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Coenzyme B_6 dependent novel bond cleavage reactions

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Abstract: Coenzyme B_6 is an important cofactor participating in many diverse biotransformations. While the function of this coenzyme in enzyme catalysis has been well established, recent studies have led to two novel coenzyme B_6-dependent reactions whose catalyses involve unique mechanistic variants of usual coenzyme B_6 chemistry. These are a C-O bond cleavage and a C-C bond cleavage reaction catalyzed by CDP-6-deoxy-L-*threo*-D-*glycero*-4-hexulose-3-dehydrase (E_1) and 1-aminocyclopropane-1-carboxylate deaminase (ACC deaminase), respectively. Enzyme E_1 is the only known pyridoxamine 5'-phosphate (PMP) dependent iron-sulfur containing dehydratase, and it is one of a few B_6-dependent enzymes with a radical mechanism. ACC deaminase is a pyridoxal 5'-phosphate (PLP) dependent enzyme which acts on a cyclopropane substrate, and catalyzes the cleavage between the C_α-C_β bond. Current knowledge concerning the properties and mechanisms of these unusual enzymes is summarized in this paper.

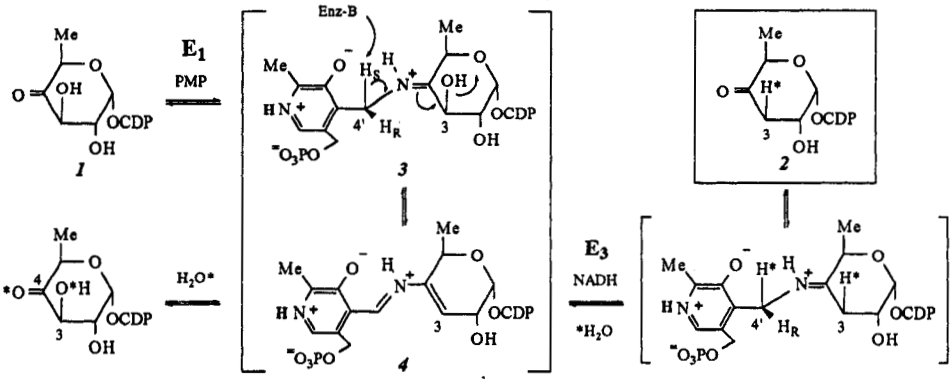
As a coenzyme, vitamin B_6 phosphate exhibits a remarkably versatile array of catalytic roles, ranging from transaminations, racemization, β- and γ-elimination/substitution, decarboxylation, etc (1). The achievement of these catalyses by B_6-dependent enzymes involves a common recurring theme, relying on the electron-withdrawing capability of this cofactor to stabilize the anion generated at the α-C of the substrate-coenzyme complex. The transiently delocalized electrons are later used for the cleavage and/or formation of covalent bonds. While extensive knowledge about the genetics, structure, function, and mechanism of this class of enzymes have been accumulated over the years, several recent studies have revealed a few new traits of coenzyme B_6 that are beyond the scope previously established for its catalyzed reactions. For example, the traditional role of coenzyme B_6 has been varied by lysine-2,3-aminomutase which utilizes PLP to catalyze a radical rearrangement of the α-amino group of lysine (2). A B_6-dependent aminotransferase was also implicated in the conversion of cytosylglucuronic acid to blasticidin S (3). The role of this as yet to be isolated enzyme in blasticidin biosynthesis has been hypothesized not only to act as a transaminase but also to catalyze the dideoxygenation at C-2' and C-3' of the glucuronate residue. In this paper, two other unusual cases of coenzyme B_6 dependent reactions will be presented: one involved in C-3 deoxygenation of a 4-keto-6-deoxyhexose and the other in the ring opening of a cyclopropane amino acid, both of which have been the subjects of our research in recent years. The current information regarding their modes of action will be summarized herein.

