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Structure of ACC synthase inactivated by the mechanism-based inhibitor L-vinylglycine

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Abstract L-Vinylglycine (L-VG) is both a substrate for and a mechanism-based inhibitor of 1-aminocyclopropane-1-carboxylate (ACC) synthase. The ratio of the rate constants for catalytic conversion to α-ketobutyrate and ammonia to inactivation is 500/1. The crystal structure of the covalent adduct of the inactivated enzyme was determined at 2.25 Å resolution. The active site contains an external aldimine of the adduct of L-VG with the pyridoxal 5′-phosphate cofactor. The side chain γ-carbon of L-VG is covalently bound to the ε-amino group of Lys273. This species corresponds to one of the two alternatives proposed by Feng and Kirsch [Feng, L. and Kirsch, J.F. (2000) L-Vinylglycine is an alternative substrate as well as a mechanism-based inhibitor of 1-aminocyclopropane-1-carboxylate synthase. Biochemistry 39, 2436–2444] and presumably results from Michael addition to a vinylglycine ketimine intermediate.
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1. Introduction

1-Aminocyclopropane-1-carboxylate (ACC) synthase (EC 4.4.1.14) catalyses the rate-determining step in the biosynthesis of ethylene [1], a plant hormone that promotes fruit ripening, and is involved in a number of other developmental processes, including senescence and wound healing [2]. This dimeric enzyme, which converts (*S, S*)-*S*-adenosyl-L-methionine (SAM) to ACC, utilizes pyridoxal 5′-phosphate (PLP) as a cofactor and belongs to fold type I PLP-dependent enzymes. ACC is subsequently oxidized to ethylene in a fast step by ACC oxidase [1]. Three crystal structures of *Malus domestica* (apple) ACC synthase are available, that of the unliganded enzyme [3] and those of the complexes with the potent inhibitor L-aminoethoxyvinylglycine (AVG, Fig. 1) [4] and an aminooxy analogue of the substrate (AMA, Fig. 1) [5]. An unliganded and an AVG-inhibited structure of *Lycopersicum esculentum* (tomato) ACC synthase have also been published [6]. These structures helped to prepare a plausible model for the complex with the natural substrate, SAM [7]. ACC synthase also catalyses the conversion of L-vinylglycine (L-VG) to α-ketobutyrate and ammonia, with a k_{cat} value of 1.8 s^{-1} and a K_m value of 1.4 mM [8]. The resulting k_{cat}/K_m value is only 600-fold less than that for the diffusion-controlled reaction with SAM. L-VG is additionally a mechanism-based inhibitor of the enzyme with one inactivation event occurring per 500 catalytic turnovers. The inactivated form of the enzyme is a covalent adduct of Lys273 to the L-VG external aldimine [8,9]. The role of L-VG as an inhibitor of ACC synthase was discovered [10] long before it was realized that it is an alternative substrate of the enzyme. The covalent adduct of the L-VG external aldimine was characterized spectroscopically as a species with an absorption spectrum similar to that of the WT enzyme and a λ_{max} red-shifted by 10 nm to 432 nm. Spectroscopic data and biochemical considerations could not, however, determine if the covalently inactivated enzyme resulted from attack of Lys273 to the β- or to the γ-carbon of the cofactor-bound L-VG [8]. To resolve this ambiguity, we determined the crystal structure of the inactivated complex.

