Review

Structure, catalytic activity and evolutionary relationships of 1-aminocyclopropane-1-carboxylate synthase, the key enzyme of ethylene synthesis in higher plants

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Both ethylene and the enzymes of ethylene synthesis are subjects of intensive scientific investigation. The present review discusses structure, catalytic activity and evolutionary relationships of 1-aminocyclopropane-1-carboxylate synthase, identified for the first time in ripening tomato in 1979. This enzyme is responsible for the conversion of S-adenosyl-L-methionine to 1-aminocyclopropane-1-carboxylic acid, which is the key step of ethylene synthesis in higher plants. The role of this enzyme (especially in the fruit ripening) was demonstrated in 1991 in transgenic tomato plants, expressing 1-aminocyclopropane-1-carboxylate synthase antisense RNA. On the basis of mutagenesis and crystallization of the enzyme, new data were provided on the three-dimensional structure and amino-acid residues which are critical for catalysis. The control of ethylene production is of great interest for plant biotechnology because it can delay senescence and overmaturation. These processes are responsible for large loss of vegetables and fruit on storage. Detailed structural and biochemical data are necessary to help design 1-aminocyclopropane-1-carboxylate synthase inhibitors, whose application is expected to have immense agricultural effects.

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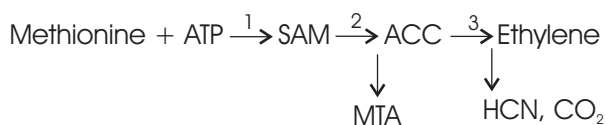
Ethylene, the simplest among the well-characterized plant hormones, occurs in the gas state under normal physiological conditions. Its production ranges from almost 0 up to 500 nl/g of plant tissue per h but its biological activity is observed even when its concentration is very low (as little as 10–100 nl/l of air) (Theologis, 1992). For example, ethylene effects include promotion of fruit ripening, flower senescence, petal and leaf abscission, inhibition of seedling elongation, and stimulation of root initiation (Lincoln *et al.*, 1993; Abel *et al.*, 1995; Clark *et al.*, 1997; Kamachi *et al.*, 1997; Trebish *et al.*, 1997; Nakatsuka *et al.*, 1998; Barry *et al.*, 2000; Ishiki *et al.*, 2000; Llop-Tous *et al.*, 2000). Ethylene production in plant tissues in response to biotic and abiotic stress (wounding, cold, anaerobiosis, Cd, ozon, pathogens, etc.) is a signalling mechanism of profound physiological consequences (Spanu *et al.*, 1993; Avni *et al.*, 1994; Olson *et al.*, 1995; Lelievre *et al.*, 1997; Schlagnhaufer *et al.*, 1997; Tuomainen *et al.*, 1997; Zarembinski & Theologis, 1997; Kathiseran *et al.*, 1998; Arteca & Arteca, 1999; Tatsuki & Mori, 1999; Kato *et al.*, 2000).

The ethylene biosynthetic pathway of higher plants involves the following steps:

1) Methionine adenosyltransferase (ATP:methionine *S*-adenosyltransferase, EC 2.5.1.6) catalyzes the conversion of methionine into *S*-adenosylmethionine (SAM), reaction 1;

2) 1-Aminocyclopropane-1-carboxylate synthase (*S*-adenosyl-L-methionine methylthioadenosine lyase, EC 4.4.1.14) catalyzes the synthesis of 1-aminocyclopropane-1-carboxylic acid (ACC) from SAM, reaction 2;

3) ACC is converted to ethylene by ACC oxidase, reaction 3 (Yang & Hoffman, 1984; Kende, 1993; Zarembinski & Theologis, 1994).

$$\text{Methionine + ATP} \xrightarrow{1} \text{SAM} \xrightarrow{2} \text{ACC} \xrightarrow{3} \text{Ethylene}$$
$$\downarrow \qquad\qquad \downarrow$$
$$\text{MTA} \qquad \text{HCN, CO}_2$$

ACC synthase is the key regulatory enzyme in ethylene synthesis in higher plants (step 2).

The regulatory effect of steps 1 and 3 is not so important because SAM is constantly synthesized and also utilized by other reactions, such as methylation and polyamine synthesis, whereas ACC oxidase activity is most often constitutive (Botha & Whitehead, 1992; Li *et al.*, 1992).

Besides ACC, ACC synthase produces 5′-methylthioadenosine (MTA). MTA is recycled into methionine by the Yang cycle (Yang & Hofman, 1984; Zarembinski & Theologis, 1994). This cycle allows high rates of ethylene production without high intracellular concentrations of methionine. It involves the following steps:

5′-methylthioadenosine (MTA) →

5′-methylthioribose (MTR) →

5′-methylthioribose-1-phosphate (MTR-1-P) →

2-keto-4-methylthiobutyrate (KMB) →

methionine

ACC may be also converted to 1-(malonyl-amino)cyclopropane-1-carboxylic acid (MACC) by ACC *N*-malonyltransferase. Malonylation of ACC can decrease free ACC level in plant tissues and is irreversible under physiological conditions. HCN is one of the products during the conversion of ACC to ethylene but higher plants possess an effective system of its detoxification (Yip & Yang, 1988).

Ethylene controls its own synthesis and in higher plants two systems of ethylene production operate. System 1 operates in all vegetative tissues and in the fruit before its ripening, system 2 during the ripening of climacteric fruit and during petal senescence. Ethylene shows auto-inhibitory effect in system 1 but auto-stimulatory effect in system 2 (Barry *et al.*, 2000).

Because of large loss of fruit, flowers and vegetables due to ethylene's effects on ripening and senescence, attempts have been made to prevent or delay fruit ripening in a reversible manner. ACC synthase activity has been demonstrated by antisense RNA experiments in to-

matoes as the rate-limiting step in ethylene synthesis. Transgenic tomatoes expressing ACC synthase antisense RNA do not ripen unless exposed to exogenous ethylene (Rottmann *et al.*, 1991; Theologis *et al.*, 1993). Transgenic tomatoes with introduced ACC deaminase gene are characterized by a reduced ACC level which results in delayed fruit ripening (Klee *et al.*, 1991; Theologis *et al.*, 1992).