CDP-6-deoxy-L-*threo*-D-*glycero*-4-hexulose-3-dehydrase (E_1)

Enzyme E_1 is a homo-dimeric protein (49 KDa per subunit) requiring a pyridoxamine 5'-phosphate (PMP) and an adrenodoxin/putidaredoxin-type [2Fe-2S] center per monomer for full activity (4). This enzyme, together with its reductase (CDP-6-deoxy-L-*threo*-D-*glycero*-4-hexulose-3-dehydrase reductase, E_3), plays an essential role in C-O bond severance in the biosynthesis of 3,6-dideoxyhexoses (5). Specifically, E_1 and E_3 catalyze the conversion of CDP-6-deoxy-L-*threo*-D-*glycero*-4-hexulose (**1**) to the C-3 deoxygenated product, CDP-3,6-dideoxy-D-*glycero*-D-*glycero*-4-hexulose (**2**). As depicted in Scheme 1, the mechanism of E_1 catalysis has been established to be initiated by the formation of a Schiff base (**3**) between PMP and the C-4 keto group of the substrate (4-6), followed by the abstraction of the pro-*S* proton from the C-4' position of the resulting adduct **3** (4b) to trigger the expulsion of the C-3 hydroxyl group leading to a $\Delta^{3,4}$-glucoseen intermediate **4** in the active site of E_1. While intermediate **4** has never been isolated, the reversible nature of its hydration was demonstrated by isotopic incorporation with $[^{18}O]H_2O$ (4a). Furthermore, replacement of the 3-OH group by a solvent hydrogen in the E_1-E_3 coupled reaction has been observed to proceed with retention of configuration (7). In sum, these results imply that the overall catalysis in the active site of E_1 is likely a suprafacial process occurring on the *si* face of the PMP-substrate complex (**4**), as is the case with most other coenzyme B_6-dependent enzymes (1). In fact, sequence align-

ments revealed a clear relationship between E_1 and other PLP/PMP enzymes (8). One significant difference highlighted by the comparisons is the replacement of a highly conserved lysine by a histidine at position 220, which has been shown to be the active-site base responsible for C-4' proton abstraction (8a). Such a single point mutation may have converted E_1's progenitor from a normal PLP-dependent enzyme to a unique PMP-dependent dehydrase.

Scheme 1

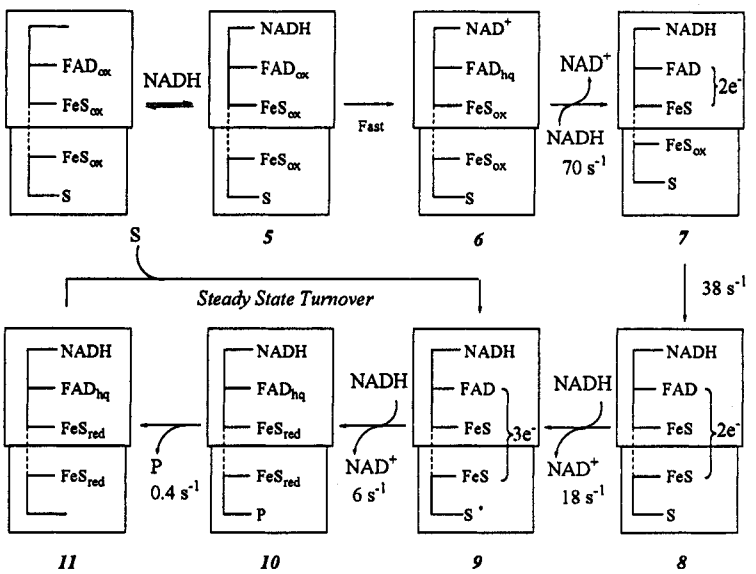


While the C-O cleavage of **1** is achieved in a dehydration step directly mediated by the PMP coenzyme in E_1, the final C-3 deoxygenation product (**2**) is formed only after NADH and E_3 are added to the reaction mixture (5). Removal of the [2Fe-2S] center of E_1 impairs its ability to promote the final product formation (4c). This requirement of the [2Fe-2S] center, which is an obligatory one-electron carrier, for product formation, strongly implicated the intermediacy of free radicals in the reaction. Indeed, EPR study of the chemically reduced E_1-substrate complex confirmed the presence of a Lorentzian-type absorption ($g \approx 2$) which is indicative of an organic radical (9). The participation of the PMP cofactor in a deoxygenation reaction is unique, but the involvement of a radical mechanism truly places E_1 in a class by itself.

