Reaction of 1-Amino-2-methylenecyclopropane-1-carboxylate with 1-Aminocyclopropane-1-carboxylate Deaminase: Analysis and Mechanistic Implications †

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Reaction of 1-Amino-2-methylenecyclopropane-1-carboxylate with 1-Aminocyclopropane-1-carboxylate Deaminase: Analysis and Mechanistic Implications†

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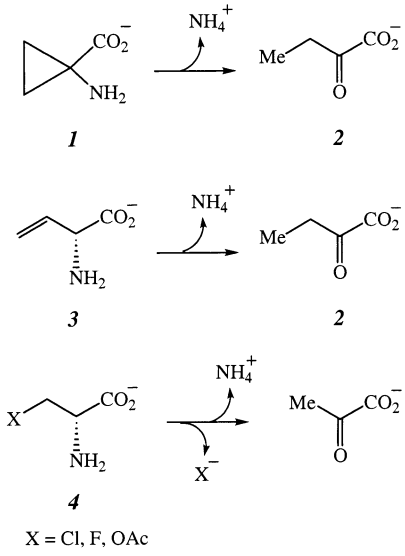
ABSTRACT: 1-Aminocyclopropane-1-carboxylate (ACC) deaminase is a pyridoxal 5′-phosphate (PLP) dependent enzyme which catalyzes the opening of the cyclopropane ring of ACC to give α-ketobutyric acid and ammonia. In an early study of this unusual $C_\alpha-C_\beta$ ring cleavage reaction, 1-amino-2-methylenecyclopropane-1-carboxylic acid (2-methylene-ACC) was shown to be an irreversible inhibitor of ACC deaminase. The sole turnover product was identified as 3-methyl-2-oxobutenoic acid. These results provided strong evidence supporting the ring cleavage of ACC via a nucleophilic addition initiated process, thus establishing an unprecedented mechanism of coenzyme B_6 dependent catalysis. To gain further insight into this inactivation, tritiated 2-methylene-ACC was prepared and used to trap the critical enzyme nucleophiles. Our results revealed that inactivation resulted in the modification of an active site residue, Ser-78. However, an additional 5 equiv of inhibitor was also found to be incorporated into the inactivated enzyme after prolonged incubation. In addition to Ser-78, other nucleophilic residues modified include Lys-26, Cys-41, Cys-162, and Lys-245. The location of the remaining unidentified nucleophile has been narrowed down to be one of the residues between 150 and 180. Labeling at sites outside of the active site is not enzyme catalyzed and may be a consequence of the inherent reactivity of 2-methylene-ACC. Further experiments showed that Ser-78 is responsible for abstracting the α-H from D-vinylglycine and may serve as the base to remove the *β*-H in the catalysis of ACC. However, it is also likely that Ser-78 serves as the active site nucleophile that attacks the cyclopropane ring and initiates the fragmentation of ACC, while the conserved Lys-51 is the base required for *β*-H abstraction. Clearly, the cleavage of ACC to α-ketobutyrate by ACC deaminase represents an intriguing conversion beyond the common scope entailed by coenzyme B_6 dependent catalysts.

1-Aminocyclopropane-1-carboxylate (ACC)[1] deaminase, originally isolated from *Pseudomonas* grown on ACC (**1**) as the sole nitrogen source, is a pyridoxal 5′-phosphate (PLP) linked catalyst with a unique capacity to break the cyclopropane ring of ACC to give α-ketobutyric acid (**2**) and ammonia (Scheme 1) (*1*). Although the catalytic roles of PLP are amazingly versatile, the common theme among the various catalytic transformations of PLP-dependent enzymes is the ability of this cofactor to act as an electron sink,

Scheme 1



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[1] Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; DTNB, 5,5′-dithiobis(2-nitrobenzoate); 2-methylene-ACC, 1-amino-2-methylenecyclopropane-1-carboxylic acid; DEAE, diethylaminoethyl; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; IPTG, isopropyl thio-*β*-galactoside; LB, Luria−Bertani; PCR, polymerase chain reaction; PLP, pyridoxal 5′-phosphate; PMSF, phenylmethanesulfonyl fluoride; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TCPK, L-1-tosylamido-2-phenylethyl chloromethyl ketone; TMS, tetramethylsilane; WT, wild type.

