Enzymatic Mechanisms

Structural Analysis of 1-Aminocyclopropane-1-Carboxylate Deaminase: Observation of an Aminyl Intermediate and Identification of Tyr294 as the Active-Site Nucleophile**

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Dedicated to Professor Christopher Walsh on the occasion of his 60th birthday

1-Aminocyclopropane-1-carboxylate (ACC) deaminase is a pyridoxal phosphate (PLP)-linked enzyme that catalyzes the conversion of ACC (**1**) into α-ketobutyrate (**2**) and ammonia (Scheme 1).[1] In most other PLP-dependent reactions the formation of an α-carbanionic intermediate is the initiating event,[2] but the ring cleavage catalyzed by ACC deaminase

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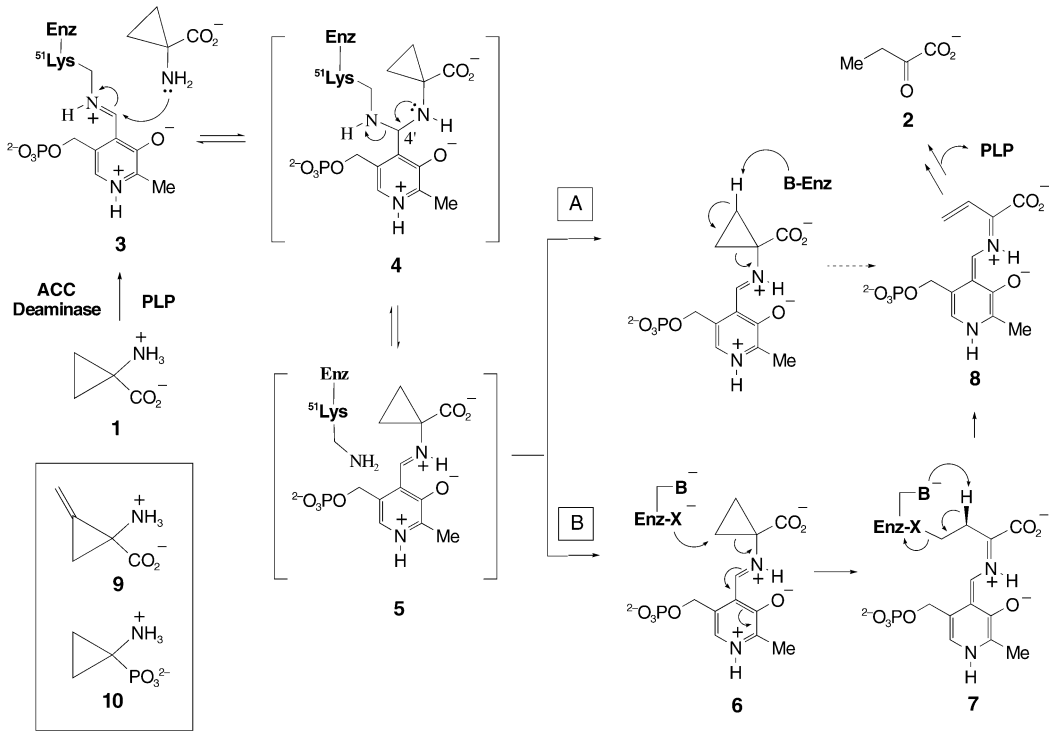

Scheme 1. Proposed mechanisms of ACC deaminase. Enz = enzyme, X^- = nucleophile, B^- = base.

cannot proceed through such an intermediate because ACC does not have an abstractable α-H atom. An early study in which the α-anion equivalent of the vinylglycyl-PLP aldimine **8** was shown to be an intermediate in ACC fragmentation suggested two possible mechanisms for the ring scission.[3,4] As depicted in Scheme 1, the reaction could involve a direct β-proton abstraction that initiates cyclopropane cleavage (Route A), or it might proceed by a nucleophilic addition that opens the ring, followed by β-proton abstraction to give **8** (Route B). We recently synthesized 1-amino-2-methylenecyclopropane-1-carboxylic acid (**9**), which has a more electrophilic ring than ACC, as a tool for studying the mechanism of this unusual reaction. We found **9** to be a better substrate than ACC for the enzyme.[5–7] This result strongly suggests that Route B is the reaction course. This conclusion is strengthened by the fact that compound **9** irreversibly inhibits ACC deaminase and the inactivation can be rationalized by a mechanism analogous to Route B.[6,7]

Various labeling and mutagenesis results implicated one of the two active-site residues (Ser78 or Lys51) as the nucleophile (Enz-X^-) that attacks the cyclopropane ring and initiates the fragmentation of ACC as shown in Scheme 1 (Route B).[7] Although the crystal structure of the enzyme from the yeast *Hansenula saturnus* without substrate/product (or their analogues) in the active site had been solved when the structural study described herein was initiated,[8] little mechanistic information could be deduced from this crystal structure. To gain more insight into this intriguing coenzyme B_6 dependent reaction, we determined the crystal structure of the *Pseudomonas* ACC deaminase in a complex with a known tight-binding inhibitor, 1-aminocyclopropanephosphonate (ACP; **10**).[9] We report herein our structural results along with mutagenesis data, which together provide strong evidence that Tyr294 is the active-site nucleophile involved in the ring-cleavage step.