Since E_3 is an indispensable component of the C-3 deoxygenation machinery, effort has also been devoted to characterize this monomeric (36 kDa) protein which contains one mol of FAD and a plant-ferredoxin type [2Fe-2S] center (10). Sequence alignments established a strong relationship between E_3 and other iron-sulfur flavoproteins in the ferredoxin-$NADP^+$ reductase (FNR) family (11). Like other members in its family, E_3 transfers reducing equivalents from NADH to a variety of one-electron acceptors, including O_2, with varying degrees of efficiency (10b). Removal of the [2Fe-2S] center impairs the ability of E_3 to catalyze final product formation in the presence of E_1 and substrate. By stopped-flow spectroscopic experiments, the kinetic mechanism of this E_3 mediated electron transfer was fully characterized (12). Interestingly, after the FAD is reduced to the hydroquinone form, the subsequent electron transfer to the [2Fe-2S] center was found to be pH-dependent. Spectroelectrochemical studies substantiated this pH-dependence by showing that the redox potentials of both the FAD and the [2Fe-2S] center change with respect to pH (13). Overall, the proposed electron transport sequence is consistent with the role of E_3 as a $2e^-/1e^-$ switch, and the evidence provides compelling support for a radical mechanism in the C-3 deoxygenation of **1**. In light of the fact that a PMP-glucoseen adduct (**4**) is the ultimate acceptor receiving electrons from E_3, the catalytic role of E_3 in the biosynthesis of 3,6-dideoxy sugars clearly constitutes a novel example of biological deoxygenation.

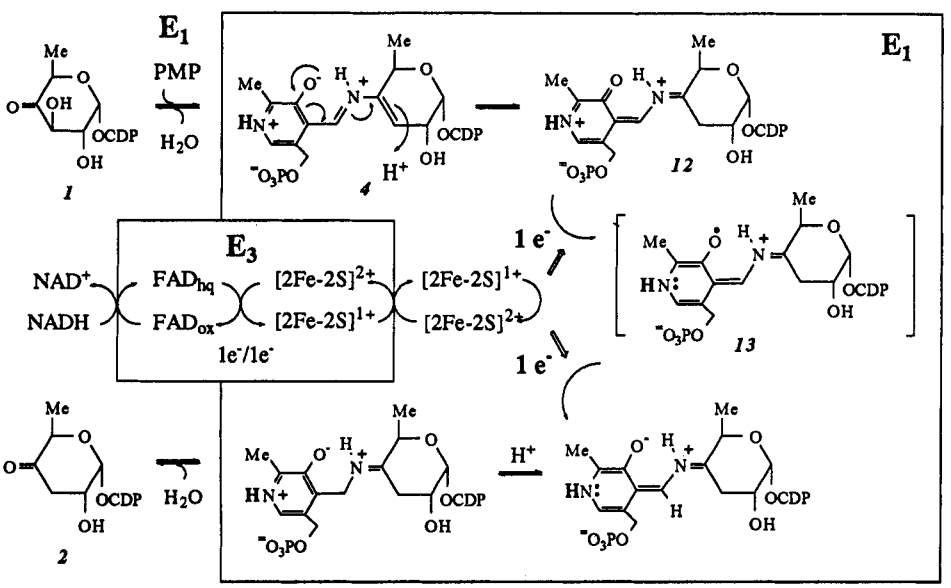
On the basis of results gathered from stopped-flow spectrophotometry and freeze-quench EPR spectroscopy experiments, a mechanism of electron transfer in the E_1-E_3 coupled reaction can be proposed (Scheme 2) (12,14). First, NADH binds to E_3 and forms a charge-transfer complex with the oxidized FAD (**5**, FAD_{ox}). Chemical modification studies identified Cys296 as a possible residue which participates in the stabilization of this charge-transfer complex (11a). The bound NADH then transfers a hydride and reduces FAD_{ox} to the hydroquinone form (**6**, FAD_{hq}). Subsequently, electrons are shuttled one at a time from the hydroquinone through the [2Fe-2S] centers of both E_3 and E_1 to reduce the PMP-glucoseen intermediate bound in the E_1 active-site as a Schiff base (**7** to **10**). Such a one-electron transfer process is expected to produce transient radicals, and two kinetically competent organic radicals formed during this process have been observed using both stopped-flow spectrophotometry and freeze-quench EPR spectroscopy (12,14). While the first radical is clearly the flavin semiquinone, the nature of the second radical is less well-characterized. Nonetheless, the available evidence supports the assignment of the second radical as being a phenoxyl radical with its unpaired spin localized mainly on the C-3 oxygen of the PMP in the coenzyme-

