Mechanistic Studies of 1-Aminocyclopropane-1-carboxylate Deaminase: Unique Covalent Catalysis by Coenzyme B_6

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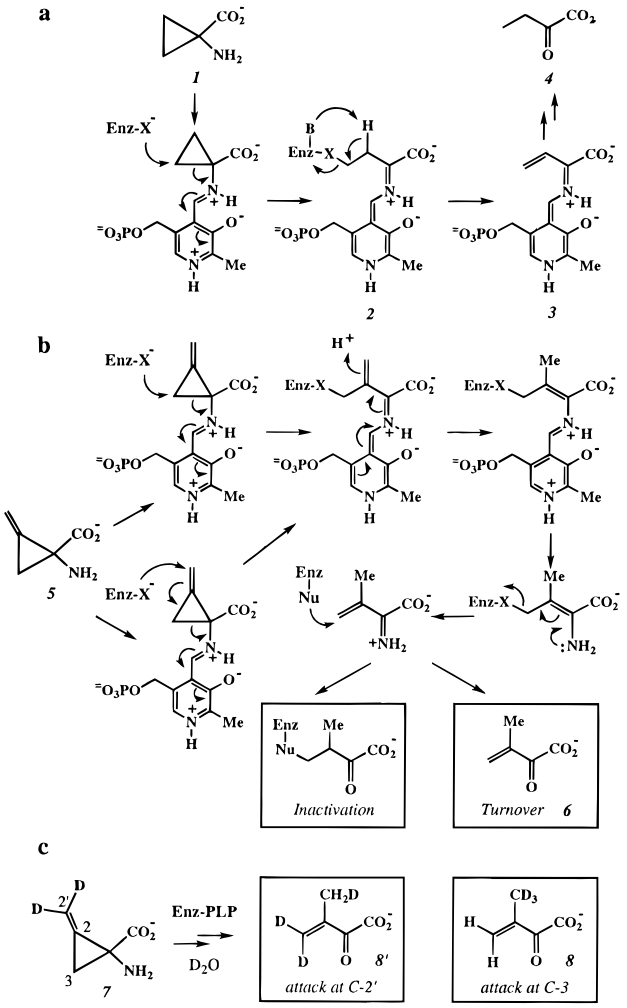
The 1-aminocyclopropane-1-carboxylate (ACC) deaminase, produced by *Pseudomonads* when ACC (**1**) is the sole nitrogen source, is a PLP-linked catalyst with a unique capacity to break the cyclopropane ring leading to the formation of α-ketobutyric acid (**4**).[1] Since ACC bears no abstractable α-H and the carboxyl group is retained in the product, the ring opening must be initiated without obvious accessibility to an α-carbanionic intermediate which is the common entry of catalysis shared by PLP-dependent enzymes.[2] Precedence for such $C_\alpha-C_\beta$ bond cleavage among PLP-linked catalysts is found only in serine hydroxymethyl transferase, in which the $C_\alpha-C_\beta$ bond is cleaved through a retro-aldol type process.[3] However, such a mode of action is apparently not feasible in the ACC case, and, thus, ACC deaminase is likely to adapt an unusual mechanism to accomplish this unique ring fragmentation reaction.

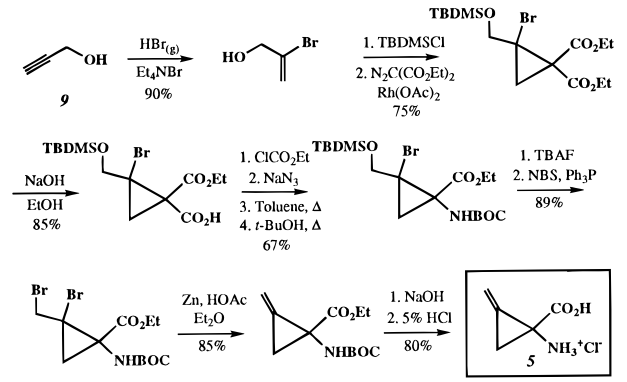
Early studies have established the competence of a variety of cyclic and acyclic amino acids as alternate substrates or inhibitors for ACC deaminase.[4] It has also been shown that the α-anion equivalent of the vinylglycyl-PLP aldimine (**3**) is a key intermediate in ACC fragmentation.[4] Although these results, in conjunction with further work using labeled substrates, had provided essential mechanistic insights into the sequence of events occurring during ACC conversion (Scheme 1),[5] the actual mode of cyclopropane fragmentation, the most intriguing and challenging facet of this catalysis, remained unresolved. Two mechanistic variants had been hypothesized: (A) nucleophilic addition to open the ring, followed by β-proton abstraction (Scheme 1(a)); (B) direct β-proton abstraction to initiate cyclopropane cleavage.[4b,6] Route B is highly unlikely since it requires initiation of catalysis by abstraction of an inert proton from the ring C−H group and such an anion induced ring cleavage is stereoelectronically unfavored. However, route A is also thought-provoking, since it invokes a unique covalent catalysis considered novel in coenzyme B_6 chemistry. Clearly, the action of ACC deaminase imposes a challenge to our understanding of the diverse roles of the B_6 coenzyme in biological transformations.

