Crystal Structures of 1-Aminocyclopropane-1-carboxylate (ACC) Synthase in Complex with Aminoethoxyvinylglycine and Pyridoxal-5’-Phosphate Provide New Insight into Catalytic Mechanisms*  

Qing Huai‡, Yuanhong Xia‡, Yongquan Chen‡, Brian Callahan‡, Ning Li§, and Hengming Ke‡‡†  

From the ‡Department of Biochemistry and Biophysics and Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, North Carolina 27599-7260 and the §Department of Biology, The Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong  


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Abstract

Ethylene is a plant hormone that profoundly influences the growth and development of higher plants such as the germination of seeds, ripening of fruits, abscission of leaves, and senescence of flowers (1–4). In the pathway of ethylene biosynthesis in higher plants (1), the precursor methionine is first converted to S-adenosylmethionine (SAM) by methionine adenosyltransferase, followed by the conversion of SAM to 1-aminocyclopropane-1-carboxylate (ACC) by ACC synthase (EC 4.4.1.14). Finally, ACC is oxidized to ethylene by ACC oxidase. For the above pathway, conversion of SAM to ACC is the rate-determining step. Thus, ACC synthase is the key enzyme in the regulation of ethylene biosynthesis in higher plants.

ACC synthase (ACS) requires pyridoxal-5’-phosphate (PLP) or vitamin B6 as the cofactor for its catalysis and thus is a member of PLP-dependent enzymes. The PLP-dependent enzymes catalyze a wide variety of reactions in the metabolism of amino acids including transamination, racemization, deamination, decarboxylation, and elimination or replacement of β and γ carbons. They share a limited sequence homology and have diversified catalytic specificities (5–8). Since the first crystal structure of aspartate aminotransferase (AAT; Ref. 9), three-dimensional structures of PLP-dependent enzymes have been determined at an explosive speed in the last decade, and over 200 crystal structures have been deposited in the Protein Data Bank to date. The structures of PLP enzymes are classified into four types of fold (10). Extensive biochemical and structural studies reveal that PLP-dependent enzymes reserve a common mechanism of catalysis (10). However, it remains unknown what is the determinant to define specificities of PLP enzymes. The crystal structures of tomato ACS containing PLP and competitive inhibitor aminoethoxyvinylglycine (AVG), as reported in this paper, suggest that Tyr152 plays a key role on the γ elimination, thus providing insight into the individual pathway of ACS catalysis.

MATERIALS AND METHODS

Purification of ACC Synthase—The truncated ripening-induced tomato ACS with amino acids 1–439 (485 amino acids for the full-length ACS) was inserted into the expression vector pET11d and overexpressed in Escherichia coli BL21 (11). ACS was purified using a slightly modified protocol of Li and Matteo (12). Briefly, after cell lysis by French press, ACS was separated by ammonium sulfate fractionation and column chromatography of DEAE ion-exchange, hydroxyapatite, and Sephadex G75. A typical yield from 2 liters of cell culture was 20–30 mg of active ACS with a purity of >95% as judged by the SDS-gel.

Crystallization and Data Collection—Crystals of tomato ACS complexed with cofactor PLP were grown by the vapor diffusion method. The protein drop contained the ACS-PLP mixture in a ratio of 1:1:1. The well buffer was 20 mM sodium cacodylate, pH 6.0, 0.2 mM Li2SO4, and 19–23% polyethylene glycol 3350. Yellow diamond-shaped crystals reached a typical size of 0.3 × 0.3 × 0.3 mm3 in 1 week, but the color faded quickly, presumably as a result of PLP hydrolysis. The crystals have the space group P6121 with cell dimensions of a = b = 123.2 Å and c = 212.8 Å and contain two molecules in the crystallographic asymmetric unit.

The ACS-PLP-AVG complex was prepared by mixing ACS, PLP, and AVG in a molecular ratio of 1:2:2 and crystallized by vapor diffusion against a well buffer of 0.1 M sodium citrate, pH 5.6, 18% polyethylene glycol 3350, 50 mM MgCl2, and 10% ethylene glycol. The crystals have the space group P6121 with cell dimensions of a = b = 123.1 Å, c = 168.6 Å, and one molecule per crystallographic asymmetric unit.

