Stereochemical course of the reaction catalyzed by the pyridoxal phosphate-dependent enzyme 1-aminocyclopropane-1-carboxylate synthase

(S-adenosylmethionine/deuterium-labeling/vinylglycine/enzyme mechanism)

KONDAREDDI RAMALINGAM†, KANG-MAN LEE†, RONALD W. WOODARD†‡, ANTHONY B. BLEECKER§, AND HANS KENDE§

†College of Pharmacy, The University of Michigan, Ann Arbor, MI 48109; and the ‡Michigan State University—Department of Energy Plant Research Laboratory, Michigan State University, East Lansing, MI 48824

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ABSTRACT  (±)-S-adenosyl-DL-(3R*,4S*),[3,4-2H2]-methionine [a 1:1 mixture of (3R,4S) and (3S,4R)] and (±)-S-adenosyl-DL-(3R*,4R*),[3,4-2H2]methionine [a 1:1 mixture of (3R,4R) and (3S,4S)] were synthesized from (Z)-[1,2-3H2]jethene, to give a 1:1 mixture of (R,R)- and (S,S)-1-chloro-2-[(methylthio)[1,2-3H2]jethene, followed by alkylation with sodium acetamidomalonate and hydrolysis to give an equal mixture of four stereoisomers of [3,4-2H2]methionine [(2R,3R,4S), (2R,3S,4S), (2S,3R,4S), and (2S,3S,4R)]. The other four stereoisomers of [3,4-2H2]methionine were prepared from (E)-[1,2-3H2]jethene. The two sets of stereoisomers of [3,4-2H2]methionine were chemically converted to S-adenosylhomocysteine, methylated to give the corresponding (±)-S-adenosyl-DL-methionines, and then incubated with 1-aminocyclopropane-1-carboxylate synthase partially purified from tomato (Lycopersicon esculentum, L.) pericarp tissue. The stereochemistry of the resulting samples of 1-aminocyclopropane-1-carboxylic acid was determined by comparison with the 1H NMR of the chemically synthesized and regio- and stereospecifically deuterated compound. The results indicate that the hydrogens at the β carbon of the methionine portion of S-adenosylmethionine do not participate in the reaction and that the ring closure occurs with inversion of configuration at the γ carbon of the methionine portion of S-adenosylmethionine, probably through a direct Sn2-type displacement of the S'-methylthio-S'-deoxygenadenosine moiety by a carbamion equivalent formed at the α carbon of the methionine portion of S-adenosylmethionine.

Although the compound was first synthesized by Perkin in 1884 (1), it was not until 1957 that Burroughs (2) isolated the cyclopropanoid amino acid 1-aminocyclopropane-1-carboxylic acid (ACC) from cider apples and perry pears. In 1979, Adams and Yang (3) demonstrated that this cyclic amino acid was the key intermediate in the bioconversion of methionine to ethylene, an endogenous plant hormone responsible for fruit-ripening as well as a number of other regulatory plant processes (4). It was suggested that conversion of S-adenosyl-L-methionine (AdoMet), a biologically activated form of methionine (5), to ACC was the rate-limiting reaction in the biosynthesis of ethylene (6). The enzyme that catalyzes the cyclization, ACC synthase, was first isolated from tomato pericarp by Boller et al. (7) and was shown to require pyridoxal phosphate as cofactor (8).