Scheme 2



substrate Schiff base (9), and a highly conjugated sugar-PMP quinonoid skeleton (**13**) has been suggested for this radical species based on its spectral characteristics (14). It is important to note that this radical is likely generated by a one-electron reduction of the quinone methide intermediate (**12**) derived from tautomerization of **4** (Scheme 3). After the second electron transfer, the resulting two-electron reduced Schiff base is hydrolyzed to release the product and end one catalytic cycle (**10** to **11**). It should also be pointed out that during the first turnover two additional NADH molecules are needed to prime the enzyme complex for steady-state catalysis (cycling between **9**, **10**, and **11**). Such priming is common for redox systems like P450 enzymes and dioxygenases.

Scheme 3



Significantly, E_1 is the best characterized coenzyme B_6-dependent catalyst that uses PMP without the usual prerequisite oscillation with PLP. Other hydro-lyases that employ coenzyme B_6 as a cofactor, such as serine and threonine dehydratase, are all PLP-dependent and their reactions involve the formation and stabilization of an α-anion typical for β-elimination catalyzed by this class of enzymes. Clearly, the PMP in the E_1-E_3 system also behaves like a normal B_6 coenzyme in the dehydration of CDP-6-deoxy-L-*threo*-D-

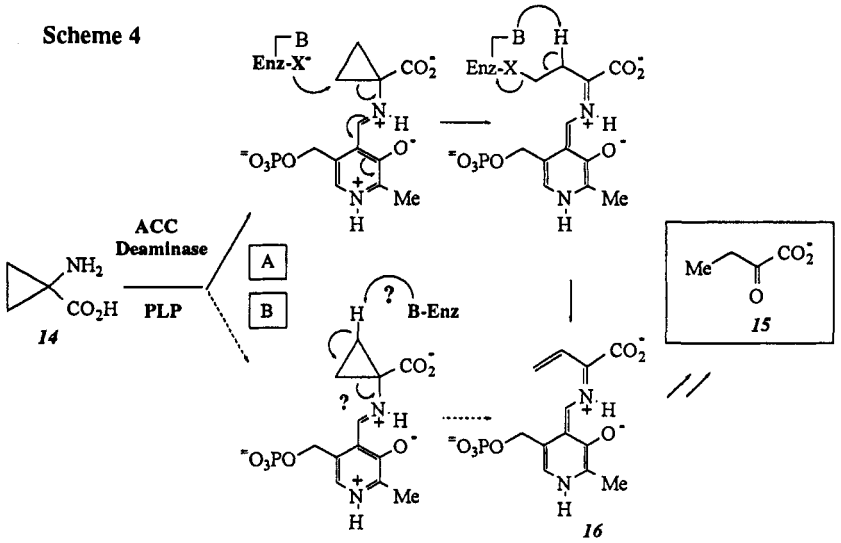
glycero-4-hexulose (**1**). However, what sets E_1 apart is that the E_1-bound PMP also mediates unprecedented one-electron redox chemistry. The combined dehydration and one-electron redox capability place E_1 in its own class, with the C-3 deoxygenation a unique example of a C-O bond cleavage event. Our studies of this reaction have added novel radical chemistry to the known functionality of PMP. It is important to point out that coenzyme B_6 is not commonly perceived as a redox cofactor, but it has long been known to mediate two-electron redox reactions, such as the transaminase reaction which is in fact a two-electron redox process. Thus, the above results not only expand our knowledge of the catalytic diversity of coenzyme B_6, but also emphasize the less recognized redox nature of this common cofactor.