The diffraction data for the ACS-PLP and ACS-PLP-AVG crystals were collected, respectively, on a Rigaku RAXIS IV phosphorus imaging plate system and on beamline X23C of Brookhaven National Synchrotron Light Source at 100 K (Table I). The cryosolvents were prepared by the addition of 10–20% ethylene glycol into the crystallization buffers.

The data were processed with the program HKL (13). The data were processed with the program HKL (13).

Crystal Structures and Comparison—The ACS-PLP-AVG structures were deposited in the Protein Data Bank with codes 18528 and 18529.

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‡ To whom correspondence should be addressed. Tel.: 919-966-2244; Fax: 919-966-2382; E-mail: hke@med.unc.edu.

† The abbreviations used are: SAM, S-adenosylmethionine; ACC, 1-aminocyclopropane-1-carboxylate; ACS, ACC synthase; PLP, pyridoxal-5’-phosphate; AAT, aspartate aminotransferase; AVG, aminoethoxyvinylglycine; TSB, tryptophan synthase β subunit.
structure was determined by the molecular replacement program AMoRe (14) using a monomer of apple ACS as the initial model (15). The rotation and translation search revealed clearly a solution with a correlation coefficient of 0.452 and an R factor of 0.439 for 3672 reflections between 4 and 8 Å resolution. The binary ACS-PLP structure was determined by using the refined ACS-PLP-AVG structure as the initial model. Our program SUDOTR (16) detected a pseudo translation approximately half-c unit cell for the two molecules in the crystallographic asymmetric unit of the ACS-PLP crystal. Thus, two solutions with the same orientation but half-difference yielded a correlation coefficient of 0.353 and an R factor of 0.489 for 7329 reflections between 4 and 8 Å resolution. The model was rebuilt by the program O (17) and refined by CNS (18). The final refinement yielded R factor/Rfree of 0.214/0.289 for 0.353 and an R factor of 0.489 for 7329 reflections between 4 and 8 Å resolution. The binary ACS-PLP complex, however, the electron density for PLP was poor, and the most recognizable pieces of density were two strong peaks associated with Arg286 and Arg412, respectively.

Overall Structure—The monomer of tomato ACS contains two domains and is similar to the structure of apple ACS (15). The αβα sandwich domain comprises a central seven-strand β-sheet flanking with nine α-helices (residues 82–322 or H3–H11, Fig. 2), whereas the αβ domain consists of five β strands and five α-helices (residues 11–19, 25–77, and 323–438). In the dimeric form, N-terminal residues 11–19 contact with the C-terminal helix H14 (Fig. 2B). The significance of the interactions is not clear but may be important for conformation stabilization and catalysis as suggested by the biochemical study (11, 12). The crystallographic asymmetric units contain a dimer in the ACS-PLP crystal and a monomer in the ACS-PLP-AVG crystal (Fig. 2). The structural superposition revealed Rms deviations of 0.58 Å for the backbone atoms between ACS-PLP-AVG and ACS-PLP and of 0.41 between two monomers of the ACS-PLP dimer, indicating that the inhibitor binding does not induce substantial changes of the enzyme conformation.

It has been a general observation that the dimeric form is a basic catalytic unit of most PLP-dependent enzymes (10). Many PLP-dependent enzymes have cofactor PLP and substrate binding to the active site of one molecule and also interacting with the residues from the second molecule. This physical sharing of an active site by two monomers within a dimer is direct evidence for the active dimeric unit. In addition, cytosolic AAT undergoes a conformational change upon the substrate binding (21, 22), and glutamate-1-semialdehyde aminotransferase shows asymmetric catalysis, in which PLP forms a covalent bond with Lys278 in only one molecule of the dimer (23). This mutual dependence and allosteric communication between two subunits within a dimer further support the argument that a dimer of PLP enzymes is a catalytic unit.

ACS is physically a dimer in the crystal structures of both tomato and apple (15), indicating a functional dimeric form. However, characteristics of the ACS structures lead us to sug-

### RESULTS AND DISCUSSION

**Quality of the Structures**—The electron density map showed that the most portions of the structures were well ordered in both ternary ACS-PLP-AVG and binary ACS-PLP complexes.

