] and will be only briefly updated here. A major topic of this paper is the mechanism of action of lysine 2,3-aminomutase, which catalyzes a reaction that is analogous to adenosylcobalamin-dependent rearrangements but is not cobalamin-dependent. The lysine 2,3-aminomutase reaction will be shown to be mechanistically analogous to B_{12}-type rearrangements and to involve the generation of an adenosylmetal cofactor whose structure is not known but that has properties and functions in common with adenosylcobalamin. A second topic of this paper is the mechanism of action of methane monooxygenase, which is not a cytochrome P-450 monooxygenase but instead contains a μ-oxodiiron complex as the oxygenation cofactor. The methane monooxygenase reaction will be shown to proceed by a mechanism that is analogous in part to the cytochrome P-450 monooxygenase reactions, in that substrate radicals appear to be intermediates, but differs in that carbocations appear also to be intermediates, at least in the reaction of one substrate.

I state with emphasis at the outset that many enzymatic reactions are poorly understood in mechanistic terms, and reactions other than those described in this review will no doubt be found to proceed by mechanisms involving organic radicals. Many oxidases and oxygenases catalyze reactions involving C–H bond cleavage at functionalized carbon or benzylic carbons. These reactions involve cofactors such as metals, flavins, pyrroloquinoline quinone (PQQ), pteridines and other

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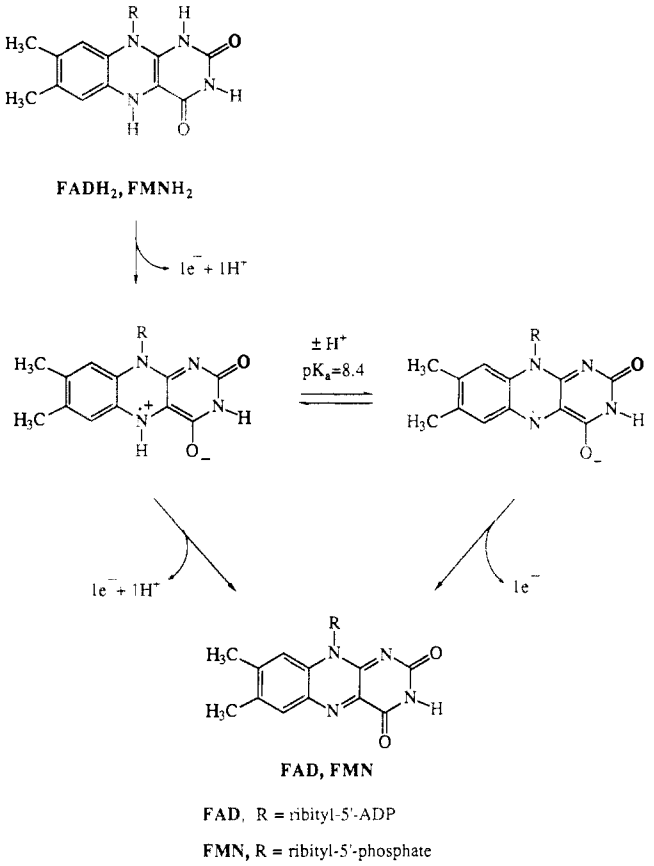


Figure 1. Structures of oxidized, reduced, and semiquinone forms of the isoalloxazine ring in flavin coenzymes.

special molecules that can catalyze reactions by mechanisms involving radical intermediates. Many of these reactions are under intensive investigation in various laboratories, where evidence for organic radicals as intermediates is accumulating. The main emphasis in this review will be on reactions involving cleavage of C–H bonds at alkane-type carbon atoms that are unfunctionalized and unactivated.

II. Organic Radicals in Biochemistry

A. Classical Systems: Semiquinones

The most widely known organic radicals in biochemistry are the semiquinone radicals of flavin coenzymes and coenzyme Q (CoQ). The unpaired electron in the flavin semiquinones is delocalized through the isoalloxazine ring, and that in the semiquinone form of Co Q is delocalized through the quinone system. The stabilities of these radicals, the structures of which are shown in Figures 1 and 2, are well-known.

The raison de etre for the presence of flavin coenzymes and CoQ in the membrane-bound electron-transport systems of all living cells is almost certainly the fact that they readily undergo one-electron-transfer reactions, in which the semiquinone radicals are intermediates. In aerobic metabolism the ultimate oxidant of nutrient molecules is O_2, which is paramagnetic and undergoes reduction in one-electron steps. Energy metabolism in cells invariably involves one-electron transfer via metal-containing cofactors, which mediate the reduction of O_2 in one-electron steps.

The flavin coenzymes have the capacity to undergo both two-electron and one-electron redox reactions; and

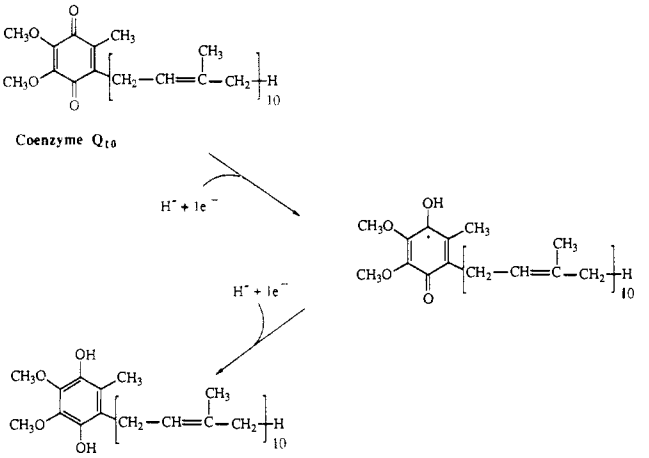


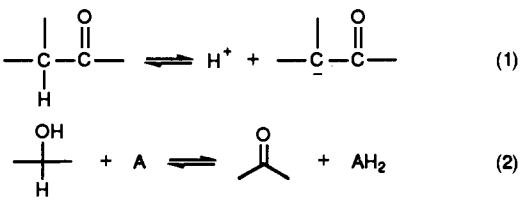
Figure 2. Structures of the oxidized, reduced, and semiquinone forms of coenzyme Q.

this property allows them to act as the switch point between the two-electron redox reactions that occur in the cytosol of cells to the one-electron-transfer reactions in the membranes, where O_2 is reduced by the metal-mediated electron-transport system. The membrane-bound electron-transport pathway consists of multiprotein complexes containing metal cofactors and flavins. The flavins are reduced by dihydroflavins in two-electron reductions by NADH and other two-electron reductants from the cytosol. The dihydroflavins then reduce metal cofactors, which are one-electron acceptors, in one-electron steps via the flavin semiquinones.

Electron transfer from one multiprotein complex to another in the electron-transport pathway is mediated by CoQ. CoQ is reduced to $CoQH_2$ by the metal cofactors associated with two of the multiprotein electron-transfer complexes in one-electron steps, via the CoQ semiquinone. $CoQH_2$ then migrates within the plane of the membrane to a third electron-transfer complex and reduces the metal cofactors in that complex, again in one-electron steps via the CoQ semiquinone. The resulting cascade of electron-transfer steps releases energy that is captured by the system in the form of ATP. The flavin semiquinone and CoQ semiquinone radicals play essential roles as mediators of electron transfer in this system.

B. Cleavage of Unactivated Carbon–Hydrogen Bonds

Many of the most important enzymatic reactions entail the cleavage of carbon–hydrogen bonds. Aerobic breakdown of nutrients by cells to produce energy always involves the oxidation of those nutrients by O_2, and for all common organic nutrients carbon–hydrogen bond cleavage is a central event in their oxidation to CO_2. The metabolic pathways have evolved in such a way that most of the carbon–hydrogen bond-cleavage reactions either involve relatively acidic hydrogens, as in eq 1, or involve functional groups such as primary or secondary alcohols that can be dehydrogenated as in eq 2. In eq 1 the C–H bond is weakened by the α-carbonyl group and cleavage proceeds by the dissociation of a relatively acidic hydrogen. In eq 2 the C–H bond cleaved is part of an alcoholic group that undergoes a two-electron oxidation concomitant with the reduction of a two-electron acceptor such as NAD^+.

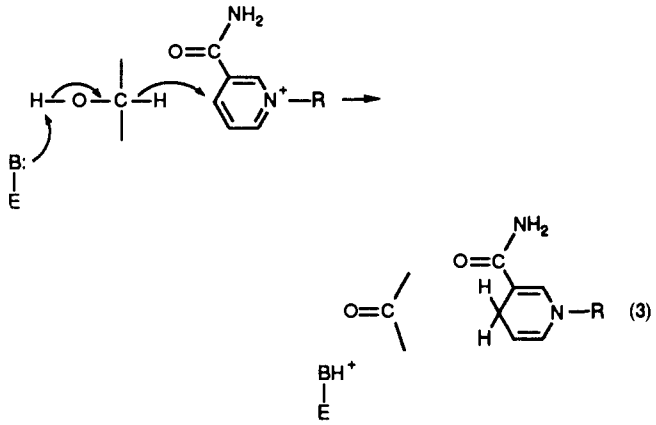


(1)

(2)

Enolizable metabolites include ketones, such as dihydroxyacetone phosphate, and esters such as acetyl coenzyme A. The pK_a values for these compounds range from about 13 to between 20 and 25, and enzyme binding interactions facilitate enolization at the active sites of enzymes under physiological conditions.

Carbon–hydrogen bond cleavage in the dehydrogenation of alcohols by eq 2 does not require or involve the dissociation of a proton. Rather, it involves the transfer of hydrogen to an acceptor. In enzymatic reactions this acceptor is generally NAD^+ or an analogous hydrogen-accepting molecule; and the reaction is facilitated by the electronic properties of the substituted nicotinamide ring, as shown in eq 3.