Our strategy to gain insight into this puzzling problem was to use modified substrates which, upon incubation with ACC deaminase, would lead to either inhibition or turnover depending on the modes of catalysis. For instance, judging from the hypothesized mechanisms, it was perceived that 1-amino-2-methylenecyclopropane-1-carboxylate (**5**, methylene-ACC) could be a mechanism-specific probe. As illustrated in Scheme 1(b), if the catalysis advances via mechanism A as predicted, reaction with **5** would lead to irreversible inactivation, accompanied by possible formation of compound **6** as a turnover product.[7] On the basis of this premise, compound **5** was synthesized by the reactions delineated in Scheme 2. Although the enantiomerically pure samples were preferred, experiments performed with a racemic mixture were able to provide ample information to

Scheme 1



Scheme 2



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(7) If the reaction proceeds via the highly unlikely route B, the enzyme may also be inactivated by **5** but with 2-oxo-3-pentenoic acid as a potential turnover product.

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distinguish these mechanisms due to the distinct outcomes of the incubation.

We observed that when ACC deaminase was exposed to **5**,[8] time-dependent inactivation occurred. This inactivation exhibited Michaelis−Menten kinetics with a k_{inact} of 1.25 h^{-1} and a K_I of 3.3 mM.[9] Since the enzyme activity was not recovered after extensive dialysis, the inactivation is clearly irreversible. Furthermore, protection from inhibition was observed in the presence of excess substrate, the effect of **5** on ACC deaminase was thus concluded to be active-site directed. A partition ratio of 8250 was deduced from a plot of the residual activity observed versus total equivalents of **5** added. 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 (**6**) by spectroscopic characterization.[10] The identity of this compound was further confirmed by comparing its spectral properties with a standard prepared by coupling ethyl oxalate with the corresponding alkenyl Grignard reagent.[10] This assignment is also supported by the fact that the integration of the methyl peak at δ 1.90 is equal to the sum of the methylene hydrogen signals at δ 6.05 and 6.20 when the incubation was conducted in deuterated buffer. The isolation of **6** as the sole turnover product is most consistent with route A as the inactivation mechanism and thus provides compelling evidence for ring cleavage of ACC as a nucleophilic-addition initiated event.

As shown in Scheme 1(b), both the ring methylene carbon (C-3) and the terminal carbon (C-2′) of the exocyclic methylene group are possible sites for the initial nucleophilic attack. While the outcomes of these two modes of attack are identical, they can be distinguished by using a probe having a dideuterated exocyclic methylene moiety such as **7**. Synthesis of labeled **7** was accomplished by the same sequence shown in Scheme 2 starting with [1,1-$^2H_2$]propargyl alcohol (**9**) which was prepared by $LiAl^2H_4$ reduction of the corresponding methyl ester. The deuterium content in the final product was estimated to be greater than 98% based on NMR integration. Analysis of the turnover product isolated from the incubation of ACC deaminase with labeled **7** revealed that the ratio between the integration of the sum of the two olefinic signals and that of the methyl signal in the NMR spectrum of **8** was 3.1:1. It is thus clear from this data that addition to C-3 is the preferred mode of nucleophilic attack in the active site of ACC deaminase.

It is worth mentioning that cyclopropanes can act as electrophiles when electron-withdrawing substituents are attached.[11] Since the role of PLP is to serve as an electron sink, the cyclopropane ring of ACC is expected to be fairly electrophilic due to double activation by both the carboxylate and the PLP-aldimine groups. Hence, such nucleophilic addition to rupture the electrophilic cyclopropyl ring of ACC is mechanistically feasible. The fact that the β-H being abstracted in the ring-opened covalent enzyme−substrate adduct (**2**) is an acidic hydrogen lends further credence to the assigned mechanism. Thus, the cleavage of ACC (**1**) to α-ketobutyrate (**4**) by ACC deaminase clearly represents an intriguing conversion beyond the common scope entailed by coenzyme B_6 dependent catalysts. Interestingly, this process, when considered in reverse, is essentially the mechanism for the PLP-enzyme ACC synthase and is clearly a highly relevant precedent.[12,13]

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(8) The ACC deaminase gene has been recently cloned, sequenced, and expressed (Sheehy, R. E.; Honma, M.; Yamada, M.; Sasaki, T.; Martineau, B.; Hiatt, W. R. *J. Bacteriol.* **1991**, *173*, 5260). A plasmid pCGN1472 containing the ACC deaminase gene has been kindly provided by Calgene. The insert of pCGN1472 was subcloned into pET17b to give a new construct pQNt23. This enzyme, isolated from the resulting overexpression *E. coli* strain pQNt23/BL21(DE3)plysS, was purified via a sequence of DEAE-Sepharose, AH-Agarose, and FPLC Mono-Q chromatography. The purified enzyme has a specific activity of 4.2 U/mg,[9] with a ratio of A_{280}/A_{420} = 4.5.

(9) A standard 1 mL assay was performed in 50 mM potassium phosphate buffer (pH 7.5) at 23 °C, containing substrate, 8−20 μg ACC deaminase, 125 μg of L-lactate dehydrogenase, and 0.1 μmol of NADH. Loss of NADH absorbance at 340 nm (ϵ 6220) was monitored. The unit of activity is defined as μmol of NADH consumption per minute. The kinetic parameters of inactivation were derived from plotting the apparent inactivation rate, obtained by incubating ACC deaminase (90 μg) with **5** of varied concentration (1−10 mM), versus inhibitor concentration.

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