**TABLE I**

| Statistics of diffraction data and structure refinement of ACC synthase complexes |
|---------------------------------|-----------------|-----------------|
| Data collection                 | ACS-PLP         | ACS-PLP-AVG     |
| Space group                     | P6,22           | P6,22           |
| Unit cell (a,c, Å)              | 123.2, 212.8    | 123.1, 106.8    |
| Resolution (Å)                 | 2.8             | 2.8             |
| Total measurements             | 218952          | 63871           |
| Unique reflections             | 23977           | 13199           |
| Completeness (%)               | 99.0 (99.9) 6   | 97.6 (76.2)     |
| Average I/σ                    | 13.2 (5.3)      | 6.0 (1.5)       |
| Rmerge                          | 0.081 (0.430)   | 0.109 (0.445)   |

**Structure Refinement**

| Bond (Å)                        | 0.0077          | 0.0072          |
| Angle                           | 1.36°           | 1.29°           |
| Number of atoms and (B) (Å²)    | 6624/54.1       | 3324/50.1       |
| Protein                         | 30/86.3         | 15/53.7         |
| PLP                             | 10/36.2         | 11/80.4         |
| AVG                             | 19/34.4         | 46/19.6         |

**Fig. 1. Stereo view on the electron density for PLP (A500) and AVG (A501).** The (2Fo – Fc) map is contoured at 1.5σ and calculated from the structure omitting PLP and AVG during refinement.
suggest that monomeric ACS may also be catalytically active. The structural comparison showed no substantial conformational changes upon the binding of inhibitor AVG. This lack of communication between two monomers of ACS is in contrast to the allosteric activation of other PLP enzymes and likely indicates the independence of two active sites in the ACS dimer. Indeed, the residues interacting with PLP and AVG and the catalytic residues identified by the sequence alignment are located entirely within an active site of a monomer, implying that a monomer of ACS may be sufficient to accomplish the catalysis. This assumption is consistent with the biochemical results that both the monomer (12, 24–26) and dimer (12, 25, 27–29) of ACS are active. Our structure supports the argument that the proteolytic cleavage of ACS into hyperactive monomeric ACS may occur in vivo upon the endogenous and exogenous stimuli such as ripening and wounding induction (12). However, we note

### TABLE II

Superposition between fold types I, II, and III of PLP-dependent enzymes

<table>
<thead>
<tr>
<th>Protein (Fold type)</th>
<th>Residues</th>
<th>Protein (Fold type)</th>
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<tbody>
<tr>
<td>ACS (Fold type I)</td>
<td>142–147</td>
<td>mODC (Fold type III)</td>
<td>149–154</td>
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<tr>
<td>181–207 (Fold type II)</td>
<td>173–199</td>
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<td>217–227 (PDB code 7odc)</td>
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<td>276–277</td>
<td>279–280</td>
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<tr>
<td>RMS deviation = 2.2 Å for backbone atoms of 63 residues</td>
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<tr>
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<td>TSB (Fold type II)</td>
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<td>144–148</td>
<td>183–187</td>
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<tr>
<td>RMS deviation = 2.2 Å for backbone atoms of 58 residues</td>
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**Fig. 2.** A, ribbon diagram of monomeric ACS. PLP is presented in the ball and stick model. The secondary structures, α-helices, are shown in light blue, β-helices in dark blue, and β strand in green, and their corresponding sequences are shown in C. B, dimeric ACS, as observed in the ACS-PLP complex, is presented in golden and green ribbons. PLP is shown as purple balls, and the substrate SAM, shown as cyan balls, was modeled into the structure.
that the side chains of Gln\textsuperscript{90} and Tyr\textsuperscript{92} from the second subunit sit \( \sim 4 \) Å away from the closest atoms of AVG. Further structural study is needed to clarify whether Gln\textsuperscript{90} and Tyr\textsuperscript{92} contribute to SAM binding and the catalysis.

**Divergent Evolution of PLP Enzymes**—Among 32 structures of PLP-dependent enzymes in the Protein Data Bank, 29 use the pyridoxal moiety of PLP for their catalysis and have been classified into four structural fold types (10). Fold type I contains a large \( \alpha\beta\alpha \) sandwich domain and a small \( \alpha\beta \) domain, and PLP mainly binds to the \( \alpha\beta\alpha \) domain, as shown in an example of tomato ACS (Fig. 2). Fold types II and IV have two \( \alpha\beta \) domains with approximately equal size, and PLP binds between two domains. Fold type III consists of a large triosephosphate isomerase barrel domain on which PLP binds and a small \( \alpha\beta \) domain. On first glance, the four families of PLP enzymes share a limited conservation of the PLP binding residues but no common structure fold. Thus, it has been a mystery how PLP binds to active sites with different geometric shapes and what the evolutionary relationship between different families of PLP enzymes is. It has been argued that different structural folding but significant conservation of the PLP binding residues represent convergent evolution (30).