It is noteworthy that the mechanism of lysine-2,3-aminomutase, another coenzyme B_6-dependent enzyme, also includes an organic radical intermediate (2). In this reaction, homolytic cleavage of 5'-adenosylmethionine generates a 5'-deoxyadenosyl radical which abstracts a hydrogen atom from the lysine substrate and initiates the radical rearrangement via the PLP cofactor to give β-lysine. Although this reaction demonstrates a B_6 coenzyme can participate in a radical process, its mechanism is quite different from the reaction catalyzed by E_1. Rather than being generated by a free radical-mediated hydrogen atom abstraction of the substrate, the radical in E_1-E_3 catalysis is generated by direct single-electron reduction of the highly conjugated cofactor-substrate complex (**4**). As mentioned above, the PMP-glucoseen Schiff base (**4**) is expected to undergo tautomerization to the quinone methide intermediate (**12**) prior to the facile reduction of this reactive quinone methide.

1-Aminocyclopropane-1-Carboxylate Deaminase

The cleavage of 1-aminocyclopropane-1-carboxylate (**14**, ACC) to α-ketobutyrate (**15**) by ACC deaminase represents another intriguing PLP-dependent reaction (Scheme 4) (15). Since ACC bears no abstractable α-H and the carboxyl group is retained in the product, the ring opening step must be initiated without obvious accessibility to a carbanionic intermediate at the α-C. Such a C_α-C_β bond cleavage is precedented in PLP-dependent enzymes only by serine hydroxymethyltransferase, which catalyzes the

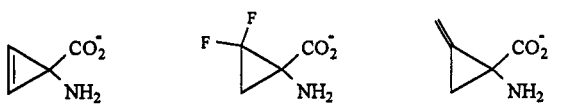
Scheme 4



interconversion of serine to glycine and a formaldehyde equivalent through a retro-Aldol type process. However, because this same route is impossible for an ACC-PLP complex, this deaminase is thus anticipated to utilize an unusual mechanism to accomplish this ring cleavage reaction. It is worth mentioning that ACC is the precursor of an essential phytohormone, ethylene (16), therefore an approach to reduce ethylene formation in plants by diverting the metabolism of its immediate precursor, ACC, will certainly alter many plant developmental processes. In fact, recent *in vivo* tests have clearly demonstrated that ACC deaminase is useful for examining the role of ethylene in many developmental and stress-related actions in plants as well as for extending the shelf life of fruits and vegetables whose ripening is mediated by ethylene (17). Clearly, study of the reaction catalyzed by ACC deaminase is important not only for understanding this unique conversion itself, but also for gaining invaluable information regarding the design of effective methods to control and regulate the ethylene biosynthetic process.

The competence of a variety of cyclic and acyclic amino acids as alternate substrates for ACC deaminase has been established by early studies (15), which showed that only the D-amino acids can be processed by this enzyme and that the α-anion equivalent of the vinylglycyl-PLP aldimine (16) is a key intermediate in ACC fragmentation (15c). The regiochemistry of ring cleavage was found to be exclusively between the α-C and the pro-*S* β-CH_2 group (18a). Further analysis also revealed that abstraction of the β-H is pro-*R* stereospecific and the reprotonation at β-C is mediated by the same enzyme base which is located at the *si* face relative to α-C (18b). Although these results have provided essential mechanistic insights into the sequence of events occurring during ACC conversion, the actual mode of cyclopropane fragmentation remained unresolved.