Microorganisms liberate ethylene as a product of metabolism of: a) 2-oxoglutarate, b) 2-oxo-4-methylthiobutyrate. These pathways do not involve ACC as the intermediate. The fungus *Penicillium citrinum* is the first reported microorganism which is able to synthesize ACC from SAM and to degrade it into ammonia and 2-oxobutyrate by the action of ACC deaminase (Jia *et al.*, 1999; Kakuta *et al.*, 2001).

REACTION MECHANISM OF THE CONVERSION OF SAM TO ACC AND MTA CATALYZED BY ACC SYNTHASE

ACC synthase belongs to the family of pyridoxal 5′-phosphate-dependent enzymes. In PLP-dependent enzymes, the formyl group at position 4 of pyridoxal phosphate forms an aldimine bond with the ε-amino group of a specific lysine residue, forming a Schiffs base within the active site of the enzyme. ACC synthases have absorption maxima between 426 and 431 nm, which is probably due to the internal Schiff base (Satoh *et al.*, 1993).

A proposed reaction mechanism — the conversion of SAM to ACC by ACC synthase — involves the following steps:

1. The active site lysine of ACC synthase forms a Schiff base (internal aldimine) with the bound PLP in unligated enzyme (Fig. 1a).

2. The amino group of the substrate SAM replaces that of active site lysine as the Schiff base partner of the cofactor (external aldimine), (Fig. 1a).

3. The C-α proton of SAM is abstracted by the ε-NH$_2$ function of active site lysine to form

a resonance-stabilized carbanion (Fig. 1a), that in turn attacs the C-γ atom (Fig. 1b) to expel 5′-methylthioadenosine (MTA), with the formation of the imine of ACC (Fig. 1b, 1c).

4. The product is released upon transimination by active site lysine to complete the catalytic sequence (Fig. 1d).

The scheme of ethylene synthesis with the atoms of methionine which are the precursors of ethylene, marked by bold type set, is presented in Fig. 1e.

THE MECHANISM-BASED INACTIVATION OF ACC SYNTHASE BY THE SUBSTRATE SAM, AND OTHER ACTIVITIES OF THIS ENZYME

ACS is inactivated by its substrate SAM in an irreversible manner (mechanism-based inactivation). ACC synthase is optimized to direct electrons from the quinonoid intermediate to the γ-carbon of its substrate — SAM (Fig. 1b), to yield ACC and MTA. SAM exists as an *S,S* or *R,S* diastereomeric mixture (isomers with respect to its sulfonium centre); only the *S,S* diastereomer yields ACC. The *R,S* form inactivates the enzyme. The inactivation of ACC synthase is a result of a nucleophilic attack on residual enzyme-bound L-vinylglycine (L-VG) originating from the β, γ-elimination of MTA from the internal SAM aldimine (Satoh & Esashi, 1986; Casas *et al.*, 1993; White *et al.*, 1994; Feng & Kirsch, 2000). A scheme of the proposed mechanism-based inactivation of ACS is shown in Fig. 2.

In vivo SAM leads to the formation of L-vinylglycine, causing alkylation and inactivation of the enzyme about once in 30000 turnovers (Oetiker *et al.*, 1997). The peptide Ser-Leu-Ser-X-Asp-Leu-Gly-Leu-Pro-Gly-Phe-Arg from SAM-inactivated ACC synthase from apple was isolated. X was a modified amino acid (alkylation product) coincident with the position of active-site Lys273 (Satoh *et al.*, 1993; Feng & Kirsch, 2000).
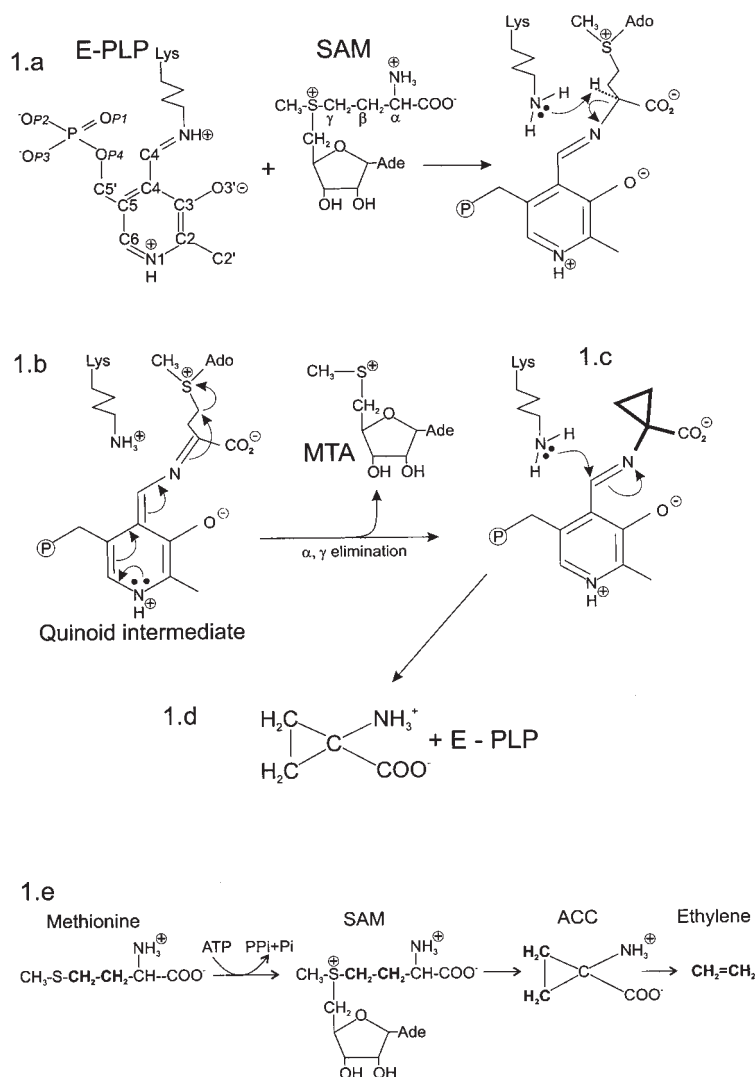
Figure 1. Scheme of reaction catalyzed by 1-aminocyclopropane-1-carboxylate synthase.
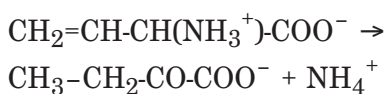
Denotations: OP1–OP4, oxygens of the pyridoxal 5′-phosphate moiety. Details in text.