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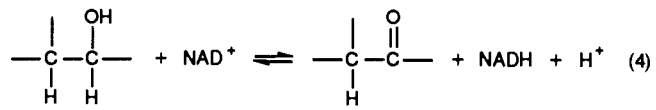
A few enzymatic reactions involve cleavage of carbon–hydrogen bonds that do not appear to be acidic but that can be made acidic by dehydrogenation of an adjacent alcoholic group according to eq 4. The enzymes



(4)

that catalyze these reactions contain NAD^+ as a tightly bound cofactor, the function of NAD^+ being to oxidize the molecule to a ketone (or an imine in the case of an amine) thus rendering the C–H bond acidic. The C–H bond cleavage and subsequent transformations ensue, and the ketone (or imine) is finally reduced back to the alcohol (or amine) by NADH, which remains enzyme-bound throughout the reaction. These reactions and the role of NAD^+ are reviewed elsewhere.[4]

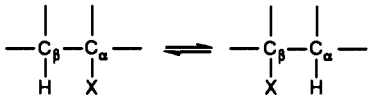
Certain enzymes catalyze reactions in which C–H bonds are cleaved that are not subject to enolizations or simple dehydrogenations such as those in eqs 1–4. These are reactions in which strong C–H bonds are broken without the benefit of any activating groups. The mechanisms by which these special reactions proceed are poorly understood by comparison with the mechanisms of enolization of ketones or the dehydrogenation of alcohols and amines. Available information indicates that they all involve organic radicals as intermediates. In each case the radical is initially generated by the abstraction of a hydrogen atom from the substrate, and the substrate radical then undergoes further transformations leading ultimately to the observed product. The hydrogen-abstracting species is in each case generated by a metal-containing cofactor that either facilitates the formation of an organic radical or reacts with O_2 to generate a paramagnetic hydrogen-abstracting species. These cofactors include heme, adenosylcobalamin (vitamin B_{12} coenzyme), cobalt, and μ-oxodiiron complexes; and they function in various ways to generate highly reactive hydrogen-abstracting species that produce substrate radicals as enzymatic intermediates.

III. 5′-Deoxyadenosyl Radical

A. Adenosylcobalamin-Dependent Reactions

The vitamin B_{12} coenzyme adenosylcobalamin is the most clearly understood of the metallocofactors that generate substrate radicals in enzymatic reactions. The adenosylcobalamin-dependent reactions are well-known and have been extensively reviewed.[1,2] Therefore, the present discussion is limited to an overview of the field, with updating of the review literature.

The adenosylcobalamin-dependent rearrangements all follow the pattern of eq 5, in which a nonacidic hydrogen and a group designated X and bonded to the adjacent carbon exchange positions. In the product,



(5)

X = –OH, $–NH_3^+$, –COSCoA, $–CH(NH_3^+)CO_2^-$, etc.

the transferred hydrogen and the group X remain in adjacent positions but have undergone cross-migration. Solvent hydrogen does not exchange with substrate hydrogen in adenosylcobalamin-dependent reactions. However, the two hydrogens bonded to the adenosyl 5′-carbon of adenosylcobalamin exchange with the hydrogen that migrates between C_α and C_β in eq 5.

1. Hydrogen Transfer and Adenosylcobalamin

The essential steps involving adenosylcobalamin and hydrogen transfer are outlined in Figure 3, in which all species remain enzyme-bound throughout the rearrangements. The reaction begins with homolytic cleavage of the cobalt–carbon bond in the cofactor to form Co(II) (B_{12r}) and the 5′-deoxyadenosyl radical. The dissociation energy for this bond is less than 30 kcal/mol.[5] It is sufficiently weak to allow it to be cleaved when a part of the energy made available by the binding of the coenzyme to its cognate enzyme is focused upon the induction of strain in this bond. Binding energy can in principle be brought to bear by the induction of steric strain in the coenzyme molecule through its binding interactions with specific subsites on the protein. Bond angle strain on the Co–C bond should weaken it sufficiently to allow it to break; however, the exact mechanism by which binding energy is utilized to this end is not known for any adenosylcobalamin-dependent enzyme. Such information might be obtained by structural analysis of any one of the enzymes, but this has so far not been achieved.

In the second step of Figure 3 the 5′-deoxyadenosyl radical abstracts the migrating hydrogen from the substrate to form a substrate radical and 5′-deoxy-

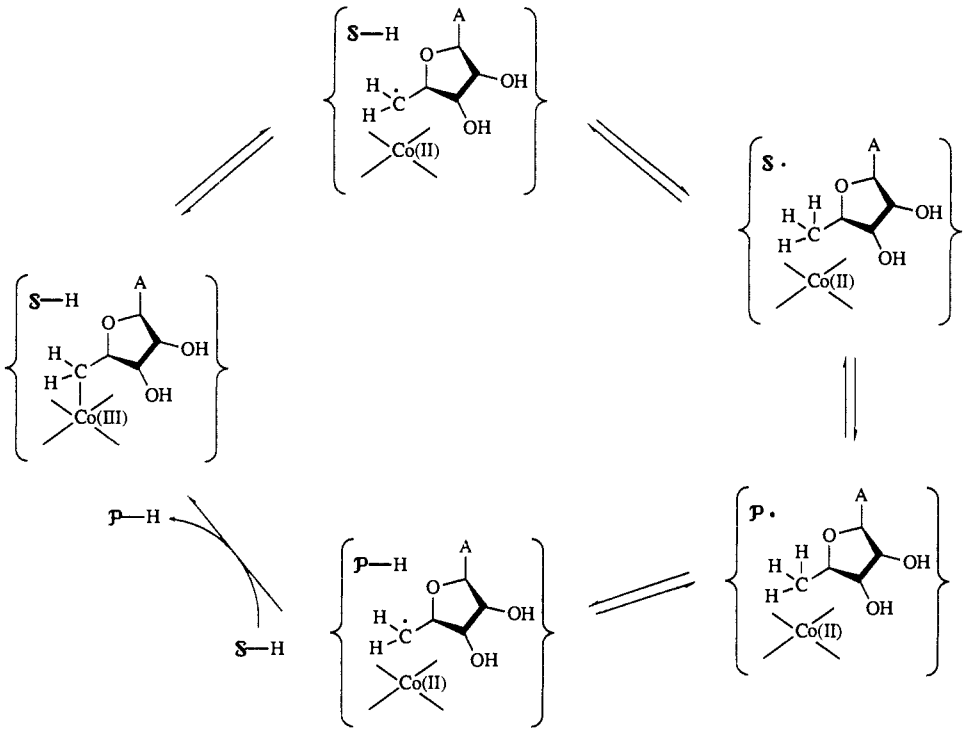
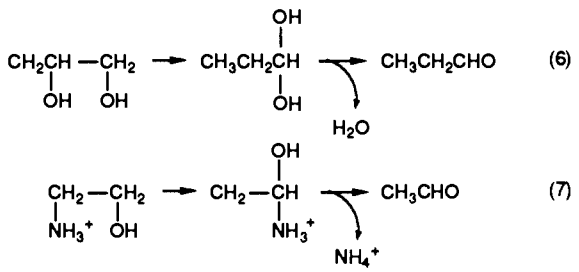


Figure 3. Role of adenosylcobalamin in generating substrate radicals and mediating hydrogen transfer in enzymatic isomerization reactions.

adenosine. In the third step the substrate radical rearranges to the product radical, and in the fourth step the product radical abstracts one of the three hydrogens from carbon 5′ of 5′-deoxyadenosine. Finally, the product dissociates, another molecule of substrate binds, and the cycle is repeated.

Figure 3 depicts the minimal mechanism for hydrogen transfer by invoking those species whose involvement is proven or very highly probable. The figure is not intended to exclude more complex schemes. For example, the 5′-deoxyadenosyl radical could be in equilibrium with one or more enzymatic radicals that may intervene between the adenosyl radical and the substrate. The extraordinarily large kinetic isotope effects in the reactions of tritium-labeled substrates for diol dehydrase and ethanolamine deaminase can be interpreted in this way.

The evidence supporting Figure 3 is derived largely from mechanistic studies of the enzymatic reactions, primarily the reactions 6 and 7 catalyzed by diol dehydrase and ethanolamine deaminase. Stereochemistry



(6)

(7)

for the diol dehydrase reaction is not shown; the enzyme accepts both (*R*)- and (*S*)-propanediol as substrates, and the configuration at C-2 determines which of the two diastereotopic hydrogens at C-1 is subject to transfer at C-2. Briefly stated, the following evidence, originally advanced by R. H. Abeles and his associates in studies of diol dehydrase, supports Figure 3: (a) The two hydrogens bonded to carbon 5′ of the adenosyl moiety of adenosylcobalamin enter a pool with substrate hydrogen and become subject to transfer to the product. This suggests that the cobalt–carbon bond is transiently cleaved and 5′-deoxyadenosine is an intermediate. (b) Hydrogen transfer from the substrate to the product proceeds via a pathway that allows either intramolecular or intermolecular hydrogen transfer in a given catalytic cycle. Again, this is consistent with 5′-deoxyadenosine as an intermediate. (c) EPR studies show that both Co(II) and organic radicals are transient intermediates. The organic radicals are derived from substrates. (d) Experiments with poor substrates show that 5′-deoxyadenosine is produced during the ethanolamine deaminase and diol dehydrase reactions, reversibly in the case of ethanolamine deaminase. (e) As stated above, the cobalt–carbon bond is weak, and it is further weakened and cleaved when adenosylcobalamin and other aldylcobalamins are bound at the active site of diol dehydrase. These facts mandate a mechanism incorporating the essential features of Figure 3 as the minimal hydrogen-transfer mechanism. The actual mechanism may prove to be more complex, but the basic principle of Figure 3 is solidly based on experimental evidence.

2. Rearrangements of Radical Intermediates

The rearrangement mechanisms, that is the interconversions $\mathcal{S}^{\bullet} \rightleftharpoons \mathcal{P}^{\bullet}$ in Figure 3, vary among the adenosylcobalamin-dependent reactions, somewhat in contrast to the unity in the hydrogen-transfer mechanisms. Very little is known from enzyme studies about the radical rearrangement. However, some of the rearrangements have been chemically modeled, and there is little doubt that they would proceed at the enzymatic active sites.