A comparison of tomato ACS with structures in the Protein Data Bank using the program Dali (19) results in a list of 51 structures that have a Z score better than 2 and are considered to share significant structural homology. Among these structures, 19 are PLP-dependent enzymes, and 18 belong to fold type I. The best superimposition occurred between tomato and apple ACSs (15) that share sequence identity of 57% and show a Z score of 60.8 and an RMS deviation of 0.817 Å for 403 C-\( \alpha \) atoms. A graphic display of the superimposed tomato and apple ACS structures revealed very similar conformations of PLP and the active site residues. Fold type I of PLP enzymes has been divided further into subgroups Ia and Ib (31). Enzymes in subgroup Ia but not Ib show large conformational changes upon the binding of substrate or inhibitors (10, 22, 32). Tomato ACS superimposes on subgroup Ib better than Ia. For example, the superposition of tomato ACS on *Thermus thermophilus* AAT (33) of subgroup Ib showed a Z score of 37.9 and an RMS deviation of 2.4 Å for 350 of 380 total residues, whereas the superposition on pig AAT in subgroup Ia has a Z score of 15.4 and an RMS deviation of 3.5 Å for 274 of 413 total residues. Thus, tomato ACS apparently belongs to subgroup Ib, in consideration of the good superposition and the absence of conformational change upon inhibitor binding.

On the other hand, the program Dali surprisingly detected a common fold between ACS in fold type I and mouse ornithine decarboxylase (34) in fold type III, showing a Z score of 2.2 for 135 amino acids in comparison. Superposition of the common fragment of ACS on that of mouse ornithine decarboxylase with the CCP4 program LSQKAB yielded an RMS deviation of 2.2 Å for the backbone atoms of 63 residues (Table II, Fig. 3A). This structural homology encouraged us to reexamine the structural homology of fold type I to other fold types of PLP enzymes. Indeed, a manual superposition identified a common folding motif between ACS in fold type I and tryptophan synthase \( \beta \) subunit (TSB, Refs. 35 and 36) in fold type II. Superposition of the common motif of ACS on that of the TSB subunit by the program LSQKAB resulted in an RMS deviation of 2.2 Å for the backbone atoms of 58 residues in comparison (Table II and Fig. 3B). Because the common motifs identified above also exist in the structures of other members of fold types I, II, and III, the homology suggests that the PLP-dependent enzymes evolved divergently from a common origin. Based on the fact that the common structural motif in ACS and mouse ornithine decarboxylase contains significant numbers of residues for PLP and

![Fig. 4. Schematic presentation of PLP and AVG binding at the active site. The dotted lines represent the hydrogen bonds. In three-dimensions, Tyr\textsuperscript{152} stacks in a parallel manner over the pyridine ring of PLP, and the \( \alpha \)-amino of AVG is \( \sim 4 \) Å away from the C4’ of PLP.](http://www.jbc.org/content/journals/10.1074/jbc.382130201)
substrate binding, we argue that this motif could serve as a core fold for the PLP-dependent enzymes. However, we note that the fragment of TSB barely comprises PLP binding residues. The explanation is unclear, but loss of the conservation could be the consequence of divergent evolution.

**Conserved Catalytic Mechanism of PLP-dependent Enzymes**—Despite the fact that PLP-dependent enzymes catalyze a wide variety of reactions with different specificities, their catalytic mechanisms have been considered to possess two common steps: an internal aldimine covalent intermediate with lysine and an external aldimine with substrate, followed by individual pathways dependent on the specificity of catalysis (10). PLP contains five binding components: C4′, N1, and O3 of pyridine, the stacking of pyridine ring, and the phosphoryl group. The lysine residue forming the internal aldimine with C4′ is absolutely conserved among all members of PLP-dependent enzymes. The structure-based sequence alignment shows good conservation on the residues for the formation of hydrogen bonds with N1 and O3 of pyridine and for hydrophobic stacking against the pyridine ring, suggesting an overall conservation of the catalytic mechanism of PLP-dependent enzymes.