Enzymes that contain pyridoxal-P as the prosthetic group catalyze a variety of reactions at the α, β, and γ carbons of α-amino acids by one of several basic mechanisms (9). With the exception of reactions occurring at the γ carbon, the mechanism and stereochemistry of these enzyme reactions have been studied extensively (10). Within the set of pyridoxal-P enzymes catalyzing reactions at the α carbon, ACC synthase seems to represent a unique subset. In the γ-elimination reactions studied to date, the accepted mechanism is as follows: the loss of a γ-carbon substituent is generally facilitated by tautomerization of the α-diketimine to the ketimine and subsequent formation of a β-carbanion that can assist in the elimination of the γ-leaving group, thus forming a β,γ-unsaturated imine intermediate (11). The presence of this vinylglycine-type intermediate is consistent with experimental evidence obtained from previously studied γ-elimination reactions (12–14) in that one of the β-hydrogens is stereospecifically exchanged. However, the conversion of this β,γ-unsaturated imine to ACC by ACC synthase (see Fig. 4, mechanism A), if concerted, would violate Baldwin’s rules, which disallow the concerted process of three-member-ring formation by addition of a carbanion to a double bond in an endo fashion (15).

To gain insight into the mechanism of the reaction catalyzed by ACC synthase, we have initiated studies to probe the stereochemical events at the α, β, and γ carbons of the methionine portion of AdoMet during the cyclization process. The main stereochemical questions are (a) does replacement of the methine hydrogen of the α carbon by the new carbon bond proceed in an inversion or retention mode, (b) does the β carbon participate in the reaction mechanism and, if so, is the pro-R or pro-S hydrogen removed and does reprotonation of the β,γ-unsaturated imine occur on the re or si face of the cisoid or transoid form, and (c) does the displacement of the S'-methylthio-S'-deoxygenadenosine moiety at the γ carbon proceed with inversion or retention? In this paper, we report results that answer questions b and c.

MATERIALS AND METHODS

General. Organic chemicals were purchased from Aldrich; biochemicals, from Sigma; and E- and Z-[1,2-3H2]jethene (98.9% 3H and 96.5% 2H, respectively), from Merck Sharp & Dohme. All materials were used without further purification.

Melting points were determined with a Thomas–Hoover capillary melting-point apparatus and are uncorrected. TLC separations were carried out on Analtech silica gel plates. Evaporation under reduced pressure refers to solvent removal on a Buchi rotary evaporator at or below 40°C. 1H-NMR spectra were recorded on an IBM WP-270 (270 MHz) spectrometer. Chemical shifts are reported in ppm from

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The DL-(3R*,4R*)- and DL-(3S*,4R*)-[3,4-2H2]methionine samples were synthesized by the method of Billington and Golding (16) from E- and Z-[1,2-3H2]jethene, respectively. 1-Amino-(25*,35*)-[2,3-3H2]cyclopropene-1-carboxylic acid (trans-ACC) and 1-aminoc-(25*,3R*)-[2,3-3H2]cyclopropene-1-carboxylic acid (cis-ACC) were synthesized by the method of Ramalingam et al. (17) from Z- and E-[1,2-3H2]jethene, respectively.

5'-Adenosyl-DL-(3R*,4R*)-[3,4-3H2]Homocysteine. Small pieces of isolated DL- (3R*,4R*)-[3,4-3H2]methionine (16) (75 mg, 0.5 mmol) in liquid NH3 (25 ml) until the blue color persisted for 20 min. This solution was added to 5'-chloro-5'-deoxyadenosine (18) (189 mg, 0.66 mol) in portions, and the resulting mixture was stirred at -40°C for 6 hr. The NH3 was allowed to evaporate over 20–30 min with the last traces being removed under reduced pressure. The residue was dissolved in water (4 ml) and the aqueous solution was heated to reflux for 4 hr. The reaction mixture was adjusted to pH 6.5 with 1 M HCl and applied to a 90-ml column of Dowex 50X4-200 (NH4 form). The column was washed with water (150 ml), and then adsorbed material was eluted with 1 M NH4OH; 2-ml fractions were collected. Fractions 22–30, containing the product, were pooled and lyophilized. The crude product was purified by column chromatography on silica gel (9:0.5, reverse phase, 60, 70–230 mesh), using elution with butanol/acetic acid/water (3:2:5, vol/vol) to remove unreacted 5'-chloro-5'-deoxyadenosine. Two-milliliter fractions were collected, those showing a positive reaction with ninhydrin (fractions 30–56) were combined, and the solvent was removed under reduced pressure to yield a colorless solid. This product was homogeneous on TLC [silica gel, butanol/acetic acid/water (13:2:5)]. The product was further purified by ion-exchange chromatography on a 15-ml column of Dowex 50X4-200 (NH4 form): the column was washed with water to remove traces of acetic acid, and the purified product was eluted with 1 M NH4OH. Fractions containing the product were pooled and lyophilized to give a fluffy white solid (yield 55%): mp 216–218°C; 1H-NMR (D2O) δ 8.37 (1, C1H), 8.2 (1, C2H), 6.1 (1, J = 5.0 Hz, C1H), 4.48 (t, 1, J = 5.5 Hz, C2H), 4.48 (t, 1, J = 5.0 Hz, C3H), 4.31 (m, 1, C4H; 1H), 3.98 (d, 1, J = 6.3 Hz, C5H), 3.13 and 3.04 (m, 2, C6H), 2.75 (m, 1, C7H), 2.21 and 2.14 (t, 1, J = 7.7 Hz, C8H).

