Apple 1-Aminocyclopropane-1-carboxylate Synthase in Complex with the Inhibitor L-Aminoethoxyvinylglycine

EVIDENCE FOR A KETIMINE INTERMEDIATE*

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The 1.6-Å crystal structure of the covalent ketimine complex of apple 1-aminocyclopropane-1-carboxylate (ACC) synthase with the potent inhibitor L-aminoethoxyvinylglycine (AVG) is described. ACC synthase catalyzes the committed step in the biosynthesis of ethylene, a plant hormone that is responsible for the initiation of fruit ripening and for regulating many other developmental processes. AVG is widely used in plant physiology studies to inhibit the activity of ACC synthase. The structural assignment is supported by the fact that the complex absorbs maximally at 341 nm. These results are not in accord with the recently reported crystal structure of the tomato ACC synthase AVG complex, which claims that the inhibitor only associates noncovalently. The rate constant for the association of AVG with apple ACC synthase was determined by stopped-flow spectrophotometry (2.1 × 10^6 M^-1 s^-1) and by the rate of loss of enzyme activity (1.1 × 10^5 M^-1 s^-1). The dissociation rate constant determined by activity recovery is 2.4 × 10^-4 s^-1. Thus, the calculated K_d value is 10–20 μM.

The plant hormone ethylene, which regulates many aspects of plant growth, development, and senescence is synthesized in the Yang cycle (1). The biosynthetic precursor of ethylene, 1-aminocyclopropane-1-carboxylate (ACC), is produced by a pyridoxal phosphate (PLP)-dependent enzyme, ACC synthase (S-adenosyl-L-methionine methylthioadenosine lyase, EC 4.4.1.14). This enzyme catalyzes the α,γ-elimination of methylthioadenosine (MTA) from S-adenosyl-L-methionine (SAM) to produce ACC (Scheme 1). The production of ACC from SAM is the committed and rate-determining step in ethylene biosynthesis; therefore, the reaction and inhibition mechanisms of ACC synthase attract considerable interest for agricultural applications. Among the most potent inhibitors of ACC synthase is L-aminoethoxyvinylglycine (AVG) (2), which is widely used for plant physiology studies and agricultural applications. The structure of unliganded ACC synthase from Malus domestica to 2.4-Å resolution is available (3). Here we describe the structure of the complex of apple ACC synthase with AVG at 1.6-Å resolution together with the rate constants characterizing its formation and decomposition. The structure results disagree with those reported by Huai et al. (4) at lower resolution (2.7 Å) on the tomato ACC synthase AVG complex.

EXPERIMENTAL PROCEDURES

Protein Purification and Crystallization—WT and E47Q ACC synthase were purified for biochemical studies as described previously (5). Recombinant V45STOP apple ACC synthase was prepared as described previously (6) and crystallized at 293 K by the vapor diffusion method (sitting drop). 1 μl of protein solution containing 15 mg/ml enzyme, 50 mM HEPES, pH 7.9, 10 μM PLP, 1 mM DTT, and 4 mM AVG was mixed with an equal amount of reservoir containing 30% 2-methyl-2,4-pentanediol (MPD) (v/v) and 50 mM MES, pH 6.5.

The space group of the crystals is C2 with cell parameters a = 103.3 Å, b = 61.1 Å, c = 77.1 Å, and β = 123.4°. The asymmetric unit contains a monomer of ACC synthase, corresponding to a solvent content of 38%.

Data Collection and Processing—The data to 1.6 Å were collected at 100 K using a MAR Research (Hamburg, Germany) imaging plate at the Swiss-Norwegian Beamline at the European Synchrotron Radiation Facility (Grenoble, Switzerland). Data were reduced with DENZO and other programs of the CCP4 suite (7).

Structure Solution and Refinement—The structure was solved by molecular replacement with AMoRe (8) using one subunit from the native apple ACC synthase structure (Protein Data Bank code 1BSG) (3). The structure was refined with X-PLOR and CNS (9), alternating cycles of torsion angle-simulated annealing and individual B-factor refinement with sessions of manual rebuilding with O (10). The data between 20 and 1.6 Å were used. No σ-cutoff was applied. A mFo – DF map calculated after the first round of simulated annealing and thermal refinement exhibited clear difference density corresponding to a covalent adduct between the PLP cofactor and AVG (Fig. 1). A model for the adduct (residue code PPG) was built into the density and included in the refinement. Subsequently, 332 water molecules and a molecule of MPD were added to the model. Refinement was performed with X-PLOR 3.85 with topology and parameter files TOPP/CHSDX.PRO and PARHCSDX.PRO (11).