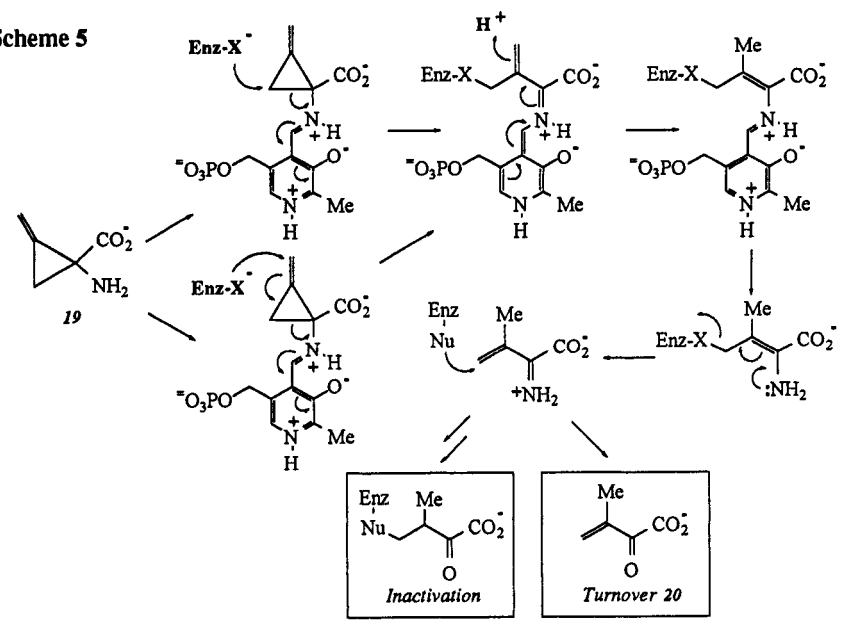
Two mechanistic variants of this interesting catalysis have been proposed: (A) nucleophilic addition to open the ring followed by β-proton abstraction; (B) direct β-proton abstraction to initiate the ring cleavage (Scheme 4) (15,18,19). Two aspects associated with mechanism B make it problematic - the proton being abstracted is kinetically inert ($pK_a \geq 40$) and the anion-induced ring scission is stereoelectronically unfavored. Mechanism A, on the other hand, invokes a covalent catalysis and has no precedence in coenzyme B_6 biochemistry. In order to distinguish between these possibilities, we have synthesized a few ACC derivatives, such as 1-aminocyclopropene-1-carboxylate (17) (20), 1-amino-2,2-difluorocyclopropane-1-carboxylate (18), and 1-amino-2-methylenecyclopropane-1-carboxylate (19) as probes to study the



17 *18* *19*

mechanism of this unique reaction. Our results showed that compound **17** is a substrate for ACC deaminase (21), while the enzyme surprisingly fails to recognize compound **18** (22). However, when ACC deaminase was exposed to **19**, time-dependent irreversible inactivation occurred, exhibiting a k_{inact} of 1.25 h^{-1}, a K_I of 3.3 mM, and a partition ratio of 8250 (23). Since protection from inhibition was observed in the presence of excess substrate, the effect of **19** on ACC deaminase was concluded to be active-site directed.