A chemical reaction that models the rearrangement part of the diol dehydrase and ethanolamine deaminase

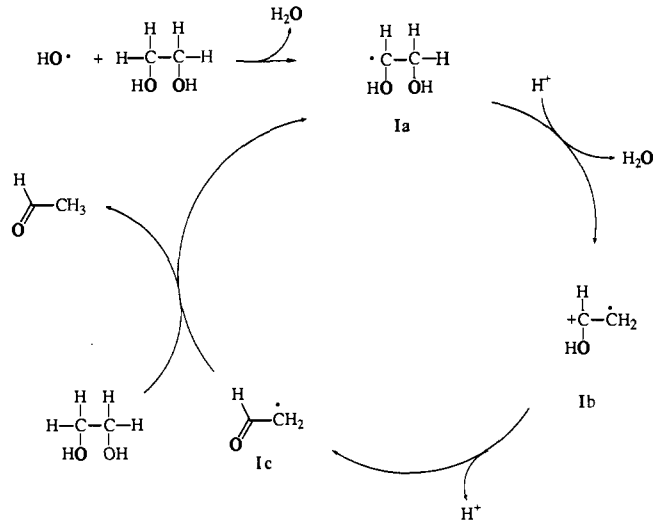


Figure 4. Chemical model for radical formation and rearrangement that is relevant to the diol dehydrase and ethanolamine deaminase reactions.

reactions is the hydroxyl radical initiated, acid-catalyzed decomposition of ethylene glycol to acetaldehyde according to Figure 4. Radical chain initiation by HO• generates radical Ia, which can undergo acid-catalyzed dehydration to the oxy cation radical Ib,[6,7] a favored species owing to stabilization of the positive charge by the hydroxyl group and to delocalization of the positive charge and the unpaired electron. The oxy cation radical can lose a proton to form the acetaldehyde radical Ic, which abstracts hydrogen from another molecule of ethylene glycol to continue the cycle.

The rearrangement phases of the reactions catalyzed by diol dehydrase and ethanolamine deaminase are probably analogous to the elimination of water in Figure 4, where the leaving groups are H_2O and NH_3, respectively, with the additional provision that the leaving groups are held at the active sites through enzymatic binding interactions and, in the next step, undergo addition to the adjacent carbon enroute to the products. In the enzymatic reactions, the 5′-deoxyadenosyl radical initially abstracts substrate hydrogen and 5′-deoxyadenosine ultimately returns the hydrogen to the product. The function of the 5′-deoxyadenosyl radical in the enzymatic reactions obviates the need for the hydroxyl radical in the model reaction. Moreover, readdition of the leaving groups to adjacent carbons with the formation of aldehyde hydrate radicals or aminal radicals as the initial rearrangement species, followed by hydrogen transfer from 5′-deoxyadenosine to the radicals, obviates the need for a chain reaction such as that of the chemical model in Figure 4, which is difficult to control at an enzymic active site. Elegant stereochemical studies mandate the return of the leaving group in the diol dehydrase reaction to form propionaldehyde at the active site prior to the release of products from the enzyme.[8]

Figure 5 outlines the rearrangement steps for the diol dehydrase reaction. The adenosyl radical initiates the rearrangement by abstracting a hydrogen from carbon 1 of the substrate to form a substrate radical in the first step. With the assistance of acid catalysis by an enzymic general acid A–H, the substrate radical eliminates H_2O to form the oxy cation radical. In the third step H_2O is added to carbon 1 with abstraction of a proton

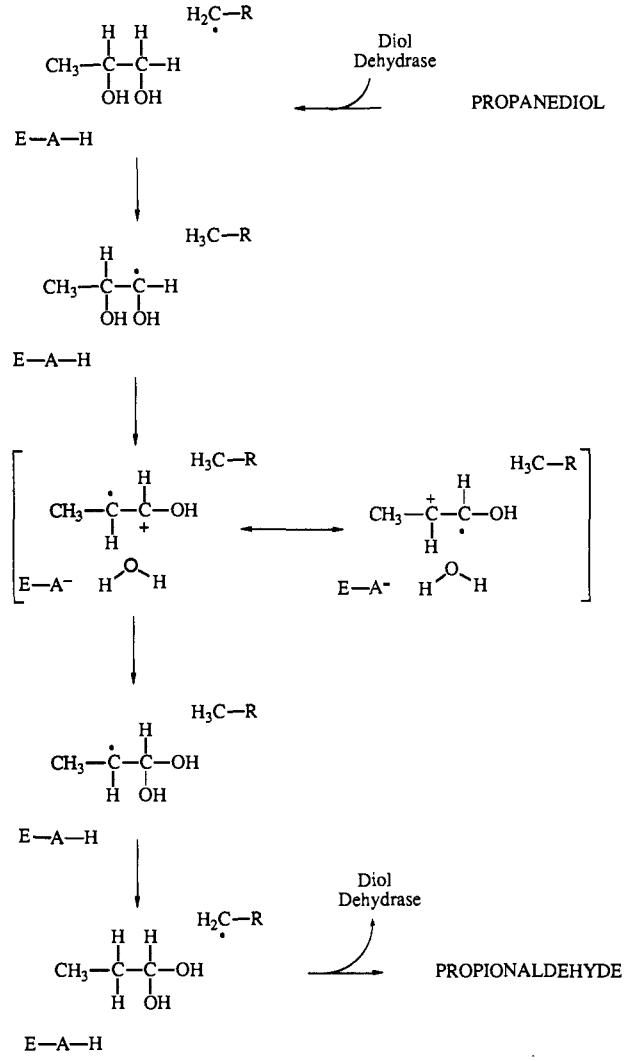
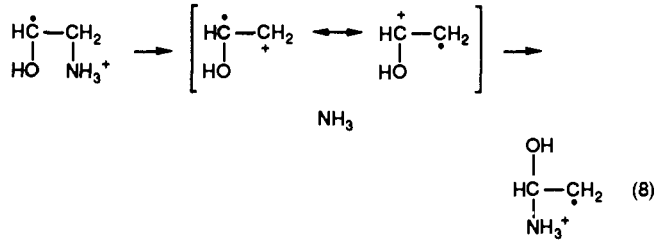


Figure 5. Role of the 5′-deoxyadenosyl radical in the diol dehydrase reaction.

from water by the conjugate base of the general acid. This step produces the product radical, which abstracts a hydrogen from the 5′-methyl group of 5′-deoxyadenosine. Propionaldehyde hydrate is then dehydrated to propionaldehyde and water and released from the enzyme.

The ethanolamine deaminase reaction is thought to proceed by an analogous mechanism, with the exception that the elimination of NH_3 and its readdition to the oxycation radical shown in eq 8 should not require general acid–base catalysis.



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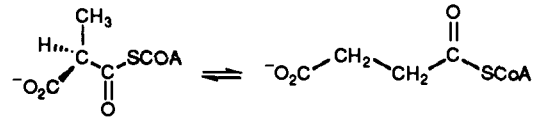
Methylmalonyl coenzyme A mutase catalyzes the adenosylcobalamin-dependent isomerization of methylmalonyl-CoA to succinyl-CoA (eq 9). As in all



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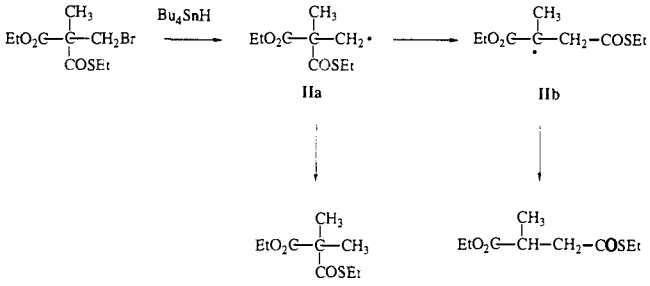


Figure 6. Chemical model for the isomerization catalyzed by the methylmalonyl-CoA mutase.

B_{12}-dependent rearrangements, the coenzyme mediates hydrogen transfer via the intermediate formation of 5′-deoxyadenosine. Little else is known about the enzymatic reaction. The enzymatic hydrogen transfer proceeds with retention of configuration at the terminus of hydrogen migration with methylmalonyl-CoA as the substrate. However, in the reaction of the poor but true substrate ethylmalonyl-CoA stereospecificity is lost, which is indicative of an intermediate radical.[9]

Radicals such as those presumed to be intermediates in the methylmalonyl-CoA mutase reaction spontaneously undergo rearrangements that are analogous to the isomerization of methylmalonyl-CoA to succinyl-CoA. Two types of experiments support this contention. Upon photolysis, the complexes [2,2-bis(ethoxycarbonyl)propyl]cobaloxime and [2,2-bis(ethoxycarbonyl)ethyl]cobalamin produce the corresponding diesters of succinate and methylmalonate.[9-11] Photolysis of the Co–C bond is known to cleave it homolytically to generate the relevant organic radical, and the photolysis products include rearranged species of the type observed in the enzymatic reaction. However, in experiments in which the organic radical is generated from a cobalt complex, one cannot be certain whether the Co(II) species is also generated by photolysis is a spectator or a participant in the rearrangement of the radical. It is conceivable that Co(II), which is redox-active, might act as a redox partner with the organic radical to generate other transient organic species that actually undergo the carbon skeletal rearrangement.

A clear demonstration that Co(II) need not be a participant in the rearrangement and probably was a spectator in the photolysis experiments was provided by the experiment outlined in Figure 6.[12] In this experiment diester radical IIa was generated from the corresponding bromide with tetrabutyltin hydride; and the two major products of radical quenching were the unrearranged 2,2-dimethylmalonyl diester and the rearranged 3-methylsuccinyl diester. Inasmuch as cobalt was absent under the reaction conditions, radical IIa must have rearranged spontaneously to methylsuccinyl radical IIb prior to quenching.

The rearrangement in Figure 6 provides chemical validation that the analogous radical generated from the methylmalonyl-CoA by hydrogen abstraction from the methyl group would rearrange to the analogous succinyl-based radical and, by hydrogen abstraction from 5′-deoxyadenosine, to succinyl-CoA. Thus, radical $\mathcal{S}^{\bullet}$ in Figure 3 should be analogous to IIa in Figure 6, and $\mathcal{P}^{\bullet}$ in Figure 3 should be analogous to IIb in Figure 6.