Early attempts to determine the three-dimensional structure of the *Pseudomonas* ACC deaminase were unsuccessful.[8] However, we were able to crystallize this protein in the presence of ACP (**10**) and solve the structure of the ACP–protein binary complex.[10] The electron density map showed that the PLP coenzyme is covalently attached to Lys51 at C-4′, as in the holoenzyme (data not shown). To our surprise, there is also a covalent bond between the C-4′ atom of PLP and the amino group of ACP (Figure 1A). Such an aminyl adduct (**4**) has long been proposed as an intermediate that occurs during the interchange between the internal aldimine (formed by PLP and the Lys anchor; **3**) and the external aldimine (formed by PLP and the substrate; **5**) in PLP-dependent catalysis. However, proof of the existence of this adduct has been elusive because of the transient nature of this intermediate.[11] Direct observation of this species was achieved only recently in a structural study of histidinol phosphate aminotransferase.[12] Our detection of the aminyl adduct shown in Figure 1A is only the second example of the direct observation of such an intermediate. It is conceivable that the tight binding of ACP[9] is partially a result of the trapping of the PLP coenzyme in the middle of the Schiff base exchange process.

The structure shown in Figure 1A not only reveals a generally undetected intermediate of the PLP-dependent reaction but also sheds new light on the catalytic mechanism of ACC deaminase. Ser78, which was previously thought to be the nucleophile (Enz-X^- in Route B) involved in the ring-opening process or the base (Enz-B^- in Route B) responsible

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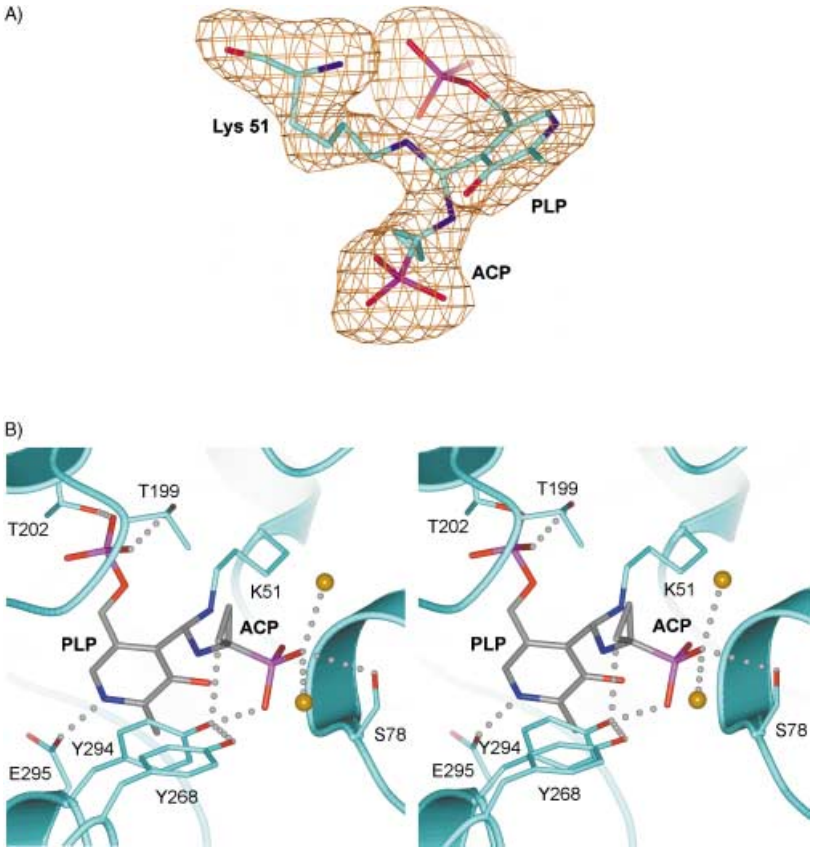