The half-life of ACS was found to be 25 min in mung bean hypocotyl segments treated with auxin but 30–40 minutes in wounded green tomato pericarp, and 114 min in ripening tomato pericarp. The mechanism-based inactivation of ACC synthase plays a pivotal role in the rapid decrease in the enzymatic activity that was observed in plant tissue (Satoh & Esashi, 1986; Satoh *et al.*, 1993).

ACC synthase reveals some additional catalytic activity apart from the preferred physiological role. L-Vinylglycine is an alternative substrate as well as a mechanism-based inhibitor of ACS. The high molar ratio of L-VG/ACS (over 5000) is necessary for a complete inactivation of ACS.

L-vinylglycine + ACS-PLP →
vinylglycine-PLP-ACS →
alkylated inactive ACS + PLP

The majority of L-VG (98.2%) is converted to a product other than the inactive enzyme. The primary reaction is the conversion of L-VG to α-ketobutyrate and ammonia:

$$CH_2=CH-CH(NH_3^+)-COO^- \rightarrow$$
$$CH_3-CH_2-CO-COO^- + NH_4^+$$

The L-VG deaminase activity of ACC synthase yields the products α-ketobutyrate
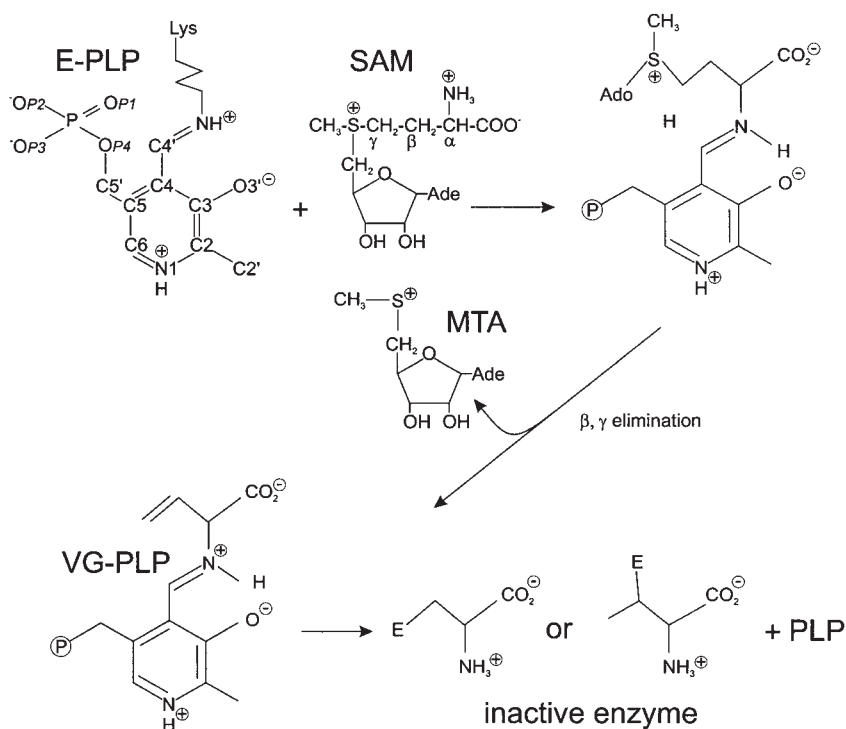
Figure 2. Model of mechanism-based inactivation of 1-aminocyclopropane-1-carboxylate synthase by its substrate SAM.

Denotations: OP1–OP4, oxygens of the pyridoxal 5′-phosphate moiety; VG-PLP, vinylglycine-PLP. Details in text.

and ammonia with a k_{cat} value of 1.8 s^{-1} and a K_m value of 1.4 mM (Feng & Kirsch, 2000).

Feng *et al.* (2000) investigated the aminotransferase activity of ACC synthase from apple and characterized it as non-α-ketoglutarate-coupled. PLP is reductively aminated to PMP (pyridoxalamine) by transamination of an amino acid to its corresponding α-keto acid:

amino acid + ACS-PLP $\rightarrow$

keto acid + ACS-PMP
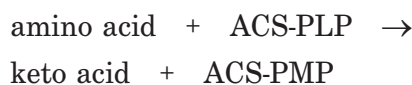
The enzyme exhibits substrate specifity in transamination with the preference of L-Ala > L-Arg > L-Phe > L-Asp. Transamination of L-Asp and L-Phe by ACC synthase is very slow. Addition of excess pyruvate converts the PMP form of the enzyme back to the PLP form. The PMP form of ACC synthase is quite unstable. This unstability would generate apoenzyme which would capture PLP to restore the physiologically active form of ACS.

INHIBITORS OF ACC SYNTHASE ACTIVITY

ACC synthase activity may be reversibly regulated by various substances associated with the methionine-recycling pathway, SAM metabolism, polyamine synthesis and natural and chemical analogs of SAM or inhibitors of pyridoxal phosphate-linked enzymes.

MTA and KMB, intermediates of the methionine-recycling pathway inhibit the enzyme activity in an uncompetitive and noncompetitive fashion, respectively (Hyodo & Tanaka, 1986).