No known radical rearrangement is strictly analogous to the interconversion of β-methylasparatate and glutamate, reaction 10, which is catalyzed by glutamate

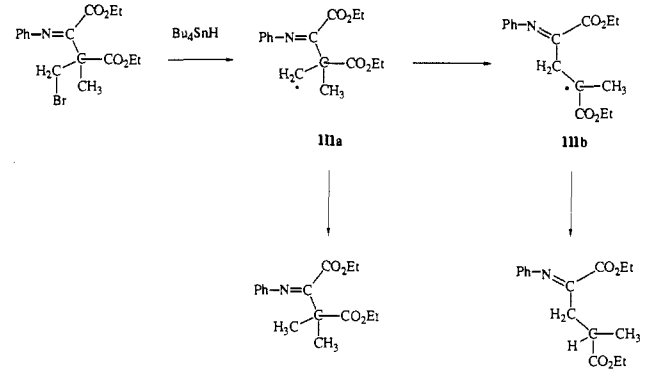
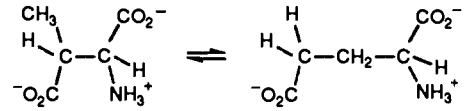


Figure 7. Carbon skeletal rearrangement that may be analogous to radical rearrangements initiated by the 5′-deoxyadenosyl radical in B_{12}-dependent reactions.

mutase, the first enzyme found to be adenosylcobalamin-dependent. A rearrangement shown in Figure 7



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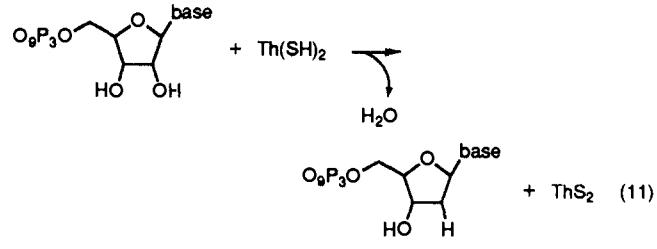
and described in a recent paper has been suggested as a possible model for the glutamate mutase reaction.[13] A significant and possibly important difference between the interconversion of glutamate and β-methylaspartate and the rearrangement in Figure 7 is that the enzymatic reaction involves amino acids, whereas an imino ester radical is required for the isomerization in Figure 7. The imine group in Figure 7 may participate directly in the isomerization mechanism; however, no such participation is possible in the enzymatic reaction unless the substrate is converted to an imine at the active site. Imine formation would require a cofactor at the active site such as pyridoxal 5′-phosphate or another aldehydo or ketonic cofactor. The enzyme contains no pyridoxal 5′-phosphate. It is not known whether another ketonic cofactor such as a pyruvoyl or a ketoglutaryl group may be present. If such a cofactor should be discovered, and if it formed an imine with glutamate, and if the α-hydrogen of glutamate were removed by an enzymic general base and shielded at the active site from exchange with the medium prior to being returned to the product, the rearrangement in Figure 7 can be accepted as a model for the glutamate–β-methylaspartate interconversion. In any case, the reactions in Figures 6 and 7 show that organic radicals can undergo carbon skeletal rearrangements that are similar to the enzymatic reactions.

3. Reduction of Ribonucleotides

The ribonucleotide reductase of *Lactobacillus leichmanii* catalyzes reaction 11, the reduction of ribonucleoside triphosphates to deoxyribonucleoside triphosphates, where Th symbolizes thioredoxin, a small protein with two closely associated and redox-active sulfhydryl groups in an active site. In the cell, thio-



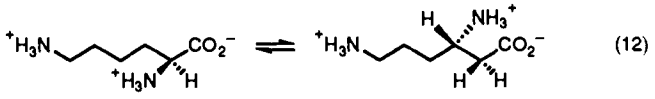
(11)

redoxin is maintained in its reduced form by the action of thioredoxin reductase, a flavoprotein that catalyzes the reduction of thioredoxin by NADPH. Ribonucleotide reductase in *L. leichmanii*, and similar enzymes in many anaerobic bacteria, are activated by adenosylcobalamin. Ribonucleotide reduction does not entail a rearrangement similar to the other adenosylcobalamin-dependent rearrangements; the mechanism of the reaction, in common with the rearrangements, involves the 5′-deoxyadenosyl radical. The 5′-deoxyadenosyl radical is thought to initiate the reaction by hydrogen abstraction from an enzymic functional group, probably a sulfhydryl group of cysteine, to form an enzyme radical that abstracts the C-3′ hydrogen from the ribonucleoside triphosphate. The substrate radical then eliminates water to form the oxy cation radical, analogous to the elimination of water in the diol dehydrase reaction (eq 7). The oxy cation radical is reduced to the 3′-radical of the deoxynucleoside triphosphate, which reabstracts a hydrogen from the enzymatic group to regenerate the enzyme radical. The reaction is catalyzed in many aerobic cells including mammalian cells by a different enzyme that utilizes a different cofactor, a μ-oxodiiron complex with an associated tyrosyl radical, to initiate the process.

B. Lysine 2,3-Aminomutase

1. Biochemistry and Molecular Properties

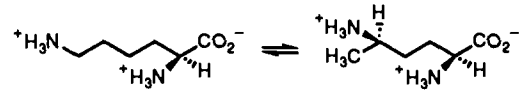
Lysine 2,3-aminomutase catalyzes reaction 12, the interconversion of L-lysine and L-β-lysine.[14,15] The molecular properties, Fe content, and activity of lysine 2,3-aminomutase originally reported by Barker and associates have been confirmed in this laboratory. The



(12)

enzyme appears to be a hexamer of subunits with a subunit M_r 47 000 and an overall M_r 280 000. Purification gives a protein that is inactive but can be activated by preliminary anaerobic incubation with Fe and pyridoxal phosphate in a reducing system, followed by addition of *S*-adenosylmethionine. The reducing system used in this laboratory consists of dithionite, dithiothreitol, and dihydrolipoate.

Reaction 12, catalyzed by lysine 2,3-aminomutase, follows the pattern of eq 5 for adenosylcobalamin-dependent reactions. Indeed, adenosylcobalamin is required for a *different* aminomutase reaction catalyzed by a *different* enzyme, β-lysine mutase, which catalyzes the further rearrangement of β-lysine to 3,5-diaminopentanoate in reaction 13.[16] Reactions 12 and 13 are



(13)

analogous and, as has recently become clear, proceed by similar mechanisms despite their different cofactor requirements. β-Lysine mutase contains pyridoxal phosphate and is activated by adenosylcobalamin; and the B_{12} coenzyme mediates hydrogen transfer and presumably facilitates radical formation, as it does in the other rearrangement reactions described above.[16] The purified lysine 2,3-aminomutase described by Barker and co-workers is not activated by adenosylcobalamin or any other known cobamide.[14] Instead, it is activated by *S*-adenosylmethionine and stimulated by Fe^{2+} and by pyridoxal 5′-phosphate. The purified enzyme also contains both Fe^{2+} and pyridoxal phosphate.

The biological significance of the lysine 2,3-aminomutase reaction is 2-fold. In the growth of *Clostridia*, with lysine as the source of carbon, energy, and nitrogen in the growth medium, this reaction is the first step in the degradation of lysine to ammonia and acetyl-CoA.[16] These molecules satisfy the needs of the organism for energy, nitrogen, and metabolizable carbon skeletons. In other microorganisms that elaborate antibiotics, β-lysine appears as an acyl substituent of a number of antibiotics;[17-19] and the only known means of producing β-lysine in nature is by the action of lysine 2,3-aminomutase on lysine.

2. Cofactors

The mechanism of action of lysine 2,3-aminomutase was enigmatic for many years. Although the enzyme catalyzes a B_{12}-type isomerization, it could not be activated by a vitamin B_{12} coenzyme. Although the enzyme contains pyridoxal phosphate and is stimulated by added pyridoxal phosphate, the known coenzymatic properties of pyridoxal phosphate did not suggest a means by which pyridoxal phosphate could facilitate the reaction via low-energy intermediates. And the mechanistic roles of Fe and *S*-adenosylmethionine as activators were unknown. The analogy to adenosylcobalamin-dependent rearrangements was strengthened by the fact that β-lysine aminomutase (also *Clostridia*) catalyzes reaction 13, is adenosylcobalamin-dependent, and also contains pyridoxal phosphate.

The enzymatic conversion of L-lysine to L-β-lysine in reaction 12 proceeds with conservation of substrate hydrogen, that is, without exchange of hydrogens with the protons of water.[14] This is consistent with a B_{12}-type rearrangement and is atypical of pyridoxal phosphate dependent reactions. The 3-*pro-R* hydrogen of lysine is transferred to the 2-*pro-R* position of β-lysine, with intramolecular transfer of the amino group from carbon 2 to carbon 3.[20]

The mechanism of action of lysine 2,3-aminomutase is currently under study in this laboratory. Recent findings on the cofactor content of the enzyme and the function of *S*-adenosylmethionine suggest that the mechanism is similar to that of adenosylcobalamin-dependent reactions, but no B_{12} derivative is involved. The studies are ongoing, and while they are incomplete, the can be summarized here to show the analogy of the lysine 2,3-aminomutase reaction with the B_{12} reactions and to show how reaction 10 is catalyzed by cofactors other than a vitamin B_{12} coenzyme.

Purification of lysine 2,2-aminomutase has been improved by working under strictly anaerobic conditions. The improvement could be attributed to the protection afforded to the iron cofactor by the absence of oxygen. The iron cofactor has been found to be an iron–sulfur center consisting of Fe and inorganic sulfide in essentially equimolar amounts. Anaerobic purification increased the Fe content, the sulfide content, and the activity of the enzyme. The enzyme was also found to contain cobalt; and when the bacteria were grown in a cobalt-supplemented medium, both the cobalt content

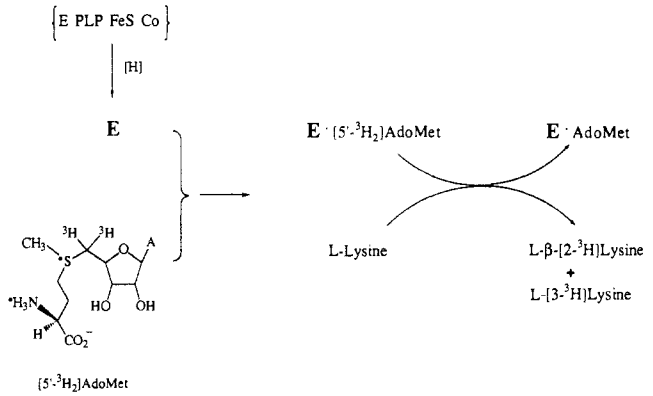


Figure 8. Role of the 5′-deoxyadenosyl group of *S*-adenosylmethionine in mediating hydrogen transfer in the lysine 2,3-aminomutase reaction.

and the enzyme activity were dramatically increased. The cobalt has been shown not to be associated with any cobamide.

