

STEREOCHEMICAL COURSE OF THE BIOSYNTHESIS OF 1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID. I. ROLE OF THE ASYMMETRIC SULFONIUM POLE AND THE  $\alpha$ -AMINO ACID CENTER

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**SUMMARY:** The substrate stereospecificity of 1-aminocyclopropane-1-carboxylic acid synthase, a pyridoxal phosphate-containing enzyme, from the pericarp tissue of *Lycopersicon esculentum* (tomatoes) was studied using the various stereoisomers of *S*-adenosylmethionine (AdoMet) at both the sulfonium pole and the amino acid center. The data indicate that only the naturally occurring isomer (-)Ado-L-Met acts as substrate ( $K_m = 20 \pm 5 \mu M$ ). Both ( $\pm$ )Ado-D-Met and (+)Ado-L-Met were inactive as substrates. The (+)Ado-L-Met ( $K_i = 15 \pm 5 \mu M$ ) was found to be a potent inhibitor of ACC synthase whereas ( $\pm$ )Ado-D-Met ( $K_i = 70 \pm 20 \mu M$ ) was less active as an inhibitor. This active isomer has the (*S*) configuration at both the sulfur and the  $\alpha$ -carbon of the amino acid portion of AdoMet.

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Relatively little information is available concerning the stereochemical course of the biosynthesis of 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase. ACC synthase, a pyridoxal phosphate (PLP)-containing enzyme, which converts *S*-adenosyl-L-methionine ((-)-Ado-L-Met) to ACC and methylthioadenosine (MTA), is the rate-limiting enzyme in the biosynthesis of ethylene (1,2). The exact mechanism of the reaction catalyzed by ACC synthase as well as the substrate stereospecificity of this enzyme remains unclear.

We have recently initiated studies to probe the substrate stereospecificity and the overall stereochemistry of the reaction catalyzed by ACC synthase from tomatoes in order to provide insight into the mechanism. In this paper, we report the results from a study in which (-)Ado-L-Met, (+)Ado-L-Met, ( $\pm$ )Ado-L-Met, ( $\pm$ )Ado-D-Met, and ( $\pm$ )Ado-D,L-Met (3) were examined as possible substrates for ACC synthase.

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**Abbreviations:** AdoMet, *S*-adenosylmethionine; AdoHcy, *S*-adenosyl-homocysteine; MTA, methylthioadenosine; ACC, 1-aminocyclopropane-1-carboxylic acid; Hepes, 4-(2-Hydroxyethyl)-1-piperazine-ethanesulfonic acid; PLP, pyridoxal phosphate.

## MATERIALS AND METHODS

**Materials.** Organic, inorganic, and biological chemicals were procured from Aldrich Chemical Company, Alfa-Ventron Corporation, and Sigma Chemical Company, respectively. Tomatoes in the "light pink" stage, vine-picked or purchased from a local vendor, were used to obtain the pericarp tissue for enzyme preparation.

**Enzyme Purification.** The unpeeled pericarp tissue of tomatoes was chopped into small pieces and allowed to stand for 3 h. at 25°C. The tissue was homogenized in a glass-Teflon homogenizer with an equal volume of 100 mM Hepes (pH 8.5), 4 mM dithiothreitol, 0.5  $\mu$ M pyridoxal phosphate and 5% polyvinylpyrrolidone (w/v). The preparation was pressed through several layers of cheesecloth and centrifuged at 15,000 xg for 30 min. The supernatant was fractionated by precipitation with solid ammonium sulfate. The fraction precipitating between 40-90% (pH 7.2) was dialyzed overnight against 2 mM Hepes (pH 8.5) containing 0.1 mM dithiothreitol and 0.2  $\mu$ M pyridoxal phosphate and the dialyzed enzyme was used without further purification.