Sinefungin (a naturally occuring antifungal antibiotic isolated from *Streptomyces griseus*), SAHC, 3dz-SAH, SIBA, 1dz-SIBA, 3dz-SIBA, 7dz-SIBA are analogs to SAM (Fig. 3). When tested with ACC synthase from tomatoes the order of decreasing inhibitory activity of the SAM analogs was as follows: sinefungin > SAHC and SAHC derivates > S-n-butyladenosine and SIBA. The inhibitors tested can be divided into two groups, those modified in the adenine moiety and those modified in the 5′

Table 1. Comparison of amino acids number (aa), molecular masses (kDa) and isoelectric points (pI) of some well-characterized ACS isoenzymes from different plants

ACS isoenzyme	aa number	Molecular mass (kDa)	pI	References
Lycopersicon esculentum				
LE-ACS1A	485	54.8	5.94	(Rottmann *et al.*, 1991)
LE-ACS1B	483	54.6	6.16	(Rottmann *et al.*, 1991)
LE-ACS2	485	54.7	7.71	(Rottmann *et al.*, 1991)
LE-ACS3	469	53.1	8.01	(Rottmann *et al.*, 1991)
LE-ACS4	476	53.5	5.40	(Rottmann *et al.*, 1991)
LE-ACS7	467	53.1	8.30	(Shiu *et al.*, 1998)
Solanum tuberosum				
ST-ACS1A	465	52.9	7.86	(Destefano-Beltran *et al.*, 1995)
ST-ACS1B	465	52.9	8.23	(Destefano-Beltran *et al.*, 1995)
ST-ACS2	441	50.2	5.74	(Destefano-Beltran, 1995)
Lupinus albus				
LA-ACS1	487	55.0	7.06	(Bekman *et al.*, 2000)
LA-ACS3	488	55.0	7.61	(Bekman *et al.*, 2000)
LA-ACS4	445	51.0	6.58	(Bekman *et al.*, 2000)
LA-ACS5	448	51.0	6.44	(Bekman *et al.*, 2000)
Vigna radiata				
VR-ACS1	484	54.6	6.0	(Botella *et al.*, 1992)
Arabidopsis thaliana				
AT-ACS1	496	54.6	7.3	(Van Der Straeten *et al.*, 1992)
AT-ACS2	496	55.6	7.3	(Liang *et al.*, 1992)
AT-ACS4	474	53.8	8.2, 8.4	(Abel *et al.*, 1995; Liang *et al.*, 1995)
AT-ACS5	470	53.3	7.5	(Liang *et al.*, 1995)
Brassica oleraceae				
BO-ACS2	491	55.0	5.9	(Pogson *et al.*, 1995)
Cucurbita pepo				
CP-ACS1A	493	55.8	7.07	(Huang *et al.*, 1991)
CP-ACS1B	494	55.9	7.68	(Huang *et al.*, 1991)
Cucurbita maxima				
CM-ACS	493	55.8	5.0	(Sato *et al.*, 1991)
Cucumis melo				
CMe-ACS1	493	56.0	6.41	(Miki *et al.*, 1995)
Citrus chinensis				
CS-ACS1	483	54.1	6.63	(Wong *et al.*, 1999)
CS-ACS2	477	53.3	6.72	(Wong *et al.*, 1999)
Pelargonium hortorum				
G-ACS1	482	54.1	6.4	(Wang & Arteca, 1995)
Oryza sativa				
OS-ACS1	487	53.1	8.2	(Zarembinski & Theologis, 1993)

substituent. The 7-nitrogen of adenine is apparently necessary for the inhibitory activity, judging from the inactivity of 7dz-SIBA. Although 3dz-SIBA was inactive, the inhibitory activity of 3dz-SAHC suggests that the 5′ substituent containing an amino group may be important for inhibition (Icekson & Apelbaum, 1983; Miura & Chiang, 1985).

SAM serves as an important common precursor for the pathway of polyamine and ethylene synthesis. The interactions between ethylene and polyamine metabolism are discussed and

many conflicting reports have been published on the effect of exogenously applied polyamines on ethylene synthesis. At physiological pH, polyamines are fully protonated and polycationic. It is possible that the amino group of amines prevent SAM from reacting with ACC synthase by interfering with the prostetic groups of the enzyme. Polyamines such as putrescine, spermidine and spermine inhibit ACC synthase activity in a noncompetitive manner. It has been suggested that polyamines also suppress the induction of the enzyme. From the physiological point of view, it is of interest to note that while ethylene is a plant-aging hormone leading to retardation of growth and promotion of senescence, polyamines delay senescence in some plant species (Hyodo & Tanaka, 1986; Botha & Whitehead, 1992).

The hydroxylamine or vinylglycine analogs are potent inhibitors of PLP-dependent enzymes activity *in vivo* and *in vitro*. The hydroxylamine analogs inactivate the enzyme forming stable oximes with PLP.

Several vinylglycine analogs are shown below.

Rhizobitoxine	$HOCH_2$-CH (NH_2)-O-CH=CH-CH (NH_2)-COOH
AVG	NH_2-CH_2-CH_2-O-CH=CH-CH (NH_2)-COOH
MVG	CH_3-O-CH=CH-CH (NH_2)-COOH

AVG has the strongest inhibitory effect on ACC synthase among the vinylglycine analogs tested (Yang & Hoffman, 1984). Rhizobitoxine [2-amino-4-(2-amino-3-hydropropoxy)-*trans*-but-3-enoic acid] is synthesized by the legume symbiont *Bradyrhizobium elkanii* and the broad-host-range plant pathogen *Burkholderia andropogonis* (Yasuta *et al.*, 1999). The bacterial toxin induces foliar chlorosis of the host plant but the role of the toxin in symbiosis and pathogenicity has not been resolved. It was first isolated from root nodules produced by *Rhizobium japonicum* in soybean. Rhizobitoxine inhibits β-cystathionase and

ACC synthase in the methionine and ethylene biosynthetic pathway, respectively.

Aminoethoxyvinylglycine (AVG) was isolated from a *Streptomyces* strain, the methoxy analog (MVG) was discovered in fermentation broth of *Pseudomonas aeruginosa* (Mattoo *et al.*, 1979). These unsaturated enol ether amino acids are potent inhibitors of ethylene synthesis in higher plants. It is interesting that both symbiotic and pathogenic bacteria produce ACC synthase inhibitors structurally and enzymologically similar to rhizobitoxine so as to control the ethylene-induced plant response that would prevent a successful infection.