Thus, the enzyme activity increases with increasing iron, sulfide, and cobalt content, showing that all three are important for activity. On the basis of enzyme activity data and analyses for iron and sulfide, the maximum Fe and S appear to 12–13 atoms of each per protein hexamer. The maximum cobalt is not yet known, but it is greater than 3 atoms per hexamer. The purified enzyme also contains 5.5 molecules of pyridoxal phosphate per hexamer. The low-temperature electron paramagnetic resonance spectrum of the purified enzyme consists of a complex signal for the iron–sulfur complex that is unlike any other FeS signal that has been reported in the literature.[21] This complex is under intensive investigation.

3. *Role of S-Adenosylmethionine*

In addition to the FeS clusters, Co, and pyridoxal phosphate, the enzyme is activated by *S*-adenosylmethionine after being incubated anaerobically with a reducing system. The function of *S*-adenosylmethionine in activating this enzyme is coming into focus because of studies of the hydrogen-transfer mechanism carried out in this laboratory.[22,23] Activation by *S*-adenosylmethionine allows the adenosyl moiety to act as the mediator of hydrogen transfer, evidently in the same capacity as that of the adenosyl moiety of adenosylcobalamin in the B_{12}-dependent rearrangements. The following facts support this view: (a) Activation of the enzyme by *S*-[5′-$^3H_2$]adenosylmethionine followed by addition of lysine leads to the appearance of tritium in both lysine and β-lysine in amounts stoichiometrically equivalent to the amount of *enzyme* in the reaction mixture. This is shown in Figure 8 as an irreversible transfer that reflects the actual experimental conditions in the relevant experiments and for illustrative purposes. Figure 8 describes the experimental findings when the amount of lysine exceeds the amount of enzyme and *S*-[5′-$^3H_2$]adenosylmethionine by several-thousand-fold in stoichiometric terms. The system actually approaches equilibrium with respect to both the formation of lysine and β-lysine and the partitioning of tritium between these two molecules. (b) The enzyme is maximally activated by the addition of 4–6 mol equiv of *S*-adenosylmethionine to the hexameric enzyme.[24] (c) Maximum tritium transfer to lysine

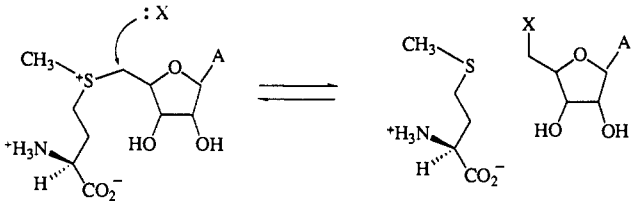


Figure 9. 5′-Deoxyadenosyl transfer from *S*-adenosylmethionine to an enzyme-bound cofactor of lysine 2,3-aminomutase to generate the active adenosyl cofactor.

and β-lysine occurs with 4–6 mol equiv of *S*-[5′-$^3H_2$]-adenosylmethionine per hexameric enzyme molecule.[24] (d) Tritium in both diastereotopic 5′-adenosyl positions of *S*-adenosylmethionine is transferred to lysine. (e) Very gradual irreversible degradation of *S*-adenosylmethionine accompanies catalytic turnover, and the products released from the enzyme in equimolar amounts are methionine and 5′-deoxyadenosine.[24] In the presence of excess *S*-adenosylmethionine to methionine and 5′-deoxyadenosine.

The available information on activation by *S*-adenosylmethionine suggests the following: First, activation by *S*-adenosylmethionine is practically irreversible; that is, *S*-adenosylmethionine, once bound, is not normally released from the enzyme at a significant rate compared with the rate of substrate turnover. Second, activation involves cleavage of the 5′-deoxyadenosyl moiety from *S*-adenosylmethionine. Third, the cleaved 5′-deoxyadenosyl group functions in the same way as that of adenosylcobalamin in the B_{12}-dependent rearrangements, that is, to generate a substrate radical and to mediate hydrogen transfer. Fourth, the gradual release of methionine and 5′-deoxyadenosine from the active site is an infrequent and probably adventious event, the observation of which supports the proposal that 5′-deoxyadenosine is produced in the course of the reaction and may be an intermediate in the hydrogen-transfer pathway.

In view of the fact that the 5′-deoxyadenosyl group of *S*-adenosylmethionine mediates hydrogen transfer in the same way as the 5′-deoxyadenosyl group of adenosylcobalamin, it is very likely that the mechanism of the lysine 2,3-aminomutase reaction is similar to the adenosylcobalamin-dependent rearrangements. The presence of cobalt in the enzyme further supports this concept. According to this view, activation of the enzyme by *S*-adensoylmethionine proceeds with cleavage of the adenosyl group away from methionine and with its attachment to another cofactor, perhaps cobalt, in such a way as to allow the 5′-deoxyadenosyl radical to be reversibly generated. The initial cleavage of *S*-adenosylmethionine is most likely to be a polar adenosyl transfer reaction, that is, an alkylation. This is shown in Figure 9, where the unknown adenosyl acceptor is designated X. In biochemical reactions *S*-adenosylmethionine almost always reacts as an alkylating agent to transfer the methyl group to a nucleophile; and *S*-adenosylmethionine is the principle biological source of methyl groups. The chemistry of adenosyl transfer is very similar to methyl transfer, and all evidence indicates that in the case of lysine 2,3-aminomutase it is the adenosyl group that undergoes the alkylation reaction.

The adenosyl acceptor, :X in Figure 9, is probably one of the other cofactors, and its adenosylated form is most

likely a species that can allow the bond between X and the 5′-deoxyadenosyl group to undergo reversible homolytic cleavage to the 5′-deoxyadenosyl radical. The chemical nature of the species X is currently under study in this laboratory. Several hypotheses regarding the nature of the adenosylated cofactor can be considered and are being tested. At this time it is not known whether X in Figure 9 is cobalt or another species, or even whether more than one adenosylated cofactor may be involved. It seems probable, from consideration of the hydrogen-transfer results, that some form of adenosyl cofactor is involved that can generate a 5′-deoxyadenosyl radical that can abstract the 3-*pro-R* hydrogen atom from L-lysine and transfer a hydrogen to the 2-*pro-R* position of L-β-lysine. This cofactor is almost certainly not *S*-adenosylmethionine itself, since the carbon 5′-sulfur bond is too strong to undergo homolytic scission under the conditions of the enzymatic reaction. The adenosyl group is very likely transferred to another cofactor, perhaps cobalt, under the activating conditions to form a species with a weak bond that can reversibly dissociate to a 5′-deoxyadenosyl radical.

4. *Role of Pyridoxal Phosphate*

The question of the function of pyridoxal phosphate in the lysine 2,3-aminomutase and β-lysine mutase reactions is interesting. The two reactions are similar and appear to proceed by similar mechanisms, probably via radical intermediates. Pyridoxal phosphate dependent reactions typically involve carbanionic intermediates rather than radicals. In enzymatic reactions pyridoxal phosphate almost always promotes the formation and the reactions of carbanions derived from amino acid substrates; and until very recently pyridoxal phosphate has not been thought to be involved in stabilizing radicals at enzymic active sites. However, there is no chemical reason why pyridoxal phosphate should not be involved in radical-type reactions, since it can in principle stabilize radicals by delocalizing unpaired electrons. The presence of pyridoxal phosphate as a coenzyme of the aminomutase reactions forces one to consider whether, in these particular reactions, pyridoxal phosphate may stabilize organic radical intermediates.

A question that arises is whether radical stabilization by pyridoxal phosphate would be advantageous in the aminomutase reactions. The ethanolamine deaminase reaction also involves the 1,2-migration of an amino group, yet pyridoxal phosphate is not involved in this rearrangement. There are important differences between the amino 1,2-migrations in the aminomutase reactions on one hand and the ethanolamine deaminase reaction on the other. The key intermediate in the ethanolamine deaminase reaction is the oxy cation radical in eq 8. The special stability of the oxy cation radical is due to the presence of oxygen in the diol dehydrase and ethanolamine deaminase substrates. Oxy cation radicals cannot be formed in the aminomutase reactions, since are no hydroxyl substituents in the reacting carbons, so the special stabilities of oxy cation radicals cannot come into play in facilitating the 1,2-migrations of amino groups.

In the aminomutase reactions 12 and 13 some means other than the elimination of ammonia from the initial radical intermediates appears to be required for the

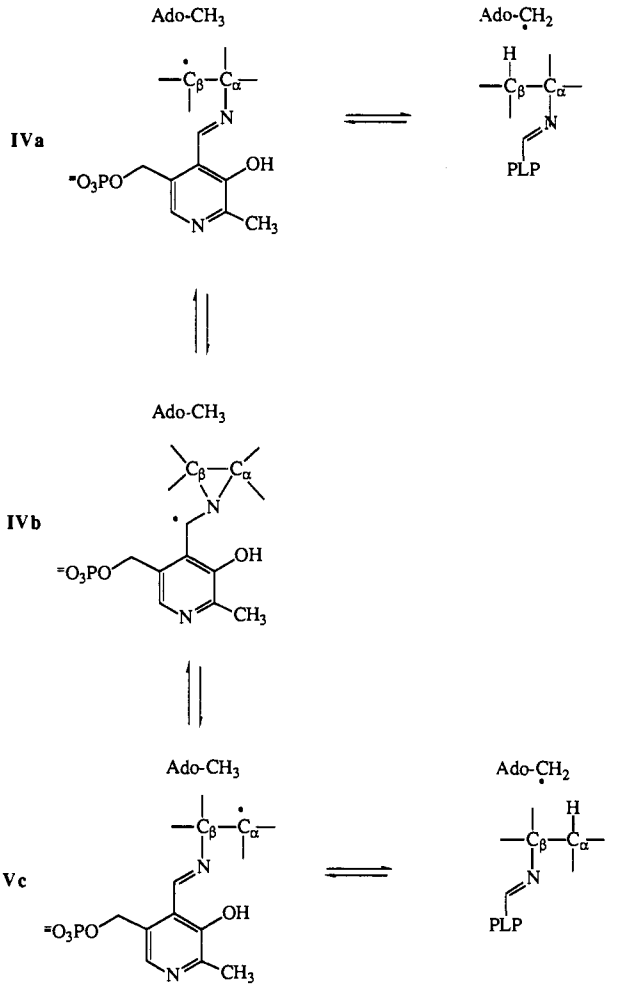


Figure 10. Roles of the 5′-deoxyadenosyl radical and pyridoxal phosphate in catalyzing the conversion of lysing to β-lysine at the active site of lysine 2,3-aminomutase.