Fig. 1. Time-dependent formation of ACC from trans-AdoMet, determined by subjecting 30-µl aliquots of the incubation mixture to the assay procedure of Lizada and Yang (21). Final yield was 3.5 µmol of ACC (85%).
RESULTS

In order to obtain mechanistic information from a stereochemical study of an enzyme, four distinct tasks are necessary: (i) the synthesis of stereospecifically labeled substrates of known absolute configuration, (ii) enzymatic conversions of these substrates into the enzyme reaction products, (iii) analysis of these products to determine the resulting configuration of the stereospecifically labeled center(s) in the product, and (iv) interpretation of the results.

The synthesis of the required AdoMet samples, (−)-S-adenosyl-L-(3R*,4R*)-[3,4-2H2]methionine and (−)-S-adenosyl-L-(3R*,4R*)-[3,4,5-2H3]methionine, started, respectively, from Z- and E-[1,2-2H3]jethene, which were converted into 1-chloro-2-(methylthio)ethanes via reaction with methanesulfonyl chloride. Reaction of the chlorides with the sodium salt of diethyl acetalmidomalonate followed by acid hydrolysis gave the respective racemic methionine (16). Each of the two methionine samples produced consisted of a mixture of four stereoisomers. The enzymatic activation, via AdoMet synthetase, of either of these stereospecifically deuterated methionine mixtures into the corresponding AdoMet samples would have led to the desired species, (−)-S-adenosyl-L-(3R*,4R*)-[3,4-2H2]methionine and (−)-S-adenosyl-L-(3R*,4R*)-[3,4,5-2H3]methionine, in which both the sulfonium center and the amino acid center would have possessed the natural configuration, 2S, S(S) (22, 23). However, the laborious process of obtaining sufficient quantities of AdoMet synthetase and the relatively low yields in this enzymatic reaction forced us to activate the above methionines chemically, thus creating two mixtures, (±)-S-adenosyl-D-L-(3S*,4R*)-[3,4-2H2]methionine (cis-AdoMet) and (±)-S-adenosyl-D-L-(3R*,4R*)-[3,4,5-2H3]methionine (trans-AdoMet), each containing eight stereoisomers (2S = 16 total possible). However, it has been shown (24) that ACC synthase utilizes only naturally occurring AdoMet, which contains the L α-amino acid center in the methionine portion and the S configuration at the sulfonium center [S(S)]. Therefore only two isomers of the eight in each mixture will be transformed into ACC (i.e., those with the "S" configuration at both the sulfur center and the α-amino acid center: (−)-S-adenosyl-L-(3R*,4S*)-[3,4-2H2]methionine and (−)-S-adenosyl-L-(3R*,4R*)-[3,4,5-2H3]methionine). The two stereoisomeric samples of AdoMet (all eight stereoisomers in each mixture) were incubated separately with ACC synthase purified from tomatoes by the method of Acaster and Kende (20). Although the specific activity of the enzyme was quite low and each mixture contained several isomers of AdoMet that are known to be inhibitors (24) of ACC synthase, significant quantities of the natural AdoMet had been converted to ACC after 3 hr. The reaction products were extracted from the lyophilized reaction mixtures with 80% ethanol and purified first by preparative TLC on silica gel and then by ion-exchange chromatography. Because of the unstable nature of AdoMet in solutions at a pH > 5, attempts to reisolate the unreacted, inactive isomers of AdoMet proved futile.