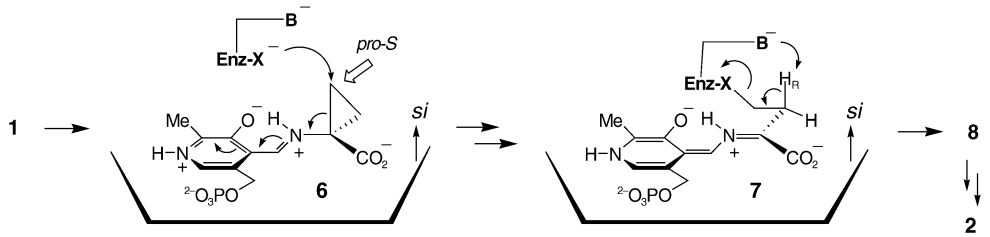
Figure 1. The active site of ACC deaminase in the ACP complex. A) The F_o-F_c difference electron densities for Lys51, PLP, and bound ACP (**10**), which show the existence of the aminyl intermediate. The map is contoured at 3 σ. B) Stereoview in which the interactions between bound ACP, PLP, and surrounding protein atoms are shown. Hydrogen bonds and close van der Waals interactions (over a distance $\leq$ 3.5 Å) are indicated by dotted lines. The two active-site water molecules are shown as yellow balls.

for the subsequent β-H abstraction,[7] was found to be distanced from the cyclopropyl ring but within H-bonding range (3.01 Å) of the phosphonate group of **10** (Figure 1B). This result suggests that Ser78 plays a primary role in substrate binding instead of directly participating in the chemical transformation. Accordingly, the large decrease in the activity caused by S78A mutation (to around 2.3% of the wild-type activity) observed in a previous experiment[7] should be attributed to a low affinity of the substrate for the active site.

Our second important observation is that Lys51, which anchors the PLP coenzyme in the active site and is a likely candidate for the attacking nucleophile in the ring-opening step,[7] is found in proximity (3.53 Å) to the pro-*S* carbon atom, rather than the pro-*R* carbon atom, of ACP (**10**) in the aminyl structure (Figure 1B).[13] The relative orientation of Lys51 is inconsistent with its proposed role as a nucleophile in catalysis because the ACC-deaminase-catalyzed ring cleavage of **1** occurs exclusively between the α-C atom and the pro-*S* β-CH_2 group of **1** (see **6** in Scheme 2).[13,14] Clearly, the previously deduced functions of both Ser78 and Lys51 in the ring-cleavage event need to be reevaluated.

Tyr294, which is hydrogen bonded to Tyr268, lies adjacent to the pro-*R* carbon atom of ACP. The 2.98 Å gap between the hydroxy oxygen atom of Tyr294 and the pro-*R* carbon atom of ACP (**10**) puts Tyr294 within reach of the cyclopropane ring (Figure 1B). This tyrosine residue is strictly conserved among the more than twenty ACC deaminase homologues known so far. It is therefore highly probable that Tyr294 is the elusive nucleophile whose addition to the pro-*S* β-CH_2 group of **1** induces ring opening. The reactivity of Tyr294 may be enhanced by hydrogen bonding to Tyr268, which is only 2.84 Å away. The catalytically more relevant structure **5** was recently observed by Ose et al. in a complex of the yeast ACC deaminase K51T mutant and ACC (**1**)[15] in which a two-phenol relay system (Y269 and Y295 in the yeast enzyme) exists that involves a similar juxtaposition of residues to that shown in Figure 1B. Comparison of the active site structure of the *Pseudomonas* enzyme (see Figure 1B) with structure **5** of the yeast enzyme[15] reveals little difference in the protein backbone and a shift of about 1 Å on the part of PLP–ACC adduct. The Tyr295 residue of the yeast enzyme is also found in proximity to the cyclopropane ring and could easily perform the proposed nucleophilic role.

To test this hypothesis, the Y294F mutant of the *Pseudomonas* enzyme was prepared. The mutant protein was determined to exhibit chromatographic behavior and spectral properties identical to those of the wild-type enzyme. However, no turnover by the mutant was detected with the lactate dehydrogenase/reduced nicotinamide-adenine dinucleotide



Scheme 2. Stereochemical course of ACC-deaminase-catalyzed ring opening and β-deprotonation.