On the other hand, the residues for the binding of the PLP phosphate moiety (Ala127, Thr228, Ser275, Ser277, and Arg286 in ACS) are poorly conserved, even for members of the AAT family that have very similar three-dimensional structures. Apparent, the PLP phosphate plays a role in the binding and orientation of the cofactor in the active site, as seen from its hydrogen-bonding network with protein residues in all the PLP enzymes. However, it is not clear whether the PLP phosphate contributes to the protonation/deprotonation status of the pyridine ring. If the phosphate is not involved directly in proton manipulation, we speculate that an artificial cofactor composed of a pyridine ring and a phosphate surrogate such as a carboxylate group might serve as a cofactor for the catalysis.

**Dissection of Roles of PLP-binding Residues**—In addition to the covalent bond with Lys278, PLP interacts with Ala127, Thr128, Tyr152, Asn209, Asp237, Tyr240, Ser275, Ser277, Lys278, and Arg286 of ACS (Fig. 4). The potential roles of these residues are discussed below.

Asp237 of tomato ACS forms a hydrogen bond with N1 of PLP (Fig. 4). This aspartate is well conserved among PLP-dependent enzymes except for serine in fold type II, glutamic acid in mouse ornithine decarboxylase of fold type III and members of fold type IV, and arginine in alanine racemase of fold type III. The function of this aspartate may stabilize the protonation status of the pyridine. For most members of the PLP-dependent enzymes, the pyridine nitrogen is probably protonated, and a general base such as a negatively charged residue or serine stabilizes the protonation state of the pyridine ring. An exception is Arg219 in alanine racemase, which forms a hydrogen bond with the pyridine nitrogen. It has been assumed that the pyridine nitrogen in alanine racemase is unprotonated (37). Thus, the sequence variation at this position probably implies a versatility of the detailed catalytic mechanisms of PLP-dependent enzymes.

Pyridine O3 of PLP forms two hydrogen bonds with Tyr240 and Asn209 (Fig. 4). Important roles for these residues have been implicated by mutagenesis, in which the Y233F mutant of apple ACS shows a 24-fold increase in K_m (27). The spatial proximity of the tyrosine to the substrate might imply its direct involvement in the protonation/deprotonation of substrate. For example, the corresponding residues Ser177 in phosphoserine aminotransferase (38) and His162 in 3-amino-5-hydroxybenzoic acid synthase (39) have been proposed to lower the pK_a of pyridine for the exchange of internal-external aldimines. Also, Tyr265 in alanine racemase has been proposed to act as a general base for nucleophilic attack on the α carbon of the substrate (37).

The apparent role of Tyr152 in catalysis is to stabilize the orientation of the pyridine ring of PLP in the active site by stacking. On the basis of this argument, it might be expected that any residues with an aromatic ring orient the pyridine ring of PLP appropriately. Indeed, the tyrosine can be replaced with tryptophan, phenylalanine, or histidine in many PLP-dependent enzymes. Another proposed role for the tyrosine is to increase the electron sink character of PLP via the π-electron interaction between the parallel rings (40). However, this proposal is weakened by the perpendicular orientation of the rings in 4-aminobutyrate aminotransferase (41), glutamate-1-semialdehyde aminotransferase (23), adenosylmethionine-8-aminooxononanooate aminotransferase (PDB entry 1dty), and 2,2-dialkyglycine decarboxylase (42). Last, an important role of the tyrosine or its correspondent may be its direct involvement in catalysis. For example, the tyrosine or its corresponding histidine abstracts a proton from the α-amino group for formation of the external aldimine in cystathionine β-lyase (Tyr111; Ref. 43), cystathionine γ-lyase (Tyr101; Ref. 40), ornithine decarboxylase (His233; Ref. 44), and cystine C-S lyase (His114; Ref. 45) or serves as an acid-base catalyst in NiFS-like protein (His95; Ref. 46). The neighboring of Tyr152 to AVG in the ACS-PLP-AVG structure implies a dual role for Tyr152, direct participation in catalysis (see below) and stacking stabilization of the pyridine ring orientation. Indeed, substitution of the tyrosine by asparagine in ACS resulted in a loss of 95–100% of enzymatic activity (47).