The final model was validated by WHAT_CHECK (12) and Procheck (13). The final refinement statistics are given in Table I. The atomic coordinates have been deposited with the Protein Data Bank with entry code 1MTY.

Spectroscopic Characterization of the AVG-PLP Complex—Steady-state absorbance spectra were recorded with an Agilent Technologies 8453 UV-visible diode array spectrophotometer. Absorbance values at 421 and 341 nm (the PLP-aldimine and ketimine species, respectively) were measured during the course of reactivation of AVG-inactivated ACC synthase using changes in protein concentration over time by normalization of the absorbance of each sample at 280 nm.

Rapid reaction studies monitoring the rate of formation of the PLP-aldimine adduct by normalization of the absorbance at 280 nm.

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‡ The abbreviations used are: ACC, 1-aminocyclopropane-1-carboxylate; AVG, L-aminoethoxyvinylglycine; CBL, cystathionine β-lyase; DTT, dithiothreitol; MPD, 2-methyl-2,4-pentanediol; MTA, 5′-methylthioadenosine; PLP, pyridoxal 5′-phosphate; SAM, S-adenosyl-L-methionine; TAPS, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]aminol-1-propanesulfonic acid; WT, wild-type; MES, 4-morpholineethanesulfonic acid; MOPS, 4-morpholinopropanesulfonic acid.
ketoamine species upon incubation of WT and E47Q ACC synthase with AVG were carried out with a temperature-controlled Applied Photo-Physics model SF.17MV stopped-flow spectrophotometer. Ketimine formation was monitored at 341 nm, and data were fit to first-order kinetics.

Kinetic Characterization of ACC Synthase Inactivation by AVG—ACC synthase activity in the presence of AVG was monitored by a continuous coupled assay (14). Reactions were initiated by the addition of AVG synthase to mixtures of AVG, SAM, and coupling enzyme preequilibrated in buffer at 25 °C. Resulting reaction progress curves were fit to Equation 1 (15),

$$P = v_f + (v_o - v_f) \left(1 - e^{-kt}\right)$$  

(Eq 1)

where $P$ is the product concentration, $v_o$ and $v_f$ are the steady-state and initial reaction velocities, respectively, and $k_{obs}$ is the observed rate constant for inactivation. The rate constant for association of the enzyme with AVG was determined from a plot of $k_{obs}$ versus inhibitor concentration.

RESULTS

Structure Determination—The structure of V435 STOP apple ACC synthase, cocryocystallized in the space group C2 with AVG, was determined by molecular replacement with the unliganded enzyme structure as search model. The crystals contain one monomer of the ACC synthase dimer per asymmetric unit with the two subunits related by a crystallographic 2-fold axis. The structure was refined to 1.6-Å resolution and an R-factor of 20.5% (R-free 21.9%) with excellent stereochemical parameters (Table I). Difference Fourier map analysis from the first step of refinement revealed that AVG is bound covalently to PLP (Fig. 1). Lys-273 is clearly displaced from the cofactor. The distance between the C4A atom of PLP and the side-chain amino group of the lysine is 3.7 Å. The final refined model for the apple ACC synthase-AVG complex encompasses 424 residues, the covalent adduct AVG, was determined by molecular replacement with the unliganded enzyme structure as search model. They also solved the structure of an ACC synthase-AVG complex cocrystallized in the space group P6422 with 332 water molecules. The geometry of the refined AVG-PLP is that of a ketimine adduct with the AVG C\text{15} atom and the C4A atom of PLP exhibiting sp\text{2} and sp\text{3} hybridization, respectively.

Comparison with PDB Entry 1IAY: Contrasting Results—The present results differ significantly from those recently published by Huai et al. (4) describing a complex between tomato ACC synthase and AVG. Huai et al. (4) solved the structure of unliganded tomato ACC synthase in the space group P6\text{3}22 by molecular replacement using the coordinates of the apple ACC synthase structure (PDB code 1B8G) (3) to build the search model. They also solved the structure of an ACC synthase-AVG complex cocryocystallized in the space group P6\text{3}22 with 1B8G as a search model (4). Huai et al. (4) report that final

![Fig. 1](image1.png)

**Fig. 1.** A, mF\text{o} - DF, electron density map of the AVG-PLP complex of ACC synthase calculated after a first cycle of refinement, contoured at 3.0 $\sigma$, and prepared with program O (22). B, 2mF\text{o} - DF, final electron density map of the AVG-PLP complex, contoured at 1.0 $\sigma$, and superimposed onto the final refined model. This illustration was prepared with program DINO (www.dino3d.org).