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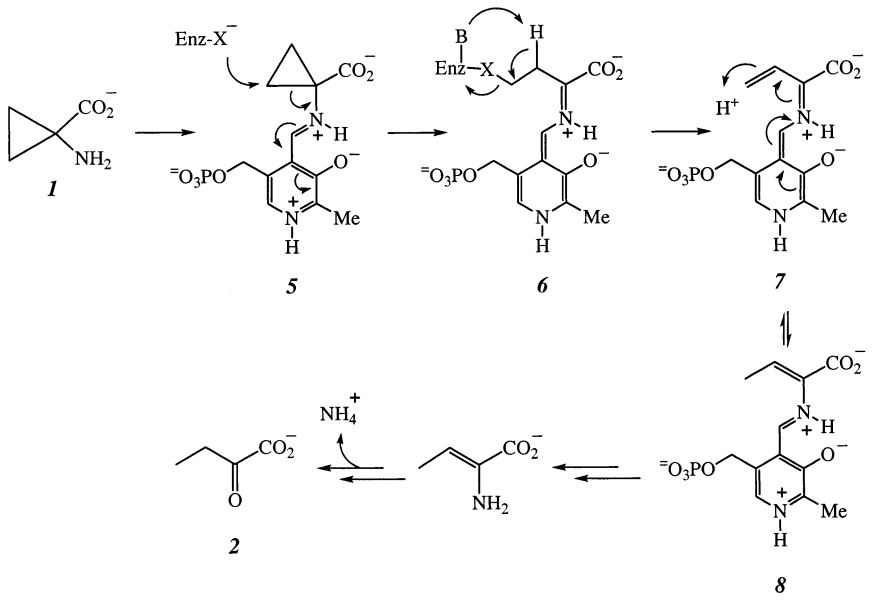
Scheme 2



temporarily storing electrons that are later used for the cleavage and/or formation of covalent bonds (*2*). The cleavage of ACC (**1**) by ACC deaminase appears to represent an intriguing conversion beyond the scope of established B_6 chemistry because the α-carbon of ACC is a quaternary carbon and the carboxyl group of ACC is retained in the product. Thus, it is puzzling how the ring opening step is initiated without obvious accessibility to a carbanionic intermediate at the α-C. Precedence for such a formal cleavage of the $C_\alpha-C_\beta$ bond of an amino acid is found only in one other PLP-dependent enzyme, serine transhydroxymethylase, which catalyzes the conversion of serine to glycine and a formaldehyde equivalent through a retro-aldol-type process (*3*). However, this route is not possible for an ACC−PLP complex. Thus, ACC deaminase must adapt an unusual mechanism to accomplish this unique ring cleavage reaction.