**Binding of AVG**—The competitive inhibitor AVG binds at a location neighboring PLP (Fig. 4). The α-amino group of AVG sits ~4 Å away from C4′ of pyridine, a promising position for the formation of a covalent external aldime with PLP. The α-carboxylate group of AVG forms three hydrogen bonds, respectively, with the backbone nitrogen of Ala54, the guanidine nitrogen of Arg412, and water. Arg412 is well conserved in PLP-dependent enzymes and has been proposed to bind the α-carboxylate group of substrate in ornithine aminotransferase (48), cystine C-S lyase (45), cystathionine β- and γ-lyases (40, 43), phosphoserine aminotransferase (38), and diaminopelargonic acid synthase (49). The critical role of Arg412 for substrate binding is supported by the observation that the R407K mutation of apple ACS increased the K_m value of SAM at least 20-fold and reduced k_cat/K_m by 4 orders (27). The amine group at the other end of AVG forms van der Waals contact with Tyr152. Overall, the orientation and interaction of AVG in ACS...
is similar to the binding of AVG in the structures of cystathionine \(\beta\)-lyase (43) and cystalysin (32) but does not form a covalent ketimine complex. Thus, the \(\alpha\)-amino and \(\alpha\)-carboxylate groups of AVG may simulate SAM binding in ACS.

Model of Substrate Binding and Putative Catalytic Mechanism—In general, the catalytic mechanism of a PLP enzyme comprises four essential stages: an internal aldime intermediate with enzyme lysine, an external aldime intermediate with substrate, substrate conversion (elimination, etc.), and transaldimation to release the product (7, 10). Although the two aldime intermediates have been well characterized for many enzymes, the details of substrate conversion remain to be illustrated. The protonation on the \(\alpha\) carbon of substrate may be stabilized efficiently by the quinonoid intermediate of pyridine conjugation among PLP enzymes. The concerted catalysis by PLP and a protein residue such as Tyr152 in ACS may represent a general mechanism accomplished corporately by both PLP and a protein residue.

Substrate SAM was modeled into the active site of ACS by simulating AVG binding. The \(\alpha\)-carboxylate group of SAM forms hydrogen bonds with Arg157 and the backbone nitrogen of Ala254, whereas the \(\alpha\)-amino group points to C4 for the formation of the external aldime (Fig. 5). The O2 and O3 atoms of the sugar of SAM form hydrogen bonds with the guanidine group of Arg157. The adenine ring of SAM is located in a hydrophobic pocket contributed by Pro206, Tyr277, Phe28, and Pro153. An important finding in the modeling is that the OH group of Tyr152 is positioned \(-3.7\) and \(3.5\) \(\text{Å}\) from C-\(\gamma\) and S of SAM. This distance may be close enough for Tyr152 to perform a nucleophilic attack on C-\(\gamma\) of SAM for the elimination. Our model is supported by the Y152N and R157G mutations that resulted in a loss of \(>95\%\) enzyme activity (47).

A putative catalytic mechanism is proposed on the basis of the above model (Fig. 6). Arg412 brings substrate SAM to the position neighboring the PLP-lysine internal aldime, and Tyr278 attracts the proton of Lys278 to promote the formation of the external aldime. Next, Tyr152 performs a nucleophilic attack on C-\(\gamma\) of SAM to break the C-\(\gamma\)-S bond, while Arg157 and Arg215 are holding SAM for the cleavage. A covalent intermediate may form between Tyr152 and C-\(\gamma\) via a mechanism similar to transmethylation reactions (50) and is then converted to ACC-aldime, a key intermediate proposed by several groups (15, 51, 52). Last, unprotonated Lys278 attacks the C4’ of PLP to release ACC (Fig. 6). Overall, our mechanism is consistent with the general scheme of the PLP-dependent catalysis (10). However, a potential nucleophilic attack by Tyr152 on the C-\(\gamma\) of SAM for the \(\gamma\) elimination is apparently unique among PLP enzymes. The concerted catalysis by PLP and a protein residue such as Tyr152 in ACS may represent a general scheme for the diversity of PLP-dependent catalyses.

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Tomato ACC Synthase Structure

Insight into Catalytic Mechanisms

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