The crystal structures of tomato ACS in complex with AVG and PLP imply that the competitive inhibitor AVG binds close to PLP. It is a good position for the formation the external aldimine with PLP. The α-amino group of AVG is located 4 Å away from C4′ of the pyridine ring of PLP. The α-carboxylate group of AVG forms the three following hydrogen bonds — with the backbone nitrogen of alanine, the guanidine nitrogen of arginine (in tomato ACS Ala54, Arg412) and water. The amine group at the other end of AVG forms a van der Waals contact with a specific tyrosine residue (in tomato ACS Tyr152). Probably the α-amino and α-carboxylate groups of AVG simulate SAM binding in ACS (Huai *et al.*, 2001).

THE STRUCTURE OF ACC SYNTHASE AND AMINO-ACID RESIDUES CRITICAL FOR ITS FUNCTION

ACC synthase, an enzyme located in the cytoplasm, is encoded by a highly divergent multigene family but the low content of ACS polypeptides (0.0001%–0.001% of plant soluble proteins) has strongly limited characterisation of these proteins. The expression of functional ACS cDNAs in heterologous systems provides an opportunity to explore these enzymes. The first ACS cDNA was cloned
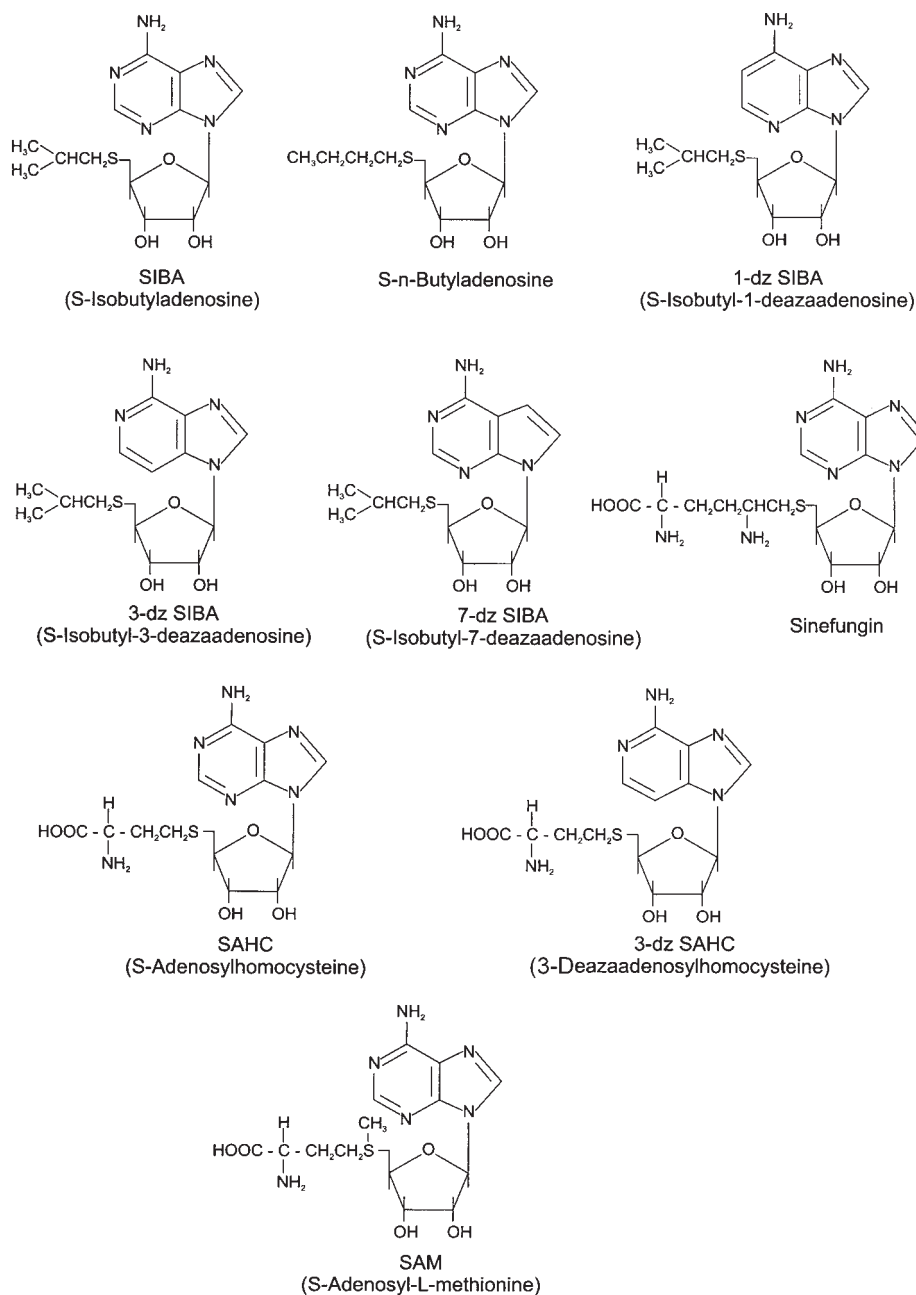
Figure 3. Natural and synthetic analogs of SAM.

from zucchini fruit tissue and overexpressed in *E. coli* and yeast (Zarembinski & Theologis, 1994; Abel *et al.*, 1995).

Despite the polymorphic character of the multigene family of ACS isoenzymes their primary structures show similar molecular size (441aa to 496aa) and seven strongly conserved regions (Wong *et al.*, 1999). ACC synthase from carnation is the only exception characterised to date. It is larger than others (516aa) because of the presence of a stretch of 18 threonine residues (ACA) at position 452–469aa encoded by repeats of the CAA sequence. The stretch of $(CAA)_{18}$ probably represents a highly repeated element within the carnation genome and is not unique to the ACC synthase gene (Park *et al.*, 1992).

A necessary feature of the polypeptide during its attachment to the ACS family is the PLP-binding site (region 5): -Ser-Leu-Ser-Lys-Asp-Leu/Met-Gly-Leu-Pro-Gly-Phe-Arg-. The highly conserved regions (regions 1 to 7)

among all plant ACS are shown below (the position of residues numbered according to the 485aa long polypeptide of LE-ACS2 from tomato).