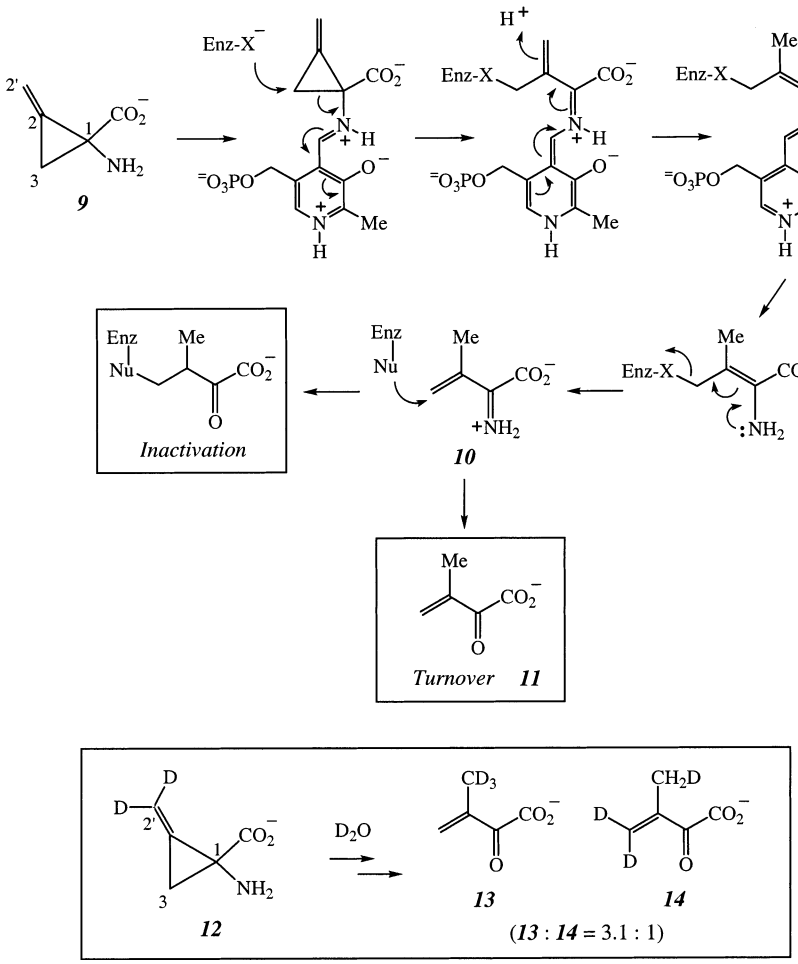
The ACC deamination process is also of agricultural significance. Since ACC is the precursor of an essential phytohormone, ethylene (*4*), an approach to reduce ethylene formation in plants by diverting the metabolism of its immediate precursor, ACC, will certainly have profound impact on many plant developmental processes. In fact, in vivo tests have clearly demonstrated that ACC deaminase is useful for examining the role of ethylene in many developmental and stress-related actions in plants and for extending the shelf life of fruits and vegetables whose ripening are mediated by ethylene (*5*). Recently, it was found that plant growth-promoting bacteria might stimulate plant growth through manipulation of the activity of ACC deaminase (*6*). Thus, studies of the reaction catalyzed by ACC deaminase are not only important for understanding this unique enzymatic conversion but will also be valuable toward the design of methods to regulate the ethylene biosynthetic process.

Early studies showed that ACC deaminase recognizes a variety of monosubstituted ACC derivatives as alternate substrates (*7*). It also catalyzes the deamination of D-vinylglycine (**3**) and the elimination of β-substituted amino acids (**4**) (see Scheme 1) (*8a*). The α-anion equivalent of the vinylglycyl-PLP aldimine (**7**, Scheme 2) has been established as the key intermediate in all cases, including the conversion of **1** to **2** (*8a*). It was found that the ring cleavage of **1** catalyzed by ACC deaminase occurs exclusively between the α-C and the *pro-S* β-CH_2 group (*9*), and the abstraction of the β-H is *pro-R* stereospecific (*10*). On the basis of these and other observations, a mechanism initiated by a nucleophilic addition to open the ring (**5** → **6**) followed by β-proton abstraction (**6** → **7**) has been proposed for the catalysis of ACC deaminase (Scheme 2) (*8*−*11*). It should be pointed out that Lys-51, which binds PLP in ACC deaminase (*12*), is expected to play a significant role in ACC turnover because the K51A mutant, which is partially constituted with PLP, is catalytically inactive (*13*).