1,2-migrations, and it is the migration process itself that may be facilitated by pyridoxal phosphate. An attractive mechanism is outlined in Figure 10, which refers to both lysine 2,3-aminomutase and β-lysine mutase. In this mechanism the migrating amino group is bound to the aldehyde carbon of pyridoxal phosphate as an imine, and the 5′-deoxyadenosyl radical from the cofactor initiates the rearrangement by abstracting a hydrogen from the amino acid. The resulting imino radical IVa can be stabilized by cyclization with the imine nitrogen, in which the unpaired electron in IVa forms a bond to the imino nitrogen by pairing with one electron of the π-bond, forming an aziridine radical IVb, in which the unpaired electron is stabilized by delocalization through the pyridine ring. Radical IVb is a pseudosymmetrical species that can undergo ring opening in either of two directions, reversal to IVa or forward to the product related radical IVc. Thus, pyridoxal phosphate can facilitate the 1,2-migration by allowing for the formation of stabilized aziridine radical IVb. Delocalization of the unpaired electron in IVb is analogous to the stabilization of carbanions at the aldehyde carbon of pyridoxal phosphate, which is an important phenomenon in enzymatic transaminations of amino acids. Thus, in the aminomutase reactions pyridoxal phosphate nicely stabilizes the substrate radicals while facilitating 1,2-migration of the amino groups. An analogous nonenzymatic rearrangement of *N*-benzylidene(2-bromomethyl)-DL-alanine ethyl ester,

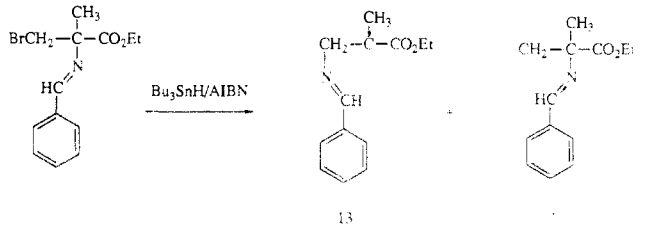


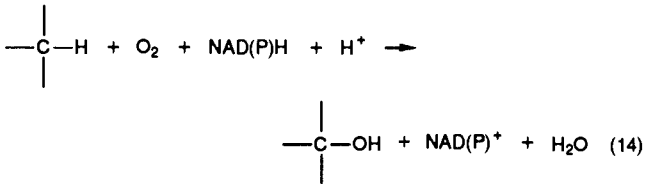
Figure 11. Radical-initiated nonenzymatic imino 1,2-rearrangement. Reaction of *N*-benzylidene(2-bromomethyl)-DL-alanine ethyl ester with Bu_3SnH in the presence of AIBN (azobis-(2-methylpropionitrile)) in refluxing benzene gives a 13:1 mixture of *N*-benzylidene-2-methyl-β-alanine ethyl ester and *N*-benzylidene-3-methyl alanine ethyl ester.

under conditions of Br abstraction to generate a free radical (Figure 11), supports the chemical rationale for the mechanism in Figure 10.[25]

Much remains to be done to clarify the mechanism of the lysine 2,3-aminomutase reaction. The nature of the adenosyl cofactor, the nature and role of the iron–sulfur cluster, the binding and function of cobalt, and the function of pyridoxal phosphate are all being intensively investigated. Whatever the outcome of these studies, it is highly probable that an adenosyl cofactor is formed, that this cofactor allows for the reversible formation of the 5′-deoxyadenosyl radical, and that this radical generates an intermediate substrate radical that undergoes the amino 1,2-migration. The amino 1,2-migration in the adenosylcobalamin-dependent β-lysine mutase reaction probably also proceeds by a similar mechanism.

IV. Oxygenation of Alkanes

The controlled oxidation of alkanes in living cells is one of the most difficult processes to conceptualize and explain in chemical terms. The usual chemical conditions for such reactions are not typical of the conditions under which living cells grow and multiply. Moreover, it is difficult to imagine how such a process can be controlled by a biological catalyst, given that the chemical processes are almost invariably chain reactions involving highly reactive intermediates. Nevertheless, enzymes that catalyze the oxidation of alkanes to alcohols exist in animals and bacteria. Enzymatic alkane oxygenation is generically defined by reaction 14, in which oxygen gas and a reducing agent, generally NADH or NADPH, react in such a way as to lead to the insertion of an oxygen atom from O_2 into a carbon–hydrogen bond of an alkane.



All known alkane monooxygenases are multiprotein complexes that consist of an oxygenase enzyme and a reductase enzyme; and there is also sometimes a third protein that is somehow involved in electron transfer. The basic oxygenation process is carried out by the action of the oxygenase enzyme subsequent to the reaction of its associated cofactor with O_2 and the reducing agent. In bacteria these enzymes enable the organisms to oxidize alkanes and to use the alkane carbon skeletons as a source of carbon and energy for cell growth and reproduction. In animals, the oxygenases have more restricted functions such as the specific oxygenations of sterols in steroid biosynthesis. They are also important in the detoxification of drugs and other foreign materials in animals. The best known and most widely distributed enzymes of this type are the cytochrome P-450 systems, which are extensively reviewed elsewhere[3] and will be briefly described in the next section.

A. Cytochrome P-450 Monooxygenases

1. Heme as an Oxygenation Cofactor

The cofactor for the oxygenase enzyme in the cytochrome P-450 monooxygenase multienzyme complexes is iron protoporphyrin IX, or heme. The reductase enzyme is a flavoprotein that transfers reducing equivalents in one-electron units from NADH or NADPH to the oxygenated heme in a process that leads in several steps to the oxygenating species at the active site of cytochrome P-450. Extensive spectroscopic and kinetic studies of many systems have led to the generalized scheme in Figure 12.[26,27] The process begins with the binding of the substrate, followed by the transfer of one electron to heme, the binding of O_2, and the transfer of a second electron to oxygenated heme. The peroxyferric heme then undergoes a transformation to the oxygenating heme in a reaction that is accom-

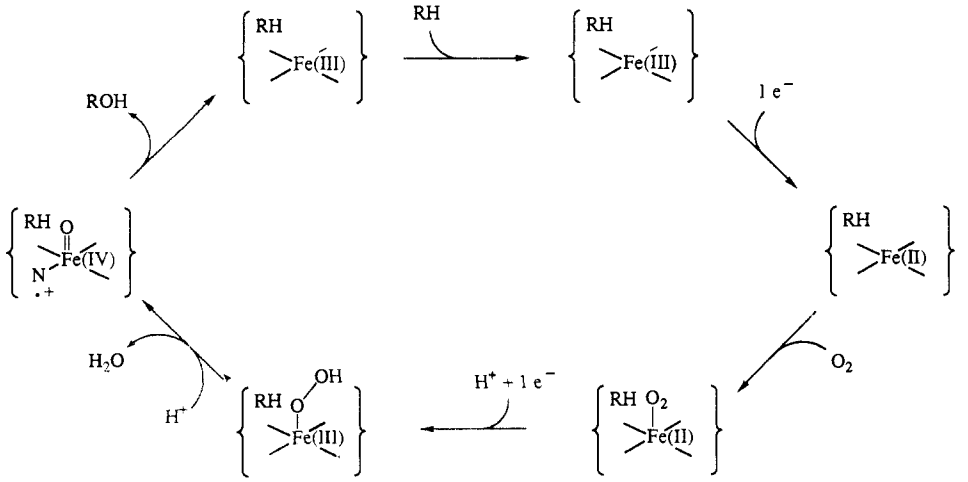
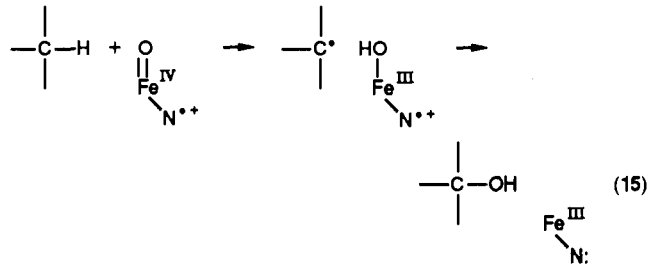


Figure 12. Steps in the formation of the oxoiron(IV) π-cation radical, the oxygenating species of cytochrome P-450.

panied by the cleavage of the peroxy ligand and the elimination of water. The oxygenating species of heme is short-lived and extremely reactive, so reactive that its structural formulation has taken a long time to deduce and is still not thoroughly understood. The most widely discussed and generally accepted structure is an oxoiron(IV) π-cation radical, in which the cation and radical center is located in the macrocyclic heme ligand and stabilized by delocalization. The central, reactive features of this structure are described in brief in Figure 12 and reaction 15. The oxygenating species has also been formulated as oxoiron(V), which differs in that the macrocyclic ligand does not participate in the redox aspect of the reaction.

2. Radicals as Oxygenation Intermediates

In the oxygenation of a substrate, the oxygenating species of cytochrome P-450 is thought to react directly with an alkane hydrogen-bonded to carbon, according to the sequence in reaction 15, to abstract the hydrogen



(15)

atom in a first step. This is followed immediately by a second step, in which the substrate radical abstracts a hydroxyl radical from the iron in heme. The mechanism is known as the rebound mechanism of oxygenation, since the second step, the rebound, quickly and irreversibly follows the first step.

The evidence supporting the oxygenation mechanism of reaction 15 is presented in other recent reviews.[26,27] Experimental support for reaction 15 is extensive and centered on two essential aspects: (a) the definition of the chemical composition of the oxygenating heme species, which is extensively documented, and (b) evidence for the intermediate formation of a substrate radical. Recent experiments with bicyclo[2.1.0]pentane as a monooxygenation substrate powerfully support the intermediacy of a radical, and they also allow the rate constant for the rebound step of reaction 15 to be estimated. Bicyclo[2.1.0]pentane is oxygenated by cytochrome P-450 with the formation of two isomeric alcohols, 2-hydroxybicyclo[2.1.0]pentane (V) and 2-cyclopenten-1-ol (VI) shown in Figure 13.[28] Alcohol V is the conventional oxygenation product, whereas alcohol VI arises through a rearrangement that almost certainly occurs at some point between carbon–hydrogen bond cleavage and oxygen insertion on the oxygenation pathway.