1	-49 Ile-Gln-Met-Gly-Leu-Ala-Glu-Asn-Gln-Leu 58-
2	-89 Phe-Gln-Asp-Tyr-His-Gly-Leu-Pro 97-
3	-136 Phe-Cys-Leu-Ala-Asp-Pro-Gly 142-
4	-205 Thr-Asn-Pro-Ser-Asn-Pro-Leu-Gly-Thr 213-
5	-274 Tyr-Ser-Leu-Ser-Lys-Asp-Met-Gly-Leu-Pro-Gly-Phe-Arg-Val-Gly 288-
6	-304 Met-Ser-Ser-Phe-Gly-Leu-Val-Ser 311-
7	-408 Pro-Gly-Trp-Phe-Arg-Val-Cys-Phe-Ala-Gln 417-

The roles of amino-acid residues critical for ACC synthase activity have been elucidated by site-directed or random mutagenesis investigations performed on ACC synthases from apple and tomato (White *et al.*, 1994; Li *et al.*, 1997; Tarun *et al.*, 1998; Capitani *et al.*, 1999; Zhou *et al.*, 1999; Huai *et al.*, 2001; McCarthy *et al.*, 2001). The residues important for catalysis and structure of ACC synthase from tomato are shown in Fig. 4 and are listed below (the numbers in brackets indicate equivalent residue in ACS from apple according to Capitani *et al.*, 1999).

Lys278 (273): active site lysine forming the Schiff base with the bound PLP in the unligated enzyme. Mutated enzymes from apple and tomato with the active-site lysine replaced by alanine have no detectable activity.

Asn209 (202), Tyr240 (233): O3 of the pyridine ring of PLP forms two hydrogen bonds with these amino-acid residues, Tyr240 attracts the proton of Lys278 to promote the formation of the external aldimine.

Tyr152 (145): stabilization of the pyridine ring of PLP and catalytic function. Tyr152 performs a nucleophilic attack to break the C-γ-S bond what leads to the formation of the key intermediate — ACC-aldimine (the OH group of Tyr152 is positioned 3.7 and 3.5 Å from C-γ and S of SAM, respectively).

Asp237 (230): hydrogen bond with N1 of PLP.

Ala127 (120), Thr128 (121), Ser275 (270), Ser277 (272), Arg286 (281): residues required for correct orientation of PLP in the active site during formation of external aldimine between SAM and PLP, hydrogen bonds with the phosphate oxygens of PLP.

Glu55 (47): putative ionic interaction between SAM and this residue positioned near the sulfonium pole of the (*S,S*)-diastereomer of SAM.

Ala54 (46), Arg412 (407): α-carboxylate group of SAM forms hydrogen bonds with the nitrogen of alanine and the guanidino moiety of arginine.

Arg157 (150): O2′ and O3′ atoms of the sugar of SAM form hydrogen bonds with the guanidine group of arginine.

Pro26 (Ser18), Tyr27 (19), Phe28 (20), Pro153 (144): these residues contribute in a hydrophobic pocket for the adenine ring of SAM.

Tyr92 (85): the complete conservation of this tyrosine residue among all known ACS suggests that it may be involved in substrate recognition. In the apple, Tyr85 to Ala85 mutation leads to an inactive ACC synthase, whereas in the tomato Tyr92 to Phe92 or Trp92 mutations produce a partially active enzyme, while substitutions Tyr92 to His92 or Leu92 inactivate the enzyme. In contrast, according to Tarun *et al.* (1998), Tyr92 residue from tomato ACS is not essential for the activity of the enzyme but it may play a role in binding the PLP cofactor and in making inter-subunit contacts in the dimeric form of ACS (Tarun *et al.*, 1998; Capitani *et al.*, 1999).

Replacement of Tyr233 with Phe233 in ACS from apple caused a 24-fold increase in K_m for SAM (White *et al.*, 1994). This tyrosine residue is not absolutely conserved in all ACS one of the ACC synthases from carnation shows a Phe residue at this position (Tarun *et al.*, 1998).

Substitution of Arg286 with Leu286 in tomato ACS results in the mutant's lower affinity for both PLP and SAM. The loss of the correct orientation of PLP at the active site of the mutant with Leu286 decreases the binding affinity of the enzyme for SAM. Moreover, the

Leu286 mutant shows the reduction in k_{cat} which suggests that Arg286 participates in the substrate catalysis. The replacement of Arg286 with Val, Thr, Ile, and Ala converts tomato ACS into mutants losing 95% to 100% of the original catalytic activity (Tarun *et al.*, 1998).

The mutation of Arg407 (tomato Arg412) to Lys407 in ACS from apple results in at least 20-fold increase in K_m for SAM (White *et al.*, 1994).

The N-termini of ACS are characterised by two highly conserved leucine and serine residues (Leu12 and Ser13 in tomato ACS, Leu4 and Ser5 in apple ACS). The removal of ten

of proteins of very similar molecular masses which differ from each other in the length of the C-truncated fragments. These values for winter squash and tomato ACS are lower by 6–8 kDa than those calculated from the corresponding cDNA. Purified wound-inducible tomato ACC synthase appears as two distinct processed polypeptides, despite the presence of protease inhibitors in the purification procedure. This suggests that the nature of the C-terminus of ACC synthase makes it susceptible to cleavage. The processing seems to occur invariably when the tissue is wounded or when cells are homogenized. The monomeric deletion mutant (tomato ACS) of the last 52aa
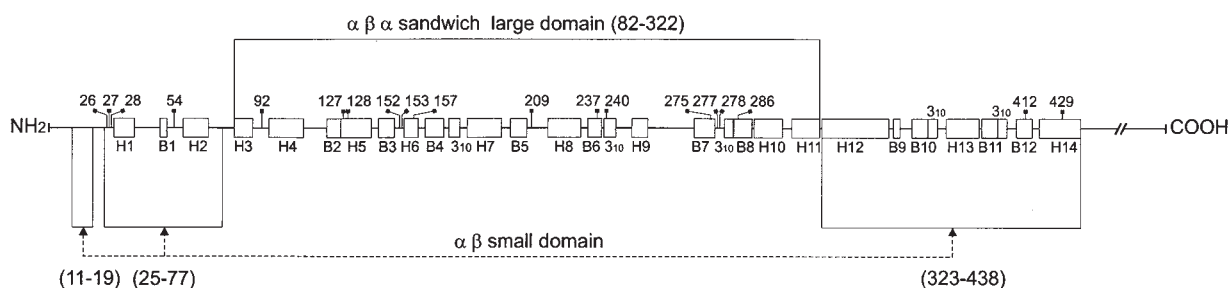