The third task involved the determination of the configuration of the ACC samples produced. The product(s) obtained from the cis- and trans-AdoMet samples should be either cis- or trans-[2,3-3H2]ACC or, potentially, a monodeuterated ACC if there is any β-hydrogen exchange. Authentic samples of cis- and trans-[2,3-3H2]ACC were prepared chemically, following previously reported procedures (17), from the same E- and Z-[1,2-2H3]jethene used to prepare the AdoMet samples used in this study. Comparison of the 3H-NMR spectra of the chemically and enzymatically prepared [2,3-3H2]ACC samples allows the determination of the configuration of the enzymatically prepared products. Although we know that, in ACC, the two hydrogens cis to the carboxylate function resonate at δ = 1.14 ppm and the two hydrogens trans to the carboxylate function resonate at δ = 0.99 (unpublished results), in the present study it does not matter which is which. Since the chemically derived cis-[2,3-3H2]ACC sample is a racemic mixture at the α position, we have the sum of the two 3H-NMR spectra of the individual isomers (i.e., one isomer with two hydrogens cis to the carboxylate and one isomer with two hydrogens trans to the carboxylate). Thus the 3H-NMR spectrum of synthetic cis-[2,3-3H2]ACC (Fig. 2) shows singlets at δ = 1.14 ppm and δ = 0.99 ppm, which, when integrated, are in a 1:1 ratio. The 3H-NMR spectrum of the synthetic trans-[2,3-3H2]ACC shows doublets at δ = 1.14 ppm (J = 7 Hz, due to the coupling of the hydrogen cis to the carboxylate function to the hydrogen trans to the carboxylate function) and δ = 0.99 (J = 7 Hz), which, when integrated, are in a 1:1 ratio. The 3H-NMR spectrum of the racemic synthetic trans isomer is again a composite of the 3H-NMR spectra of the two stereoisomers, which in this case would each give the same spectra. The small amount of undeuterated ethene that contaminates the Z-[1,2-2H3]jethene is processed both chemically and enzymatically to give what appears in the 3H-NMR spectra to be some cis-[ACC] contaminating the trans-[ACC].

The 3H-NMR spectra of the enzymatic reaction mixtures are also shown in Fig. 2. The ACC products are also racemic at the α position and thus the 3H-NMR spectra will be composites of both isomers. As can be seen by comparison with the spectra of the synthetic samples, the ACC obtained from (−)-S-adenosyl-L-(3R*,4R*)-[3,4-2H2]methionine (trans-AdoMet) had the trans configuration, whereas the ACC obtained from (−)-S-adenosyl-L-(3S*,4R*)-[3,4,5-2H3]methionine (cis-AdoMet) had the cis configuration.