![Fig. 2](image2.png)

**Fig. 2.** Formation of a 341-nm absorbing species following addition of AVG to WT ACC synthase. ACC synthase (3 $\mu$M) was added to 3.9 $\mu$M AVG in pH 6.3 buffer containing 62.5 mM MES, 62.5 mM MOPS, 125 mM 4-hydroxy-N-methylpiperidine, and 10% glycerol at 25 °C. Spectra were recorded every 60 s. The experiment was carried out at low pH in to slow the rate of conversion of the PLP cofactor from its aldimine to ketimine form.

![Table 1](table1.png)

**Table I** Data collection and refinement statistics

| Parameter | Overall | Outermost shell |
|-----------|---------|-----------------|
| Resolution range | 15–1.6 | 1.64–1.60 |
| Unique reflections | 50,540 | |
| Multiplicity | 2.8 | |
| Completeness (%) | 95.2 | 76.0 |
| $R_{sym}$ (%)$^a$ | 5.2 | 28.1 |
| Resolution range used in refinement | 15–1.6 | 1.66–1.60 |
| $R_{cryst}$ (%)$^b$ | 0.205 | 0.277 |
| $R_{free}$ (5% of the data) | 0.219 | 0.317 |
| Number of protein atoms | 3367 | |
| Number of water molecules | 332 | |
| Mean overall B-factor (Å$^2$) | 23.5 | |
| Ramachandran plot regions (%) | |
| Most favored | 89.2 | |
| Additionally allowed | 10.3 | |
| Generously allowed | 0.5 | |

$^a$Average over 1 and 2 data sets, excluding the outermost shell.

$^b$Average over 1 and 2 data sets, including the outermost shell.
refined model (PDB code 1IAY) of that complex contains no covalent adduct between the cofactor and the inhibitor. They find that AVG is bound near the entrance of the active site with its \(-\text{amino group} \) from the PLP C4A. In 1IAY, AVG binds to the protein through its \(-\text{carboxylate group}, \) which accepts two hydrogen bonds, one from the backbone nitrogen of Ala-54 and the other from a guanidinium nitrogen of Arg-412. A third hydrogen bond is donated to the \(-\text{carboxylate of AVG} \) by a water molecule. This mode of AVG binding, involving a few weak interactions, cannot be reconciled with our crystallographic results. Our refinement strategy was aimed at avoiding model bias; thus, neither cofactor nor AVG was included in the first steps of refinement against the 1.6-Å diffraction data. Simulated annealing at 5000 K ensured additionally that residual model bias from the internal aldimine of 1B8G was removed. A difference Fourier map after one cycle of individual B-factor refinement contoured at 3 \(\times \) exhibited a very clear and strong signal corresponding to the AVG-PLP covalent adduct shown in Fig. 1. Clausen et al. (16) and Krupka et al. (17) also find covalent AVG-PLP adducts in Escherichia coli cystathionine-\(-\text{lyase \( (\text{CBL}) \) and in Treponema denticola \((\text{PDB codes 1CL2 and 1C7O) \), respectively.}

\text{Biochemical Evidence for a Ketimine AVG-PLP Adduct—}\)

Incubation of ACC synthase with AVG results in the rapid disappearance of the PLP internal aldimine absorption band at 421 nm with concomitant appearance of an enzyme species that absorbs at 341 nm (Fig. 2). The extinction coefficient for the latter is \(34,600 \text{ M}^{-1} \text{ cm}^{-1} \) (18). An AVG-PLP ketimine species (Scheme 1), however, would contain an addi-
Comparison of Unliganded and AVG-inhibited ACC Synthase—Superposition of the unliganded (PDB code 1B8G) and AVG-inhibited ACC synthase structures shows that association of AVG with the enzyme does not induce large conformational changes (Fig. 6C). The root mean square deviation between the two structures is 0.7 Å. The only large-scale differences are found in the small domain. 1) A part of the small domain encompassing mainly helix α1, strand β2, helix α13, and strand β13 (nomenclature is according to Ref. 3) is slightly displaced toward the large domain of the other subunit. The program DYNDOM identifies this conformational change as a rotation of the aforementioned “subdomain” by ~7° around an axis that crosses the small domain and is approximately parallel to the dimer axis. However, whether one considers the small domain as a whole, no overall domain movement can be identified by visual inspection or by DYNDOM (19). 2) The main chain conformation at the beginning of helix α1 is rearranged so that Tyr-19 points inside the active site in the AVG-bound structure (see following paragraph).