**Enzyme Assay.** A modification of the assay previously described was employed to analyze the ACC synthase activity (4). The assay mixture consisted of 50 nmol of AdoMet, 30  $\mu$ mol of K-Hepes pH=8.5, and 700  $\mu$ l of the 40-90%  $\text{NH}_4\text{SO}_4$  ppt dialyzed enzyme preparation in a total volume of 800  $\mu$ l. After 15 min to 3 h at 30°C the reaction mixtures were cooled to 4°C in ice, 100  $\mu$ l of 10 mM  $\text{HgCl}_2$  were added to each tube, the volumes were adjusted to 1 ml for each mixture and the tubes capped with rubber septa, vortexed, and returned to the ice bath. To analyze the amount of ACC formed in each tube, the ACC was converted to ethylene by the addition of 100  $\mu$ l of NaOCl (5%)-NaOH (10 N) mixture (2:1 vol/vol.) After the addition of the bleach solution, the tube was vortexed for 30 s and a 1 ml sample from the gas phase was withdrawn through the septum cap for the gas phase determination of ethylene by a gas chromatograph equipped with a flame ionization detector. A column (45 x 0.64 cm) of 60-80 mesh  $\text{Al}_2\text{O}_3$ , at 90°C with helium as carrier gave a retention time of 25 s for ethylene. To determine the efficiency of the conversion of ACC to ethylene, a known amount of ACC (10 nmol) was added as an internal standard to another similarly incubated reaction mixture and was degraded as described above. Yields of ethylene were consistently 80-90%, and ethylene production from blank samples containing no substrate or no enzyme was less than 1% of that obtained with enzyme. Protein was determined according to Bradford (5).

**Enzyme Inhibition.** The  $K_m$  and  $K_i$  values were estimated from standard Michaelis-Menten plots. The velocity data were obtained at 10, 20, 40, 60, 80, 120  $\mu$ M AdoMet concentrations. Inhibition studies were conducted at 0, 20, 60, 100  $\mu$ M concentrations of each inhibitor.

**Syntheses**

**5'-Chloro-5'-deoxyadenosine.** This compound was prepared according to the procedure of Kikugawa and Ichino (6).

**S-Adenosyl-D,L-homocysteine.** To S-benzyl-D,L-homocysteine (1.10 g, 4.89 mmol) in 50 ml of liquid  $\text{NH}_3$  was added Na in small pieces until a blue color was maintained for 30 min. To this solution of D,L-homocysteine anion was added  $\text{NH}_4\text{Cl}$  until the blue color was just dissipated, then 1.01 g (3.5 mmol) of 5'-chloro-5'-deoxyadenosine was added slowly. The resulting mixture was stirred until all the ammonia had evaporated. The solid residue was dissolved in 10 ml of  $\text{H}_2\text{O}$  and neutralized with 5% HCl. The solution was applied to a 50 ml column of AG50W-4X cation exchange resin ( $\text{NH}_4^+$ ) and eluted first with  $\text{H}_2\text{O}$  and then 1N  $\text{NH}_4\text{OH}$ . The appropriate fractions were combined and lyophilized to yield off-white flakes, mp 206-208°C (7).

**( $\pm$ )-S-Adenosyl-D,L-methionine [( $\pm$ )Ado-D,L-Met].** The S-adenosyl-D,L-homocysteine (25 mg) was added to a stirred solution of a 1:1 (vol/vol) mixture of acetic acid:formic acid (1 ml). To this mixture was added 0.1 ml of methyl iodide and then 25 mg of silver acetate. The mixture was stirred in a foil-wrapped flask at room temperature for 4 h. The mixture was centrifuged and the supernatant extracted with diethyl ether (8). The aqueous layer was then applied to a C-18 reverse phase low pressure column (Applied Science; Adsorb-

osil-LC, 200-425 mesh) previously equilibrated with buffer A (0.2 M NH<sub>4</sub>OAc, pH=3.5). The column was eluted with buffer A and the AdoMet eluted immediately after the void volume. The appropriate fractions were lyophilized. The purity was checked by HPLC (Whatman Particil PXS 10/25 SCX); buffer 0.1 M NH<sub>4</sub>PO<sub>4</sub> pH=3.5; flow rate 3.5 ml/min; UV Detector (260 nm wavelength monitor); retention times (ml) were as follows: MTA 11.3, AdoHcy 7.5, AdoMet 50. The concentration of AdoMet was determined by UV;  $\epsilon = 12,600$  at 260 nm. S-Adenosyl-L-homocysteine [AdoHcy]. This compound was synthesized by the method described above for S-adenosyl-D,L-homocysteine but using S-benzyl-L-homocysteine or L-cystine or purchased from Sigma Chemical Co. (±)-S-Adenosyl-L-methionine [(±)Ado-L-Met]. This compound was synthesized by the procedure described above for (±)Ado-D,L-Met except utilizing S-adenosyl-L-homocysteine. (±)-S-adenosyl-D-methionine [(±)Ado-D-Met]. This compound was synthesized by the procedure described above for (±)Ado-D,L-Met except utilizing S-adenosyl-D-homocysteine. (+)-S-Adenosyl-L-methionine [(+)Ado-L-Met]. The (+)Ado-L-Met was enzymatically resolved to yield (+)Ado-L-Met using the procedure of Borchardt and Wu (9) which takes advantage of the substrate specificity of catechol O-methyltransferase. The optical purity was checked as described by Borchardt and Wu. The (+)Ado-L-Met was, however, purified as described above.