Recently, we showed that 1-amino-2-methylenecyclopropane-1-carboxylic acid (**9**, 2-methylene-ACC) is an irreversible inhibitor of ACC deaminase, and 3-methyl-2-oxobutenoic acid (**11**) is the sole turnover product upon incubation of **9** with the enzyme (Scheme 3) (*14*). Analysis of **13** isolated from the incubation of ACC deaminase with **12** having a dideuterated exocyclic methylene moiety further revealed that addition to C-3 instead of C-2′ is the preferred mode of nucleophilic attack in the active site of ACC deaminase (Scheme 3) (*14*), since alternate addition at C-2′ led to a minor isomer, **14**, with the two deuteriums in the vinyl methylene group. These observations are most consistent with an inactivation mechanism illustrated in Scheme 3 and provide compelling evidence for ring cleavage of ACC as a nucleophilic addition initiated event (Scheme 2) (*14*, *15*).

To gain further insight into this unique enzymatic reaction, a detailed mechanistic inquiry to identify the amino acid residue modified by 2-methylene-ACC (**9**) and to explore its role in catalysis was performed. Toward this goal, a tritium-labeled 2-methylene-ACC ([2′-^{3}H]-**9**) was synthesized and used to inactivate ACC deaminase. To our surprise, more than 1 equiv of **9** was found to be incorporated into the inactivated enzyme. Subsequent biochemical and mutagenesis study have led to the identification of most of the labeled residues including Ser-78, which is likely to play a significant role in ACC deaminase catalyzed reactions. Reported herein are our results and the mechanistic implications of this study.

Scheme 3



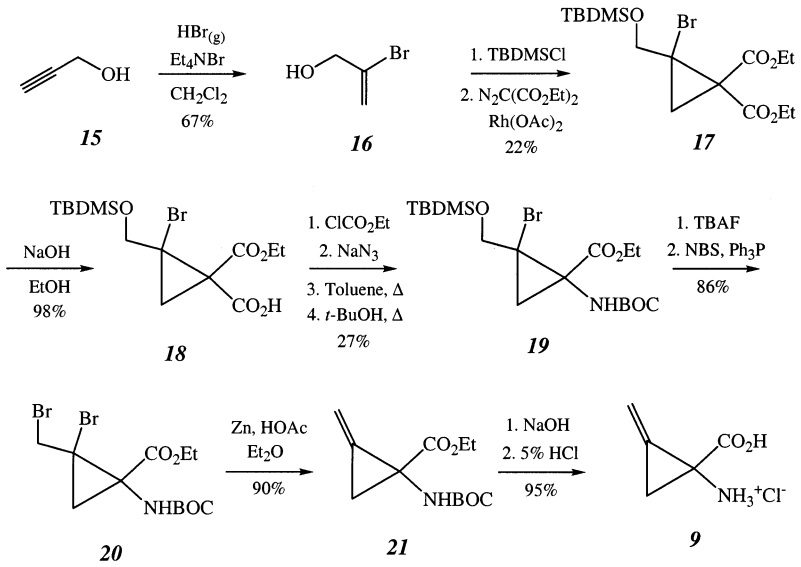
EXPERIMENTAL PROCEDURES

General. NMR spectra were recorded on a Varian 300 MHz spectrometer. Chemical shifts (δ in parts per million) are given relative to those for TMS (for ^{1}H and ^{13}C), with coupling constants in hertz (Hz). Fast atom bombardment (FAB) and chemical ionization (CI) mass spectra were recorded by the MS facility at the Department of Chemistry, University of Minnesota. Analytical thin-layer chromatography was carried out on Merck silica gel 60 GF-254 plates, and the spots were visualized either under UV light or by heating plates previously stained with solutions of vanillin/methanol/H_2SO_4 (0.75:97.75:1.5) or phosphomolybdic acid (4% in EtOH). The concentration of protein was determined by Bradford's method (*16*) using bovine serum albumin as the standard. The relative molecular mass and purity of enzyme samples were determined using SDS−polyacrylamide gel electrophoresis as described by Laemmli (*17*). The native molecular masses of these protein samples were determined by a gel filtration method reported by Andrews (*18*). Sequencing of the tryptic peptide fragments was carried out at the Protein Core Facility at the Mayo Clinic, Rochester, MN. MS analyses of the protein and the digested peptide samples were performed at the Mass Spectrometry Facility at the University of British Columbia, Vancouver. DNA sequencing was conducted by the Advanced Genetic Analysis Center of the University of Minnesota, St. Paul.