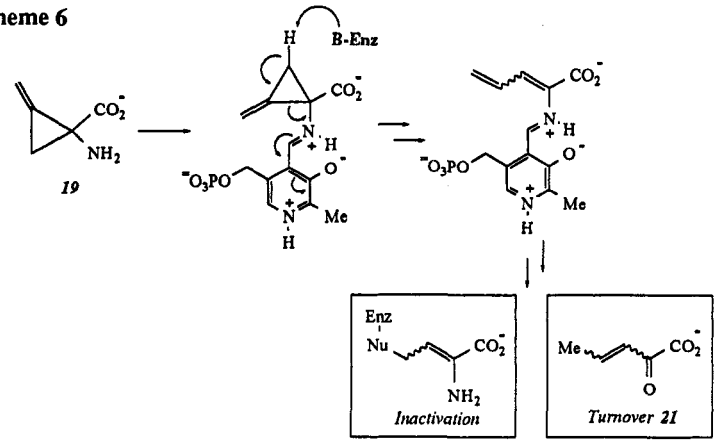
Scheme 5



As illustrated in Scheme 5, if the catalysis proceeds via mechanism A, reaction with **19** would lead to irreversible inactivation, accompanied by possible formation of **20** as a turnover product. On the contrary, if the reaction proceeds via mechanism B, the enzyme may also be inactivated but with **21** as a potential turnover product (Scheme 6). Interestingly, NMR analysis of the crude incubation mixture revealed the existence of a single turnover product which was elucidated as 2-oxo-3-methyl-3-butenoic acid (**20**) by comparing its spectral characteristics with a standard (23). The isolation of **20** as the sole turnover product is

most consistent with route A (Scheme 4) as the inactivation mechanism and therefore provides compelling evidence for ring cleavage of ACC as a nucleophilic-addition initiated event. Furthermore, analysis of the product isolated from the incubation of ACC deaminase with **19** carrying a labeled exocyclic methylene moiety revealed that addition to C-3, rather than to the terminal carbon (C-2') of the exocyclic methylene group, is the preferred mode of attack in the active site of ACC deaminase (23).

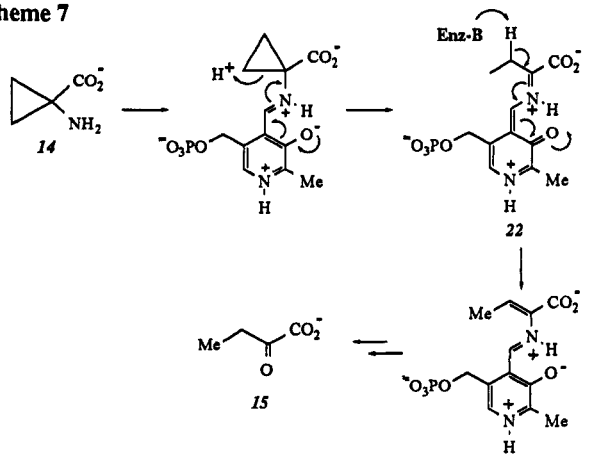
Scheme 6



It is well documented that cyclopropanes substituted with electron-withdrawing groups can act as electrophiles (24). Examples of cyclopropane-containing mechanism-based inhibitors are known in which the target enzyme activates the cyclopropane for nucleophilic addition as a result of oxidation of an appended group to a carbonyl or related species (19, 25). Since the role of PLP is to serve as an electron sink, the cyclopropane ring of ACC must be fairly electrophilic due to double activation by both the 1-carboxylate and the PLP-aldimine groups. Hence, the proposed nucleophilic addition to rupture the electrophilic ring of ACC is mechanistically feasible. Interestingly, this process, when considered in reverse, is essentially the mechanism for the PLP-enzyme ACC synthase (26).

It should be noted, however, that the above results cannot rule out a third possible route (Scheme 7, mechanism C) in which the ring opening is catalyzed by a general acid mechanism, facilitated by the tautomerization of the PLP-ACC complex (**22**) via a quinone methide intermediate (**23**) using the phenoxide oxygen of PLP as the electron donor. Such a push-pull proton-assisted cyclopropane fragmentation has been demonstrated by Jenck's model studies on phenylcyclopropane cleavage (27). A similar quinone methide species **13** postulated as an intermediate formed during C-3 deoxygenation in the active-site of E_1 is clearly a highly relevant precedent (9). Experiments designed to test this possibility are currently in progress.

Scheme 7



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