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(NADH) assay.[4,16] A subsequent large-scale incubation in which ACC (**1**, 17 mM) was mixed with Y294F protein (60 μM) in potassium phosphate buffer (650 μL) prepared in D_2O (pD 8.0) led to no product formation, as assessed by ^1H NMR spectroscopy after 24 h. In contrast, the control reaction with wild-type enzyme (5 μM) resulted in complete conversion of **1** into **2** in 2 h. The inability of the Y294F mutant to process ACC provides strong support for the assigned role of Tyr294 in catalysis.[17] As we expected, Y268F mutation also caused the loss of more than 98% of the wild-type activity.[18] Ose et al. have reported the crystal structure of the yeast ACC deaminase Y295F mutant complexed with ACC, which revealed that the bound ACC adopts a very different, nonproductive conformation in the mutant complex.[15] This result suggests that, in addition to its proposed catalytic role, the Tyr294 residue of the *Pseudomonas* enzyme (or Tyr295 in the yeast enzyme) may help to correctly position the bound substrate in the active site.

Since the hydroxy group of Tyr295 is near the amino group (3.48 Å) of ACC in the yeast enzyme complex, Ose et al. proposed that this tyrosine residue plays a role in facilitating the formation of the external aldimine **5**. In fact, the authors favored Route A as the mechanism for the yeast enzyme and assigned Lys51, whose side chain orientation could only be deduced by modeling, as the base responsible for abstraction of the β proton.[15] This interpretation clearly differs from ours and warrants additional comment. A ring-opening mechanism initiated by deprotonation is inconsistent with the results obtained from studies of 2-methylene-ACC (**9**).[6,7] Such a mechanism is also thermodynamically unfavorable because the cyclopropane ring hydrogen atoms are not acidic. In contrast, the cyclopropane ring of ACC is readily susceptible to nucleophilic attack because of activation by the electron-withdrawing 1-carboxylate group and the imine moiety of the Schiff base.[19] The fact that Tyr294 is positioned close to the β-C atom of ACC/ACP makes it an ideal candidate to assume the role of the attacking nucleophile. Moreover, a ring opening initiated by a direct β-proton abstraction is inconsistent with the pro-*R* stereospecificity determined for this β-C–H bond cleavage event (see **7** in Scheme 2),[20] since it is the pro-*S* hydrogen atom, not the pro-*R* hydrogen atom, at the pro-*S* β-C atom (in **10**) is more accessible to the proposed base, Lys51, in the enzyme–ACP (**10**) complex (Figure 1B).

In summary, structural studies of ACC deaminase complexed with the tight-binding inhibitor ACP (**10**) revealed the presence of an aminyl adduct formed by the PLP cofactor and ACP in a rare example of the capture of a transient intermediate during enzymatic turnover. Both Ser78 and Lys51 are oriented away from the pro-*S* β-C atom of the ring cleavage site (in **1**), which casts doubt on the previously proposed functions of these two residues. Most significantly, the crystal structure reveals that Tyr294, which has not been implicated as a key residue in this reaction, is probably the nucleophile involved in the cyclopropane ring opening step. This assignment is further corroborated by the finding that the Y294F mutant is totally inactive with regard to ACC turnover. These results, coupled with the observations of Ose et al.,[15] provide strong evidence in support of the hypothesis that the ring cleavage of ACC is initiated by nucleophilic addition and thus establish an unprecedented role of coenzyme B_6 in catalysis.[21] Our ongoing investigation into the structures of this enzyme in complexes with other ligands may provide additional mechanistic insight into this unusual coenzyme B_6 dependent reaction.

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[17] Tyrosine can act as a nucleophile in enzyme catalysis, for example, in tyrosine kinase reactions. For examples involving enzymes other than kinases, see: a) J. A. Mancini, R. J. Waugh, J. A. Thompson, J. F. Evans, M. Belley, R. Zamboni, R. C. Murphy, *Arch. Biochem. Biophys.* **1998**, *354*, 117–124; b) J. C. Gebler, D. E. Trimbur, R. A. J. Warren, R. Aebersold, M. Namchuk, S. G. Withers, *Biochemistry* **1995**, *34*, 14547–14553.

[18] The Y268F mutant protein was characterized and assayed by the procedures described for Y294F. The mutant retained less than 2% of the wild-type activity. While both tyrosine residues are important for catalysis, Tyr294 is a more likely candidate for the nucleophile for two reasons: Tyr294 is closer than Tyr268 to the cyclopropane ring, and all enzymatic activity is lost when Tyr294 is mutated to phenylalanine.

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[21] There are many examples in which the PLP coenzyme promotes nucleophilic addition at the β-carbon atom of the substrate–PLP complex. However, the reaction is always initiated by an α-deprotonation step, which occurs prior to the nucleophilic addition at the β-position. Such an α-deprotonation is not possible for ACC deaminase since ACC (**1**) has no α-H atom. In fact, the nucleophilic addition is the initiation step in the ACC-deaminase-catalyzed reaction, a mechanism that is unprecedented in coenzyme B_6 dependent enzymes.

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