#### RESULTS AND DISCUSSION

The enzyme preparation from the unpeeled pericarp tissue of Lycopersicon esculentum Mill. (tomato) catalyzes the synthesis of ACC from (-)Ado-L-Met in the rate-limiting step in the biosynthesis of the plant hormone, ethylene. In the present study, we have determined the substrate stereospecificity of the enzyme ACC synthase using the various diastereomers of AdoMet at the sulfonium pole and the amino acid center.

The activity of (-)Ado-L-Met in the formation of ACC compared with that of the various AdoMet stereoisomers, used at the same concentration, is shown in the time-dependent plot Figure 1. The results demonstrate that ACC synthase is capable of utilizing only the naturally occurring (-)Ado-L-Met as substrate and not (+)Ado-L-Met. Also, neither sulfonium stereoisomer of Ado-D-Met is capable of producing ACC. These being the cases, at an infinite incubation time, one would have expected the amount of ACC formed from (±)Ado-L-Met (racemic at the sulfonium pole) to equal exactly one-half that formed from (-)Ado-L-Met and similarly that (±)Ado-D,L-Met (racemic at both the sulfonium pole and the amino acid center) would form one-fourth the amount of ACC formed from (-)Ado-L-Met. However, within finite incubation periods, the activity of (±)Ado-L-Met is less than one-half that of (-)Ado-L-Met and the activity of (±)Ado-D,L-Met is less than one-fourth that of (-)Ado-L-Met.

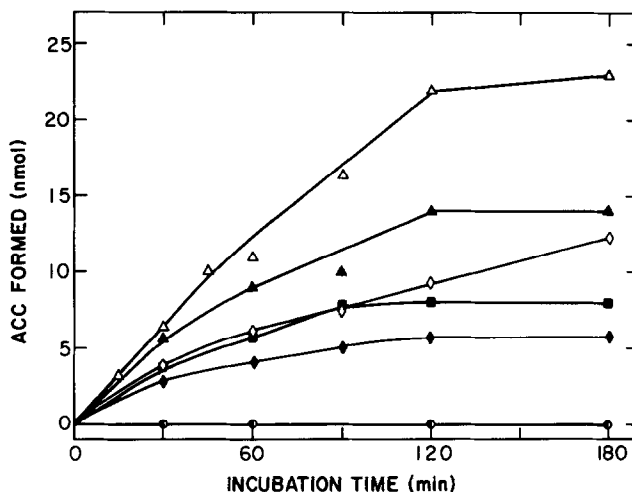


Figure 1: Utilization of the sulfonium and amino acid stereoisomers of AdoMet by ACC synthase. ACC synthase 0.5 mg, (-)Ado-L-Met 50 nmol ( $\Delta$ ), (-)Ado-L-Met 25 nmol ( $\blacktriangle$ ), (-)Ado-L-Met 12.5 nmol ( $\blacksquare$ ), ( $\pm$ )Ado-L-Met 50 nmol ( $\diamond$ ), ( $\pm$ )Ado-D,L-Met 50 nmol ( $\blacklozenge$ ), (+)Ado-L-Met 50 nmol ( $\odot$ ) and ( $\pm$ )Ado-D-Met 50 nmol ( $\bullet$ ) in a total volume of 800  $\mu$ l. Both (+)Ado-L-Met and ( $\pm$ )Ado-D-Met produced no detectable ACC and are represented by the same symbol. The amounts of ACC formed are the average of duplicate experiments. The reaction conditions and ACC assay are reported under "Experimental Procedures".