2. Materials and methods

2.1. Crystallization and data collection

V435STOP ACC synthase (ACS-1 from *M. domestica*), a truncated form of the enzyme designed to eliminate C-terminal proteolysis, was prepared by recombinant expression in *Escherichia coli* following White et al. [11]. L-VG was purchased from Sigma. Preparation of inactivated ACC synthase was carried out at 4°C: 4 mg of L-VG (MW 101.1 Da) were added to 50 µl of ACC synthase solution (6.9 mg/ml, 0.14 mM, MW 49.1 kDa) containing 50 mM HEPES, pH 7.9, 10 µM PLP, 1 mM DTT. The L-VG concentration was therefore 790 mM and the molar L-VG/enzyme ratio was 5600. Cocrystals of the ACC synthase–L-VG complex were obtained by the sitting drop method: 1 µl of the protein L-VG solution was mixed with 1 µl of precipitating solution containing 17.5% (w/v) PEG4000, 4.4 mM $NiCl_2$ and 125 mM Tris, pH 7.5. The crystals belong to space group *C*2 with cell constants $a = 103.3$ Å, $b = 59.4$ Å, $c = 79.0$ Å, $\beta = 124.2°$. Data were collected at a wavelength of 0.934 Å on an ADSC Q4R detector at the ID14-1 beamline at the European Synchrotron Radiation Facility in Grenoble (France). The crystals were flash-cooled in a cold nitrogen stream at 100 K, after flushing them for a few seconds in a cryo-protecting solution obtained by adding 15% ethylene glycol to the reservoir solution. Data processing and reduction were performed

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Abbreviations: ACC, 1-aminocyclopropane-1-carboxylate; PLP, pyridoxal 5′-phosphate; L-VG, L-vinylglycine; AVG, L-aminoethoxyvinylglycine; SAM, *S*-adenosyl-L-methionine; AMA, [2-(aminooxy) ethyl](5′-deoxyadenosin-5′-yl)(methyl)sulphonium; MTA, 5′-methylthioadenosine

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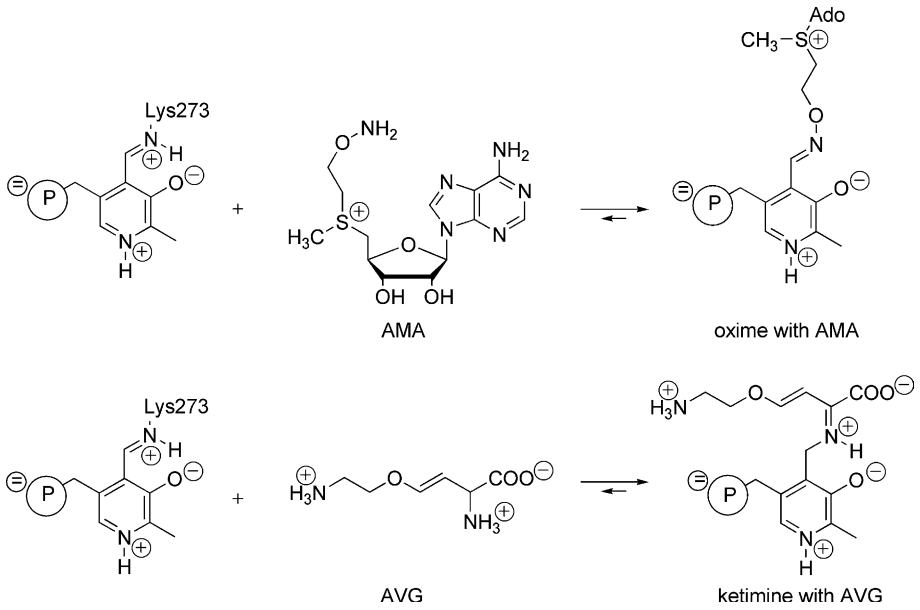

Fig. 1. Schematic summary of the reactions of ACC synthase with the inhibitors AVG and AMA. Prepared with ChemDraw.

with DENZO/SCALEPACK [12] and programs from the CCP4 suite [13]. Data processing statistics are shown in Table 1. The high-resolution cut-off for the data was set at 2.25 Å, using a completeness criterion (overall completeness better than 95% and completeness in the outer shell (2.33–2.25 Å) around 70%).