Interactions of the Ketimine Adduct with the Protein—The AVG-PLP adduct is tightly bound in the active site of ACC synthase (Kd ~10–20 µM based on the ratio of the kobs and kL values described above). Fig. 7 is a LIGPLOT scheme (20) that shows all hydrogen-bonding interactions that contribute to AVG association, and Fig. 6A presents a stereoview of the active site. The PLP moiety of the adduct retains the strong interactions between the phosphate group and the enzyme side chains that are present in the unliganded structure (PDB code 1B8G) (3). The position of the phosphate is only slightly modified with respect to 1B8G, whereas the pyridine ring of the cofactor appears to be tilted by ~16°. Thus, the phosphate moiety acts as an anchor. It is noteworthy that the formation of the external aldimine or ketimine not only tilts the cofactor but pushes the ring 1.1 Å down toward the carboxylate of Asp-230 to form a relatively strong hydrogen bond (2.7 Å) in the complex. These crystallographic results confirm the theoretical model of a reaction intermediate of ACC synthase (external aldimine with ACC) described previously (3), which predicted that such a hydrogen bond would be established upon external aldimine formation.

The carboxylate group of the AVG-PLP adduct interacts
strongly with the protein. It forms a doubly hydrogen-bonded salt bridge with Arg-407 and accepts a hydrogen bond from the carboxamide nitrogen of Asn-202. Notably, the AVG-PLP α-carboxylate receives a fourth hydrogen bond from the OH of Tyr-19. The side chain of Tyr-19 points toward the solvent in the unliganded ACC synthase structure, whereas in the ACC synthase-AVG complex there is a rearrangement involving residues 18–20 that results in the phenolic ring of Tyr-19, reaching the inner active site to interact with the AVG-PLP α-carboxylate group.

The ε-amino group of the AVG side chain in the complex makes a triple electrostatic interaction with Glu-47 and Lys-273. The side chain of Glu-47 is found in a different orientation with respect to the unliganded enzyme, and its γ-carboxylate is the pivot for the formation of this interaction. The side chain amino groups of both AVG and Lys-273 donate hydrogen bonds to the Glu-47 γ-carboxylate, which also receives another hydrogen bond from the carboxamide nitrogen of Gln-83 of the other subunit (Gln-83*). A comparison of the AVG-bound ACC synthase and CBL active sites (Fig. 6B) (16) provides a structural

Fig. 6. A, superposition of the active sites of unliganded (sea green) and AVG-bound (yellow) ACC synthase. Only selected residues are shown. B, the active sites of AVG-bound ACC synthase (gray) and cystathionine β-lyase (sea green). C, the Ca traces of unliganded (yellow) and AVG-bound (blue) ACC synthase. This illustration was prepared with DINO.
basis for the different AVG affinities of the two enzymes ($K_i = 1.1 \mu M$ for CBL; $K_d = 10-20 \mu M$ for ACC synthase). The two AVG-PLP adducts superimpose very well; however, the side chain of AVG-PLP interacts with CBL much more weakly than with ACC synthase because the AVG side chain in the CBL-AVG complex points toward the active site entrance and its amino group is held in place mainly by weaker interactions with the hydroxyl group of Tyr-111 and with two water molecules.

A molecule of the precipitating agent MPD is found at the entrance of the active site of AVG-bound ACC synthase. Interestingly, the MPD association is stereoselective. Racemic MPD was used for crystallization, but only the $R$-enantiomer is found in the present structure. The two OH groups of $R$-MPD are the points of contact. One (O4 in the crystallographic atom naming) accepts a hydrogen bond from the guanidinium group of Arg-150, whereas the second (O2) is involved as donor in a bifurcated hydrogen bond with the carboxamide oxygen atom of Gln-83* and the ether atom of AVG.

**Kinetics Describing the Association of AVG with E47Q ACC Synthase**—The addition of AVG to E47Q ACC synthase results in the formation of an AVG-ketimine adduct. The kinetics of this process were studied using stopped-flow spectrophotometry. The absorbancies of the enzyme at 341 nm (0.067 absorbance units) and 421 nm (0.074 absorbance units) prior to the addition of AVG are indicated on the ordinate.