Materials. Plasmid pCGN1472, used as the PCR template for the ACC gene, was kindly provided by Calgene (Davis, CA) (*12*). The pET-17b(+) vector and overexpression host strain *Escherichia coli* BL21(DE3)/pLysS were purchased from Novagen Inc. (Madison, WI). *E. coli* strain HB101 was acquired from Bethesda Research Laboratories (Gaithersburg, MD). Culture media were products of Difco (Detroit, MI), and DEAE-Sepharose CL-6B as well as Sephacryl S-200 HR resin were purchased from Pharmacia (Piscataway, NJ). Cloned DNA polymerase *Pfu* and GeneClean DNA purification kits were obtained from Stratagene (La Jolla, CA) and Bio 101 Inc. (La Jolla, CA), respectively. All electrophoretic reagents, except for the DNA ladders (100 bp and 1 kp) and agarose which were obtained from Gibco BRL (Grand Island, NY), were products of Bio-Rad (Hercules, CA). DNA minipreps were performed with the Wizard DNA purification kits from Promega (Madison, WI). Oligonucleotides used in the PCR amplification of desired inserts were prepared by Gibco BRL and used without further purification. Restriction endonucleases were either from Gibco BRL or Promega. Pepsin and TPCK-treated trypsin were purchased from Sigma. The synthesis of D-vinylglycine (**3**) was based on a literature procedure (*19*) for the preparation of L-vinylglycine with minor modification, while 3-methyl-2-oxobutenoic acid (**11**) was prepared by a known procedure (*20*).

Subcloning of the ACC Deaminase Gene. Two oligonucleotide primers complementary to the sequence at each end of the ACC deaminase gene were prepared to amplify the gene from the plasmid pCGN1472 (*12*). The start primer

Scheme 4



5′-CGCG**CATATG**AACCTGCAACGA-3′ contains an *Nde*I restriction site (in bold), and the halt primer 5′-GCGC-**GAGCTC**TCAGCCGTCTCGGAAGA-3′ contains an *Sac*I restriction site (in bold) immediately downstream of the stop codon. The PCR-amplified DNA fragment was purified, digested with *Nde*I and *Sac*I, and ligated into the *Nde*I/*Sac*I sites of pET17b(+). The resulting construct, pQNst23, was then used to transform *E. coli* BL21(DE3)pLysS. Methods and protocols for recombinant DNA manipulations were carried out according to the manufacturers' manuals or from general references (*21*).

Purification of the Recombinant Wild-Type ACC Deaminase. An overnight culture of *E. coli* BL21(DE3)pLysS/pQNst23, grown in LB medium supplemented with ampicillin (100 μg/mL) at 37 °C, was diluted 200-fold to 3 L of the same medium and incubated at 37 °C until the OD_{600} reached 0.6. The culture was then cooled to 24 °C, induced with IPTG to a final concentration of 240 μM, and allowed to grow for an additional 6 h at 24 °C. The cells were harvested by centrifugation at 6500*g* for 5 min at 4 °C, washed with 50 mM potassium phosphate buffer (pH 7.5), and stored at −20 °C. The typical yield was 3.3 g of wet cells/L of culture. Thawed cells (10 g) were resuspended in 50 mL of 50 mM potassium phosphate buffer (pH 7.5) supplemented with 0.6 mM PMSF and 5 mM EDTA and disrupted by sonication burst. Cell debris was removed by centrifugation at 16000*g* for 25 min. The supernatant was subjected to ammonium sulfate fractionation (20−60%). The precipitate formed in the 60% saturated solution was collected, redissolved, and purified by DEAE-Sepharose (eluted with a linear gradient of 0−0.5 M KCl in 50 mM potassium phosphate buffer, pH 7.5) followed by Sephacryl S-200 chromatography (eluted with 50 mM potassium phosphate buffer, pH 7.5). The purified protein was concentrated (Amicon YM-10) and stored at −80 °C.