In order to interpret the results at the α- and/or the γ-center of the methionine portion of the stereoisomers of AdoMet, we constructed Fig. 3 to predict which ACC would be formed from which isomer of AdoMet depending on the stereochemical events at the α- and γ-positions, without considering racemization or β-participation as possible events. Also shown are the predicted individual 3H-NMR spectra of the two possible ACC species derived from the two enzymatically active isomers of AdoMet in each substrate sample. It should be remembered that the experimentally observed
The enzymatic conversion of AdoMet in the enzymatic conversion of AdoMet to ACC appears unlikely because the 1H-NMR spectra of the ACC samples obtained from the enzymatic reactions were identical to the spectra of the ACC samples chemically synthesized. The two enzymatically active AdoMet species in each incubation mixture contained a deuterium in either the 3R or 3S β-position. Therefore, the abstraction of a β-hydrogen (without isotopic discrimination between the two stereoisomers in each of the incubation mixtures) and subsequent exchange (either partial or total) of deuterons with protons of the medium would have resulted in the mass of deuterium from the β-position and the formation of an ACC species in which the integration of the peaks at δ = 1.14 ppm and δ = 0.99 ppm would not have been 1:1. The spectral pattern would also have been quite different. Since the ratio of the two hydrogen signals in the 1H-NMR spectra of the enzymatically produced ACC samples is exactly 1:1, each substrate mixture of isomers with deuterium in the pro-3R and pro-3S position would have had

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**Fig. 3.** Potential stereospecifically labeled ACC products obtained from the mixture S-adenosyl-DL-[3S*,4R*]methionine (trans-AdoMet) (Left) and from the mixture S-adenosyl-DL-[3S*,4R*]methionine (cis-AdoMet) (Right) by various combinations of stereochemical events at the α- and γ-positions such as α-retention and γ-retention (A); α-retention and γ-retention (B): α-inversion and γ-retention (C); and α-inversion and γ-inversion (D). This scheme does not consider β-participation or racemization as possible events. For each mixture, the two enzymatically active AdoMet substrates are depicted, and the other six isomers are only listed. The 270-MHz 1H-NMR spectrum of each ACC product is shown below the structure of the product. Ad, 5'-deoxyadenosyl; D, deuterium.
to react at equal rates. If there were hydrogen abstraction at the β carbon, it would be likely to involve an isotope effect, resulting in greater conversion of the isomer carrying a normal hydrogen in the position from which the hydrogen is abstracted. Hence the ratio of the two proton signals in the product would again be different from 1:1. The only process consistent with our findings, which would involve β-hydrogen (deuterium) abstraction, would have necessitated the removal of a β-hydrogen with no isotopic discrimination, followed by 100% return of the removed hydrogen (or deuterium) again with no isotope effect. Since this is highly unlikely, the most logical explanation of the data is that there is no β-hydrogen participation.

From the 1H-NMR spectra of the enzymatically produced ACC samples, one can see that (−)-S-adenosyl-L-(3R*,4R*)-[3,4-2H2]methionine (trans-AdoMet) gave trans-ACC and (−)-S-adenosyl-L-(3S*,4R*)-[3,4-2H2]methionine (cis-AdoMet) gave cis-ACC. Based on Fig. 3, this is consistent with α-inversion, γ-inversion or α-retention, γ-inversion. Although the stereochemical events at the α carbon consistent with the experimental results are ambiguous, the results clearly demonstrate that the stereochemical events at the γ carbon involve overall inversion of configuration. Such an S1,S2 mechanism was suggested by Yang and coworkers (3, 8), albeit their prediction was not based on experimental evidence.

From our experimental evidence, we conclude that the reaction involves inversion of configuration at the γ carbon and that the reaction does not involve exchange of β-hydrogens with the solvent. These two results suggest that (i) the reaction proceeds by direct displacement of the 5'-methylthio-5'-deoxyadenosine moiety at the γ carbon by an α-carbon carbanion (see Fig. 4, mechanism B) and (ii) the reaction does not involve a vinylglycine or similar intermediate (see Fig. 4, mechanism A), unless such an intermediate is formed and further transformed (α) with 100% internal hydrogen return and (b) without any significant isotope effect in the abstraction of a β-hydrogen. This apparent lack of β-participation and direct γ-displacement makes the reaction mechanism of ACC synthase unique among the pyridoxal-P-mediated γ-replacement reactions studied to date.

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