**Table II**

| Enzyme            | $k_a$ | $k_d$ | $K_d$ (in M) |
|-------------------|-------|-------|--------------|
| WT ACC synthase   | $2,100\text{ s}^{-1}$ | $0.24\text{ s}^{-1}$ | $0.011\text{ M}$ |
| E47Q ACC synthase | $720\text{ s}^{-1}$ | $8.2\text{ s}^{-1}$ | $1.1\text{ M}$   |
| CBL               | $1.5\text{ s}^{-1}$ | $\sim 4\text{ s}^{-1}$ | $\sim 300\text{ M}$ |

*a* Reported $k_a$ values were calculated from stopped-flow spectrophotometric measurements of ketimine formation rates. $k_d$ values were obtained by monitoring recovery of the internal aldimine absorbance band following removal of AVG from reaction mixtures. CBL data are from Clausen et al. (16).
in a rapid loss of the PLP internal aldimine absorption band at 421 nm, and concomitant appearance of an absorption band at 341 nm with an extinction coefficient of $-35,200 \text{ M}^{-1}\text{cm}^{-1}$ (data not shown). The rate constants for appearance of the absorption band at 341 nm were measured as a function of AVG concentration, yielding a second-order rate constant of $7.2 (\pm 0.7) \times 10^{8} \text{ M}^{-1}\text{s}^{-1}$ for that reaction (see Fig. 3).  

The association of AVG with E47Q ACC synthase is irreversible (Fig. 8). Dialysis of AVG-bound E47Q ACC synthase against buffer containing 1 \u03bcM PLP results in a loss of the ketimine absorption band at 341 nm and recovery of the internal aldimine absorption band at 421 nm with a rate constant of $8.2 (\pm 1.7) \times 10^{-5} \text{ s}^{-1}$. The rate at which catalytic activity is recovered could not be determined because of the extremely low activity of the E47Q mutant for $\alpha_7$-elimination on SAM (5). 

**DISCUSSION**

The AVG-ACC Synthase Complex Is a Ketimine—We present crystallographic and biochemical evidence that AVG inhibits ACC synthase from *M. domestica* by forming a covalent ketimine complex with the PLP cofactor. The high resolution crystallographic evidence supported by spectroscopic and kinetic analyses cannot be reconciled with the findings of Huai et al. (4) for the complex of *Lycopersicum esculentum* ACC synthase with the same inhibitor (PDB code 1IAY). That report claims that a noncovalent complex is formed between AVG and the holoenzyme and that it is stabilized only by two hydrogen bonds. An examination of electron density for 1IAY as calculated by the Uppsala University Electron Density Server (portray.bmc.uu.se/eds/index.html) shows that the AVG ligand is associated with the density only in its carboxylate moiety (assuming that the structure factor file deposited for 1IAY and used by the server corresponds to the ACC synthase-ACC complex). Our estimated $K_d$ for apple ACC synthase-ACC is 10–20 pM. A weak non-covalent enzyme-inhibitor interaction as proposed by Huai et al. (4) would suggest a much higher $K_d$ value. Finally, the 341-nm absorption maximum is incompatible with a conjugated aldimine. The identity level between the *M. domestica* and the *L. esculentum* (LE-ACS 2) sequences is very high (56% with 67% similarity), and all of the residues that could be involved in binding are conserved; thus, the very different structural results with AVG cannot be ascribed to differences between the tomato and the apple enzyme. In our structure, the very strong affinity of AVG toward ACC synthase for crystallization experiments and Andrew Eliot for carrying out the stopped-flow experiment described in Fig. 3. We also thank Liang Feng for preparing V435STOP ACC synthase complex than it is for the corresponding WT ACC synthase complex. Although this decrease in affinity is significant, the $K_d$ for the E47Q-ACC synthase complex remains 300-fold less than that of the WT CBL complex which also lacks the Glu-47-AVG interaction, thus suggesting that other protein-inhibitor interactions such as the hydrogen bond between Tyr-19 and the $\alpha$-carboxylate group are also important for the tight binding of AVG to ACC synthase. The fact that the E47Q mutation elicits such a large reduction (−3 kcal/mol) in affinity of the enzyme for AVG supports the earlier conclusion that the carboxylate group of Glu-47 plays an important role in the recognition of the sulfonium group of the natural substrate SAM as suggested by mutational, kinetic, and modeling studies (5).

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2 The $K_d$ value of $\sim300 \text{ nm}$ for CBL was determined from the ratio of the spectrophotometrically determined dissociation and association rate constants reported by Clausen et al. (16) and differs slightly from the $K_d$ value (1.1 \u03bcM) determined by kinetic analysis of activity inhibition and reported in the same paper.
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