2.2. Refinement and validation

The coordinates of the 1.6 Å crystal structure of the ACC synthase–AVG complex [4] (PDB code 1M7Y), minus the cofactor, ligand, and side chain of Lys273, were used as a starting point for the refinement against the ACC synthase–L-VG data. Initial torsion angle simulated annealing was carried out at 5000 K to help to remove model bias from the structure. All refinement procedures were carried out with CNS [14]. Various refinement cycles, including low-temperature simulated annealing, positional and isotropic B-factor refinement were alternated with manual rebuilding using the program O [15]. Clear 2m*F*o-D*F*c electron density appeared for the PLP–L-VG adduct after the first refinement cycle. The covalent connection to the amino group of Lys273 side chain was also apparent. The PLP–L-VG adduct was manually positioned in the density and appropriate restraints were introduced for a covalent bond between the γ-carbon of L-VG and the side chain nitrogen of Lys273. Water molecules were added with an automatic search protocol in CNS, followed by manual verification in O. Refinement statistics are given in Table 1. The structure was validated using PROCHECK [16] and WHAT_CHECK [17]. Coordinates and structure factors have been deposited with the Protein Data Bank (PDB code 1YNU).

Table 1
Data collection and refinement statistics for the ACC synthase–L-VG complex

Resolution limits (Å)	30.0–2.25 (2.33–2.25)
Unique reflections	18 038
Redundancy	3.3
Completeness (%)	95.5 (69.7)
Average $I/\sigma(I)$	11.6 (3.2)
$R_{sym}(I)$ (%)	6.3 (27.7)
R_{factor} (%)	23.4 (30.2)
R_{free}[a] (%)	29.0 (34.9)
Number of atoms	3455
rms deviation from ideal geometry	
R_{msd} bonds (Å)	0.007
R_{msd} angles (°)	1.2
Ramachandran plot distribution (%)	
Most favoured	89.4
Additionally allowed	10.6
Wilson B-factor (Å^2)	42.4
Average model B-factor (Å^2)	42.1

[a]Test set: 2.8% of the reflections.

3. Results and discussion

3.1. L-VG functions both as an inhibitor of PLP-dependent enzymes and as a substrate of ACC synthase

L-VG was isolated 30 years ago from the carpophores of the fungus *Rhodophyllus nidorosus* [18] and chemically synthesized in the same year [19]. It was shown to be a mechanism-based inhibitor of aspartate aminotransferase [20] and of kynurenine aminotransferase [21]. Satoh and Yang [10] described L-VG as a mechanism-based inhibitor of ACC synthase. They also proposed that the mechanism-based inhibition of ACC synthase by SAM may proceed through β,γ-elimination of SAM leading to the formation of L-VG, which in turn would inhibit the enzyme. Recently, McCarthy et al. [7] carried out a complete kinetic study of ACC synthase, leading to a more complex picture. By using enantiomerically pure (*S, S*)- and (*R, S*)-SAM as substrates, they showed that (*R, S*)-SAM can undergo the same α,γ-elimination as the (*S, S*)-diastereomer, but it more often eliminates the same leaving group in a β, γ-process to produce a vinylglycine-related intermediate (a quinonoid). The ratio of rate constants for α,γ- and β,γ-elimination ($k_{\alpha,\gamma}/k_{\beta,\gamma}$) is ~60 for (*S, S*)-SAM [22] but only 0.4 for (*R, S*)-SAM [7]. Feng and Kirsch [8] had previously reported that L-VG is a substrate for ACC synthase, which converts it to α-ketobutyrate and ammonia. Once every 500 catalytic cycles, however, L-VG acts as a mechanism-based inhibitor of the enzyme, leading to the formation of a covalent adduct of Lys273 with the L-VG external aldimine. They suggested that the ε-amino moiety of the base responsible for the abstraction of the C_α-proton of SAM makes a nucleophilic attack at either the β- or the γ-carbon of the cofactor-bound L-VG to yield a species absorbing maximally at 432 nm (species B and A, respectively, in Fig. 2). In

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