Figure 4. Schematic presentation of tomato ACS polypeptide with marked α-helical and β-stranded secondary structure regions, residues critical for catalysis and fragments of the polypeptide representing the large and the small domain in the three-dimensional structure of enzyme.

Denotations: open blocks, α-helical regions (H1–H14), filled blocks, β-strand (B1–B12) and helix 3_{10} regions.

residues from the N-terminus of tomato ACS leads to a slight increase in ACC synthase activity but a deletion of any more residues would result in a substantial reduction in the activity. Addition of several amino-acid residues to the N-terminus does not affect the formation of the dimeric protein (Satoh *et al.*, 1993; Li *et al.*, 1996; Tarun *et al.*, 1998).

The carboxyl termini of all known ACC synthases are quite divergent and consist of a hypervariable region of 18–85 C-terminal residues (Wong *et al.*, 1999). Mutations in this hypervariable region (for tomato LE-ACS2 in the region of the last 56 residues) do not lead to any decrease in the enzyme activity.

ACS polypeptides prepared from plant tissues or overexpression systems are mixtures

C-terminal residues shows a dramatic increase in activity. Further deletion of the C-terminus through Arg429 results in a complete inactivation of the enzyme. Limited C-terminal proteolysis of ACC synthase may occur naturally and certain endogenous or exogenous stimuli cause the activation of ACC synthase by forming the enzymes with the higher specific activity. The phenomenon may be attributed to the release of proteases upon wounding and during tissue senescence (Kende, 1993; Satoh *et al.*, 1993; Li & Mattoo, 1994; Zarembinski & Theologis, 1994; Tarun *et al.*, 1998).

The subunit structure of ACS is controversial (Nakajima *et al.*, 1988; Rottmann *et al.*, 1991; Lincoln *et al.*, 1993; Oetiker *et al.*, 1997;

Huxtable *et al.*, 1998). ACS enzymes from mung bean hypocotyls and a wound-inducible ACC synthase from zucchini have dimeric forms. Satoh *et al.* (1993) report that enzymes from ripe tomato are monomers, whereas the same enzymes overexpressed from tomato ACS cDNAs occur in dimeric forms. In contrast, Li & Mattoo (1995) report that tomato LE-ACS2 isoenzyme from fruit is a dimer whereas its carboxyl terminal deletion mutants (with last 46 and 52 residues deleted) function as highly active monomers. According to Tarun & Theologis (1998) the highly active carboxyl terminal deletion mutant LE-ACS2 with last 46 residues deleted is able to dimerize.

The catalytically inactive tomato and apple ACS mutants with substitutions of the highly conserved tyrosine or lysine residues and the hyperactive mutant with 46 C-terminal amino-acid residues deleted were coexpressed in *E. coli* (Li *et al.*, 1997; Tarun & Theologis, 1998). The catalytic activity of ACS was measured. The conclusions are the following:

1) an active dimer may be formed from two different properly folded defective monomer subunits.

2) the hyperactive ACS monomer is able to dimerize with inactive monomer, which results in a reduced activity.

3) two inactive monomers of different tomato isoenzymes are able to undergo hetero-dimerization and show limited restoration of activity.

The question is whether ACS may be active both as a monomer and as a dimer. On the basis of the experimental data, we cannot distinguish these two possibilities.

The AT-ACS1 polypeptide from *Arabidopsis thaliana* is missing the highly conserved tripeptide Thr-Asn-Pro (region 4) and has no ACS activity. It is possible that AT-ACS1 may act as a dominant negative regulator of other ACS by forming heterodimers (Liang *et al.*, 1995).

Satoh *et al.* (1993) suggest that the dimeric forms of tomato ACS isoenzymes (LE-ACS2 and LE-ACS4) are more sensitive to mechanism-based inactivation than their monomers because of minor differences in the parameters of the active sites of these two forms. The concentration of SAM localized in the protoplasm in ripe tomato pericarp (higher than 100 micromolar) should almost saturate the enzyme's active site. Changes in the sensitivity of the enzyme to mechanism-based inactivation probably take part in the regulation of ethylene synthesis in plant tissue.

Despite the quite similar molecular masses of ACS isoenzymes their isoelectric points differ considerably, as shown in Table 1.

Penicillium citrinum synthesizes ACC from SAM and metabolizes ACC to ammonia and 2-oxobutyrate, not to ethylene (step 3, ACC deaminase).

$$\text{Methionine} + \text{ATP} \xrightarrow{1} \text{SAM} \xrightarrow{2}$$
$$\text{ACC} \xrightarrow{3} \text{2-oxobutyrate} + \text{ammonia}$$

The ACC synthase from *Penicillium citrinum* is a 430aa long polypeptide (a shorter C-terminus than that in plant enzymes) with molecular mass 48 kDa. The native form of the enzyme is a dimer. ACS from *P. citrinum* has K_m for SAM about 100-fold higher than plant ACS (Jia *et al.*, 1999). Five of the seven highly conserved regions (regions 1, 3, 4, 5, 7) found in plant ACS occur in ACS from *P. citrinum* and have 45–50% of residues identical with their plant counterparts. *P. citrinum* ACS contains six highly conserved residues responsible for the binding of PLP and SAM (Lys268, Tyr225, Asn194, Asp222, Arg276, Arg408) and Arg424 corresponding to the conserved Arg429 of the tomato enzyme. The highly conserved tyrosine residue — Tyr85 or Tyr92 (in apple or tomato ACS) is not conserved and Gly74 is at the equivalent position in *P. citrinum* ACS (Kakuta *et al.*, 2001).