This decreased production of ACC may be the result of several factors. One, the diastereomers of (-)Ado-L-Met have an inhibitory effect on the levels of product obtained in a given time. The apparent  $K_m$  for (-)Ado-L-Met is  $20 \pm 5 \mu\text{M}$  ( $V \approx 3 \text{ nmol} \cdot \text{mg protein}^{-1} \cdot \text{h}^{-1}$ ) whereas the apparent  $K_i$ s for (+)Ado-L-Met and ( $\pm$ )Ado-D-Met are  $15 \pm 5 \mu\text{M}$  and  $70 \pm 20 \mu\text{M}$ , respectively. Borchardt and Wu (9) have also demonstrated that (+)Ado-L-Met is an inhibitor of a number of methyltransferases. A second factor which may contribute to an under production of ACC is that synthetic ( $\pm$ )Ado-L-Met has been shown by Stolowitz *et al.* (10), using high-field  $^1\text{H}$ -nmr, to be 40% (-)Ado-L-Met and 60% (+)Ado-L-Met. Our commercial (-)Ado-L-Met samples contained 20-25% (+)Ado-L-Met whereas our synthetic ( $\pm$ )Ado-L-Met, ( $\pm$ )Ado-D-Met and ( $\pm$ )Ado-D,L-Met contained 55-65% of the (+) isomer (11). The enantiomeric enrichment of the synthetic AdoMet samples implies some asymmetric induction by the other chiral centers in the molecule. Although the ratio of the (-) isomer in the natural (-)Ado-L-Met to the (-)isomer in the unnatural ( $\pm$ )Ado-L-Met is approximately one-half, one may not assume, based on the inhibition

studies, that the relative activity of the synthetic mixtures would be one-half (or one-fourth in the case of ( $\pm$ )Ado-D,L-Met) that of the natural AdoMet.

The high specificity of this enzyme with respect to the sulfonium pole is typical of AdoMet-utilizing enzymes studied to date. Borchardt and Wu (9) have shown that transmethylation catalyzed by catechol O-methyltransferase (EC 2.1.1.6), phenylethanolamine N-methyltransferase (EC 2.1.1.28), histamine N-methyltransferase (EC 2.1.1.8) and hydroxyindole O-methyltransferase (EC 2.1.1.4) use only the natural (-) enantiomer of AdoMet as a methyl donor. The same substrate stereospecificity was found with AdoMet decarboxylase (EC 4.1.1.50) (11). Zappia and other investigators (12,13) have, however, found a total lack of substrate stereospecificity with L-homocysteine S-methyltransferase (EC 2.1.1.10) (14).

The substrate stereospecificity at the  $\alpha$ -amino acid center is not uncommon in enzymes utilizing  $\alpha$ -amino acids as substrates. At least six enzymes dealing with the synthesis or metabolism of AdoMet and/or AdoHcy have shown a preference for the "L" (S) enantiomer of the  $\alpha$ -amino acid. The synthetic enzyme which condenses adenosine with L-homocysteine to form Ado-L-Hcy and the synthetase (EC 2.5.1.6) which condenses adenosine triphosphate and L-methionine to form (-)Ado-L-Met utilize only the "L" (S) enantiomer (15) of the appropriate  $\alpha$ -amino acid. Borchardt and co-workers (16) have shown that ( $\pm$ )Ado-D-Met was inactive as a substrate for catechol O-methyltransferase, phenylethanolamine N-methyltransferase and hydroxyindole O-methyltransferase but was a substrate for histamine N-methyltransferase with a slightly lower  $V_{max}$  and a  $K_m$  value substantially higher than that for the "L" isomer. Guanidinoacetate methyltransferase (EC 2.1.1.2) also uses both  $\alpha$ -amino acid enantiomers but at different rates (13) with the rate of ( $\pm$ )Ado-D-Met being quite slow with respect to the naturally occurring isomer. Furthermore, ACC synthase is a pyridoxal phosphate-containing enzyme and most pyridoxal phosphate enzymes with the exception of racemases, are known to show an enantiomeric preference for the  $\alpha$ -amino acid center (17).

Thus, the results shown in Figure 1 in which ACC synthase utilized neither sulfonium isomer of the "D" (R)  $\alpha$ -amino acid center is not without precedent.

From this study, we conclude that ACC synthase has a rather strict geometric requirement at the active site in regard to both the sulfonium pole and the  $\alpha$ -amino acid center. These results are not surprising, since both centers are very close to the site of reaction. Both regio- and stereo-specific isotopically labeled (-)Ado-L-Met samples are being prepared to determine the exact mechanism of the reaction, and additionally, further differences in binding specificity are being explored using various derivatives of AdoMet differing at the base, sugar and amino acid portions in an attempt to design specific inhibitors of ACC synthase.

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