The structrue of VI provides an important clue to the structure of the oxygenation intermediate. Radical Va in Figure 13 is analogous to the cyclopropylcarbinyl radical and can be expected to rearrange very rapidly to radical VIa;[29] and radicals Va and VIa are the logical precursors to oxygenation products V and VI. In mechanistic scheme of Figure 13, radical Va is partitioned to the two products by a competition between the cyclopropylcarbinyl rearrangement to VIa and oxygenation to V. (Experiments with dideuterio[2.1.0]-bicyclopentane revealed no evidence for other carbon skeletal rearrangements.[28]) If the rate constant for one of these processes is known, that for the other can be calculated from the product composition. Since the product ratio of V to VI is 7:1,[28] the rate constant for oxygenation of the radical Va (k_0) should be 7 times that for the rearrangement. The nonenzymatic rate constant for the rearrangement of Va to VIa (k_R) is, according to a recent report, 2.4×10^9 s^{-1} at 37 °C; therefore, the rate constant for the oxygenation of Va should be about 2×10^{10} s^{-1} to account for the product ratio.[30] This value for k_R is very large, about 10 orders of magnitude larger than the turnover number for cytochrome P-450; and it means among other things that the steady-state levels of radical intermediates Va and VIa must be very low, too low to detect by spectroscopic methods or by spin-trapping experiments.

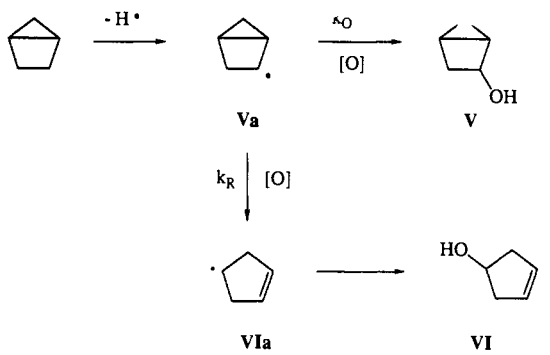
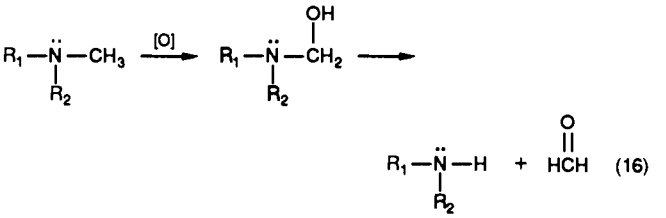


Figure 13. Radical rearrangements in the cytochrome P-450 catalyzed oxygenation of bicyclo[2.1.0]pentane.

Alkane radicals are not stabilized by delocalization of the unpaired electron, and these species, which are exemplified by the radicals in Figure 13, will probably be too unstable to exist at spectroscopically detectable levels in steady states. However, they may leave traces of their fleeting existence by undergoing rearrangements of the type shown in Figure 13 at rates that approach or even exceed the rates of subsequent steps.

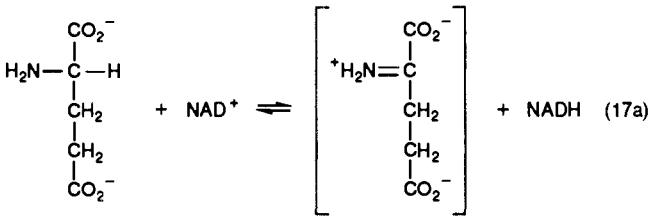
3. Oxygenation of Molecules Other than Alkanes

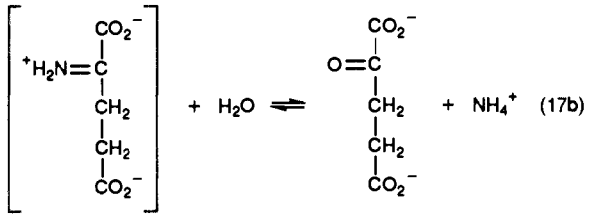
Many of the cytochrome P-450 monooxygenases that catalyze oxygenation of specific alkanes also catalyze oxygenation of structurally related alkenes to epoxides. Epoxidation of alkenes does not involve a carbon–hydrogen bond cleavage step such as is required in alkane oxygenation, since the oxygenating heme species inserts oxygen into the π-bond of the alkene. In reactions that are mechanistically similar to the oxygenation of alkanes, certain cytochrome P-450 monooxygenases catalyze the dealkylation of amines. An example is reaction 16, where oxygenation of a methyl group bonded to an amino group creates a hydroxymethylamine, an amine adduct of formaldehyde, which decomposes to the amine and formaldehyde. These reactions are important in detoxification and secondary metabolism.



(16)

It seems that something like cytochrome P-450 monooxygenases had to appear during evolution if alkane metabolism was essential or provided survival advantage to certain species. However, the dealkylations of amines would not by themselves have required the evolution of a highly reactive oxygenating species such as the oxoiron(IV) π-cation radical, since many of them can in principle be carried out by other means. As a straightforward and well-known example, glutamate dehydrogenase catalyzes the two reactions 17a and 17b,





which is a dealkylation of ammonia. The glutamate dehydrogenase reaction is a pyridine nucleotide dependent dehydrogenation that is analogous to the dehydrogenations of alcohols in reactions 3 and 4, but with the important consequence that it produces an imine rather than a ketone or aldehyde. The imine undergoes hydrolysis to ammonia and α-ketoglutarate. This reaction has the important biochemical advantage over monooxygenase-catalyzed dealkylations in that it produces rather than consumes NADH, a high-energy molecule that can be used in biosynthesis or for the production of ATP. Dealkylations of quaternary amines could not be carried out by pyridine nucleotide dependent dehydrogenases, but primary, secondary, and tertiary amines could be dealkylated in such reactions. Other well-known amine dealkylations in biochemistry are catalyzed by amino acid oxidases, which are usually flavoproteins. It may be that the cytochrome P-450 monooxygenases first appeared under the evolutionary pressure of a need to metabolize alkanes and were later recruited into a variety of other chemically less demanding roles.

B. Methane Monooxygenase

1. Molecular Properties of Methane Monooxygenase

The growth of methanotrophic bacteria on methane as their sole source of carbon and energy begins with reaction 18, catalyzed by methane monooxygenase.

$$CH_4 + O_2 + NADH + H^+ \rightarrow CH_3OH + H_2O + NAD^+ \quad (18)$$

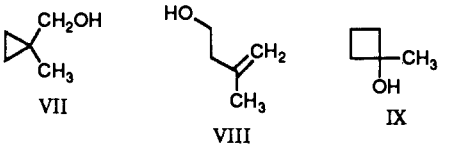
Methanol produced by methane monooxygenase is next dehydrogenated by methanol dehydrogenase to formaldehyde. Methanol dehydrogenase contains the recently discovered oxidation cofactor methoxatin, now known as pyrroloquinoline quinone (see later text). Methane monooxygenase appears in two forms in methanotrophic bacteria: one that is membrane-bound and one that is soluble in the cytosol. Little is known about the membranous enzyme. The soluble methanemonooxygenases from *Methylococcus capsulatus* (Bath) and *Methylosinus trichosporium* (OB3b) have been partially characterized and consist of three proteins as follows.[31-36] The oxygenase (component A) has an overall M_r of 220 000 and consists of three different subunits, with two copies of each ($\alpha_2\beta_2\gamma_2$). The oxygenase cofactor is a μ-oxodiiron complex with an associated organic radical that is thought to reside with an amino acid side chain. The reductase (component C) is a flavoprotein of M_4 44 000 that contains an iron–sulfur cluster (Fe_2S_2). This protein interacts with NADH and transfers electrons to the oxygenase. And a coupling factor (component B) is a small protein of M_r 16 000 that facilitates electron transfer from the reductase cofactors to the oxygenase cofactor.

2. Radicals and Carbocations as Oxygenation Intermediates

Little is known about the mechanism by which the μ-oxodiiron complex in the oxygenase activates O_2 to generate an oxygenating species. The reduced form of the cofactor reacts with O_2 to form a species that is chemically competent to oxygenate substrates; however, the structure and chemical properties of the oxygenating species are still under investigation and are not well understood.

Research on methane monooxygenase in this laboratory has been concerned with the identification of substrate-derived intermediates on the oxygenation pathway. In these studies advantage has been taken of the remarkable lack of substrate specificity exhibited by methane monooxygenase, which has been reported to accept alkanes, alkenes, and aromatic hydrocarbons with up to eight carbons as substrates.[31] Alkanes are oxygenated to alcohols, alkenes to epoxides, and phenyl rings to phenolic groups.

1,1-Dimethylcyclopropane is a good substrate for the soluble methane monooxygenase from *M. trichosporium*; and the product composition is remarkable for what it reveals about the oxygenation mechanism.[37] The products are (1-methylcyclopropyl)methanol (VII), 3-methyl-3-methylenepropan-1-ol (VIII), and 1-methylcyclobutanol (IX), which are produced in parallel is the ratios 8.1:0.6:1.3. VII is a conventional oxygen-



ation product and, as such, does not reveal much about the reaction mechanism, apart from the fact that it constitutes about 80% of the total products. It is very unlikely that several products from a single substrate in an enzymatic reaction would be formed in mechanistically unrelated processes, since enzymes catalyze specific reactions. Therefore, the simplest and most probable correct interpretation of the appearance of two other products, VIII and IX, is that they arise through the isomerization of an oxygenation intermediate that is common to all three products. Ring-opened product VIII constitutes about 6% of the total oxygenation products; and its formation is most easily related to the

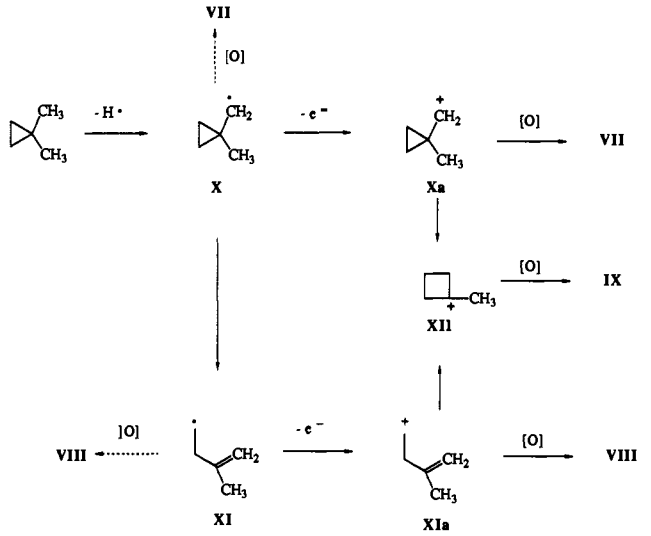


Figure 14. Radicals and carbocations as intermediates in the methane monooxygenase catalyzed oxygenation of 1,1-dimethylcyclopropane.

formation of VII by regarding them as two alternative final products derived from the substrate radical X in Figure 14. According to Figure 14, the oxygenation reaction is initiated by the abstraction of a hydrogen atom from a methyl group of 1,1-dimethylcyclopropane by a hydrogen-abstracting cofactor, most likely either the oxygenated μ-oxodiiron complex or the enzymic radical, to form the (1-methylcyclopropyl)carbinyl radical X. The intermediate X then undergoes further reaction by at least two pathways, oxygenation to VII and rearrangement to the homoallylic radical XI; and the homoallylic radical can also undergo oxygenation to VIII.