An artificial bifunctional enzyme — ACSO (90 kDa) was created by in-frame fusion of a C-terminus truncated ACC synthase from soybean and an N-terminus truncated ACC oxidase from tomato. ACSO was able to con-

vert SAM to ethylene. Native ACSO is oligomeric, about 360 kDa, suggesting that this fusion enzyme has a tetrameric form (Li *et al.* 1996).

THE CRYSTAL STRUCTURE OF ACC SYNTHASE

The crystal structure of ACC synthase from apple without the PLP cofactor and two crystal structures of tomato ACS: with PLP and with PLP and the inhibitor AVG were described (Hohenester *et al.*, 1994; Capitani *et al.*, 1999; Huai *et al.*, 2001). The structures with and without PLP represent dimeric forms but the structure that with PLP and AVG is a monomer. The residues involved in catalysis and PLP and AVG interaction are located within the active site of the monomer. The monomeric form of tomato ACS implies should be sufficient to catalysis.

A schematic presentation of the secondary structures building the large and the small domain in the three-dimensional structure of ACS from tomato is shown in Fig. 4. The large domain ($\alpha/\beta/\alpha$ sandwich domain) contains a central several strand β-sheet flanked by nine α-helices (residues 82–322). The small domain (α/β domain) comprises a five β strands and five α-helices (residues 11–19, 25–77, 323–438), (Huai *et al.*, 2001).

The full-length polypeptide from apple (52 kDa) is C-terminally truncated to a shorter form (48 kDa) by unknown proteolytic processes upon storage. Only the truncated form has been crystallized to date. ACC synthase from apple is a two-domain protein with each domain composed of a central sheet of β-strands connected by α-helices packed on both sides. The large domain of this ACC synthase contains a seven-stranded β-sheet, the central part of the small domain consists of a four-stranded antiparallel and a two-stranded parallel sheet. In the dimeric form the active site is localized close to the subunit interface, in a cleft between the domains.

Most of the strictly conserved amino-acid residues are located on the dimer surface and nearly all are clustered close to the active site (Capitani *et al.*, 1999). The crystallographic data imply that the large domain distinguished in the three-dimensional structure of ACS from apple and tomato contains the majority of the conserved regions (region 2–6). The crystal structures of ACC synthase from tomato and apple are very similar.

EVOLUTIONARY RELATIONSHIPS OF ACC SYNTHASE

The lysine residue forming the internal aldimine with C4$'$ of PLP is absolutely conserved among all members of the PLP-dependent superfamily. The enzymes which use the pyridoxal moiety of PLP for catalysis have been classified into four structural fold types (29 enzyme structures deposited in the Protein Data Bank). The four types of PLP-dependent enzymes do not have a common structure fold but a limited conservation of the PLP binding residues. The type I fold consists of a large $\alpha\beta\alpha$ sandwich domain and a small $\alpha\beta$ domain. Type I has been divided into subgroups on the basis of conformational changes upon the binding of substrate or inhibitors: subgroup Ia — large conformational changes, subgroup Ib — absence of conformational changes. The crystal structure of LE-ACS2 from tomato suggests that this enzyme belongs to subgroup Ib rather than to Ia (Huai *et al.*, 2001).

The PLP-dependent enzymes are found in four out of the six EC classes of enzymes but sequence and structural alignments indicate that ACC synthases are most closely evolutionarily related to the subgroup I of aminotransferases which includes aspartate, alanine, histidinolphosphate, tyrosine and phenylalanine aminotransferases (Alexander *et al.*, 1994; Mehta & Christen, 1994; Feng *et al.*, 2000).

There are only 12 amino-acid residues completely conserved among various amino-

transferases. All but one of them are present in appropriate places in all ACC synthases (Rottmann *et al.*, 1991; Theologis, 1992). Dimerization is necessary for the catalytic activity of aminotransferases. Aspartate aminotransferase has two active sites and each of them is formed by the juxtaposition of essential residues from both subunits (Feng *et al.*, 2000).

The closest three-dimensional structural homologues of apple ACS are *Thermus thermophilus* aspartate and *Trypanosoma cruzi* tyrosine aminotransferases, but *Bacillus* sp. aspartate and rat tyrosine aminotransferases are the most similar ones to apple ACS in the primary structure (Feng *et al.*, 2000; Capitani *et al.*, 1999). Other structures of the PLP-dependent enzymes that align well with apple ACS three-dimensional structure include pig cytosolic aspartate aminotransferase, and *E. coli* β-cystathionase and 8-amino-7-oxonanoate synthase.

ACC synthase has been found and its function is known only in higher plants and in the fungus *Penicillium citrinum*. Possible homologues of plant ACS were found in the human and pufferfish (*Fugu rubripes*) genomes. Transcripts of these genes were detected in human and *F. rubripes* cDNA libraries. The function of the encoded proteins have not been resolved yet. The human homologue of ACS has bound PLP but does not convert SAM to ACC. This homologue is capable of deamination of L-VG (Feng *et al.*, 2000; Feng & Kirsch, 2000; Peixoto *et al.*, 2000; Koch *et al.*, 2001).

Members of the PLP-dependent family are characterized by significant conservation of the PLP binding residues. It is not known whether they represent convergent evolution or evolved divergently from a common origin.

A phylogenetic tree of ACS isoenzymes from various plant species constructed on the basis of amino-acid sequence identity implies that the ACS gene divergence had taken place before monocots and dicots separation. ACS genes were then divided into three main groups (Liang *et al.*, 1992; Destefano-Beltran

et al., 1995; Subramaniam *et al.*, 1996; Trebitsh *et al.*, 1997). Expression of each of these groups is induced by similar stimuli. The multigene family of ACS reflects the evolution of a set of polypeptides which catalyse the rate-limiting step in ethylene synthesis in various plant tissues during plant growth, development or stress.

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