The third product, 1-methylcyclobutanol (IX), cannot easily be explained on the basis of the intermediate formation of substrate radicals X and XI alone. The most probable route from 1,1-dimethylcyclopropane to IX is via the 1-methylcyclobutyl carbocation XII in Figure 14, which can be expected to react quickly with any hydroxylated metal species, such as the oxygenated cofactor, to form IX. Carbocation IX could be formed by either ring expansion of the carbocation Xa or cyclization of cation XIa. Cations Xa and XIa are related to radicals X and XI by the loss of an electron from the radicals. This suggests that oxygenation of the radicals involves their oxidation by one-electron to carbocations Xa or XIa, which are then oxygenated to the final products. The formation of 1-methylcyclobutanol can then be explained by the partitioning of cations Xa or/and XI to cation XII, followed by oxygenation.

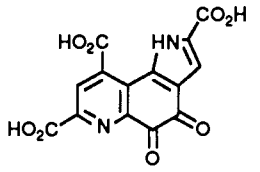
The pathways traced by the solid arrows in Figure 14 outline a mechanism by which 1,1-dimethylcyclopropane may be oxygenated to the observed products via the formation of radicals and carbocations as intermediates. The most direct pathway leads to the major product VII via radical intermediate X and carbocationic intermediate Xa, each of which can undergo rearrangements in competition with oxygenation. The rearrangements account for the other two products.

While it seems certain that radicals X and XI are intermediates, and that the carbocations are on the pathway to 1-methylcyclobutanol, it is less certain that carbocations are compulsory intermediates in the reactions of all substrates. It is conceivable that the (1-methylcyclopropyl)carbinyl radical is simply extraordinarily susceptible to electron transfer, which occurs as a side reaction and leads to the formation of cation Xa and, by oxygenation, IX as a side product. In this latter case radicals X and XI might undergo direct oxygenation, by the pathways indicated with dashed arrows, without undergoing electron transfer in an intervening step. If electron transfer is an adventitious side reaction in this case, it must be very fast to compete with the rearrangement of the (1-methylcyclopropyl)carbinyl radical, which probably occurs with a rate constant in excess of 2×10^8 s^{-1}.[29]

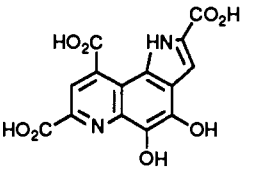
In summation, the production of VIII and IX as oxygenation products of 1,1-dimethylcyclopropane implicates both radical and carbocationic intermediates in the oxygenation mechanism. These intermediates can be connected by electron transfer, where the electron acceptor is most likely an oxygenated form of the cofactor μ-oxodiiron. The carbocations are oxygenated, presumably by the oxygenated cofactor. This mechanism differs from that for cytochrome P-450 by the involvement of a carbocation derived by electron transfer from a radical intermediate; carbocations have never been implicated in the rebound mechanism of cytochrome P-450 reactions. Therefore, the mechanism of action of the μ-oxodiiron complex in methane monooxygenase differs in definite ways from that for heme in cytochrome P-450. The chemistry of μ-oxometal complexes is still unfolding; and progress in this field will be required to develop a complete picture of the function of this cofactor in oxygenation reactions.

3. Oxidation of Methanol in Methanotrophic Bacteria

Once methane is oxygenated to methanol in methanotrophic bacteria, it is next dehydrogenated to formaldehyde by the action of methanol dehydrogenase.[38] This enzyme contains the coenzyme methoxatin, now known as pyrroloquinoline quinone (PQQ). PQQ is the



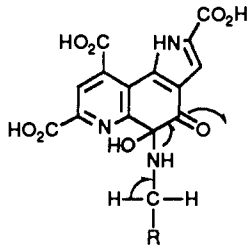
pyrroloquinoline quinone (PQQ)



$PQQH_2$

coenzyme of bacterial alcohol and amine dehydrogenases, in which the electrons derived from substrate dehydrogenation are transferred to the membrane-bound electron transport system of the cell, which are one-electron acceptors. PQQ, like flavin coenzymes, appears to have the capacity to undergo two-electron reductions by alcohols or amines to the hydroquinone from $PQQH_2$. Being a quinone/hydroquinone system, the $PQQ/PQQH_2$ system also has the capacity to undergo one-electron-transfer reactions via intermediate semiquinone radicals. Enzymes such as methanol dehydrogenase and methylamine dehydrogenase[40] utilize the dual electron-transfer capacity of the coenzyme PQQ, initially to accept two electrons in the dehydrogenation of methanol or methylamine. The $PQQH_2$ formed in the first step can then transfer two electrons to the bacterial membrane-bound electron transport pathways in two one-electron steps, with regeneration of PQQ at the enzymic active sites. In no case is the detailed mechanism of enzymatic reduction of PQQ or

its reoxidation known in detail; however, a recent thorough study of the nonenzymatic reduction of PQQ by benzylamine shows that adduct formation followed by electron transfer is the most probable and reasonable mechanism for two-electron reduction of PQQ.[41]



amine–PQQ adduct

V. Conclusion

The classical organic radicals in biochemistry are the resonance-stabilized semiquinone radical forms of flavin coenzymes and coenzyme Q, which facilitate one-electron transfer in reactions of oxygen and metals in the electron-transport chain of living cells. Flavin coenzymes and a few other analogous coenzymes such as PQQ are also similarly involved in the actions of bacterial methanol and methylamine dehydrogenases, where their coenzymatic functions seem to be related to their dual capacity to undergo both two-electron and one-electron redox processes.

Those enzymatic reactions in which unreactive carbon–hydrogen bonds in alkanes are cleaved also involve radical intermediates. However, these reactions differ from the more classical cases, in that the intermediates are much higher in energy than semiquinone radicals and require special hydrogen-abstracting forms of metal-based cofactors to cleave the carbon–hydrogen bond. In some cases the active metal cofactors do not interact directly with substrates but initiate radical formation by generating protein radicals that directly interact with substrates. The active metal-based cofactors that can initiate radical formation include adenosylcobalamin and the oxoiron(IV) π-cation-radical species of protoporphyrin IX.

Other cofactors that are functionally related to adenosylcobalamin and iron protoporphyrin IX are known to exist but have not been fully characterized or are under investigation. These include the adenosyl cofactor of lysine 2,3-aminomutase and the oxygenated forms of μ-oxodiiron complexes in methane monooxygenase and ribonucleotide reductase. Additional examples of analogous or similar complexes in enzymology are the unknown cofactors of several other enzymes, one of which is pyruvate formate lyase from *Escherichia coli* which, like lysine 2,3-aminomutase, is activated by *S*-adenosylmethionine.[42] Another is the ribonucleotide reductase of *E. coli* grown under anaerobic conditions. The ribonucleotide reductase from *E. coli* grown aerobically contains a μ-oxodiiron complex and a tyrosyl radical. The tyrosyl radical is generated by the interaction of the μ-oxodiiron complex with O_2; therefore, growth under anaerobic conditions is inconsistent with the operation of this cofactor system. Under anaerobic conditions of growth a different cofactor system is in operation, one that presumably also allows for the generation of an organic radical to initiate the reduction of ribonucleotides to deoxyribonucleotides. This alternative cofactor system includes activation by *S*-adenosylmethionine in a process that may have mechanistic features in common with lysine 2,3-aminomutase or/and pyruvate formate lyase.[43] The conversion of arginine to β-arginine in *Streptomyces griseochromogenes* appears to be similar to the lysine 2,3-aminomutase reaction described in this paper; however, the arginine-specific 2,3-aminomutase has never been purified or described as a molecular entity.[44] It remains for future research to determine whether the arginine aminomutase is similar to lysine 2,3-aminomutase or to the adenosylcobalamin-dependent β-lysine mutase.

Finally, as outlined in this paper, some new reactions of organic radicals at enzyme active sites are beginning to emerge. One example is the mechanism of oxygen insertion catalyzed by methane monooxygenase. The reductively oxygenated form of the μ-oxodiiron complex in methane monooxygenase, the structure of which is not yet known, is apparently somehow involved in generating a substrate radical, as is the oxoiron(IV) π-cation radical in cytochrome P-450. However, the detailed mechanism by which it carries out oxygen insertion into a carbon–hydrogen bond apparently differs from that of the oxoiron(IV) π-cation radical, in that the substrate radical initially formed seems, for at least one substrate, to be oxidized to a carbocation prior to the insertion of oxygen. A second example of a new mechanism is the role of pyridoxal phosphate in the amino group migrations catalyzed by lysine 2,3-aminomutase and β-lysine mutase. It seems likely that pyridoxal phosphate facilitates amino 1,2-migration by stabilizing an aziridine radical, as illustrated in Figure 10. Future research in this field will address these new mechanistic possibilities.

VI. Acknowledgment

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Registry No. Lysine 2,3-aminomutase, 9075-20-1; methane monooxygenase, 51961-97-8.

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