Site-Directed Mutagenesis and Purification of Mutants of ACC Deaminase. The C41A and C41S mutations of ACC deaminase were made using the Sculpture kit from Amersham Life Science (Piscataway, NJ). The mutagenesis primer for the C41A mutation is 5′-GCGAAACGCGAAGAC**GC-G**AACAGCGGCCTGGCG-3′, and that for the C41S mutation is 5′-GCGAAACGCGAAGAC**AGC**AACAGCGGC-CTGGCG-3′, where the sequence in bold denotes the codon change for the respective mutations. Both mutant genes were cloned into a pET17b(+) vector for protein production. The S78A and S78C mutants were prepared using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA), and the following primers were used to introduce the point mutations (where the sequence in bold denotes the codon change for the respective mutations): S78A (forward), 5′-GCGGCATTCAG**GCG**AACCAGACGCGCCAG-3′; S78A (reverse), 5′-CGCGTCTGGTT**CGC**CTGAATGCCGCCG-ATC-3′; S78C (forward), 5′-GGCGGCATTCAG**TGT**AAC-CAGACGCGCCAG-3′; and S78C (reverse), 5′-GCGCGTC-TGGTT**ACA**CTGAATGCCGCCGATC-3′. The mutant proteins were purified by the same procedure described for the wild-type enzyme.

Activity Assay. A standard 0.5 mL assay was performed in 100 mM potassium phosphate buffer (pH 8.0) at 25 °C, containing 5 mM substrate, 7.5 units of L-lactate dehydrogenase from rabbit muscle (Sigma, St. Louis, MO), 0.3 mM NADH, and an appropriate amount of deaminase (*8a*). The reaction was initiated by the addition of enzyme solution, and the decrease in absorption of NADH was monitored at 340 nm (ϵ_m = 6220 $M^{-1}\cdot cm^{-1}$). To determine the kinetic parameters of the catalysis, the assay was performed as described above using a fixed amount of deaminase, while the ACC concentration was varied from 1 to 14.0 mM. The experimental data were fit into the Michealis−Menten equation to determine the K_m and k_{cat} values.

*Synthesis of 1-Amino-2-methylenecyclopropane-1-carboxylic Acid (**9**) (14).* The synthesis of 2-methylene-ACC (**9**) was accomplished according to the reaction sequence outlined in Scheme 4. The cyclopropane ring was constructed via a rhodium acetate catalyzed [2 + 1] insertion of diethyl diazomalonate to a protected 2-bromo-2-propen-1-ol (**16**) (*22*). The resulting product **17** was converted to an acyl azide intermediate which, upon refluxing with toluene and *t*-BuOH, led to the formation of the protected cyclopropylamine **19** via a Curtius rearrangement (*23*). The *tert*-butyldimethylsilyl (TBDMS) protecting group in **19** was removed, and the exposed primary hydroxyl group was converted to the

corresponding dibromide **20** by treatment with *N*-bromosuccinimide (NBS) and triphenylphosphine. Subsequent debromination using zinc dust in acetic acid generated the exocyclic double bond in **21**. Removal of the protecting groups was achieved using NaOH followed by 5% aqueous HCl to give **9**, isolated as a hydrochloride salt.

*2-Bromo-2-propen-1-ol (**16**).* A suspension of tetraethylammonium bromide (46 g, 0.22 mol) in CH_2Cl_2 (200 mL) cooled at 0 °C was bubbled with hydrogen bromide until the white solid dissolved. This brown-yellow solution was warmed to room temperature, treated with propargyl alcohol (**15**, 12 mL, 0.21 mol), and heated at reflux for 5 h. The reaction mixture was then cooled and chilled in an ice−water bath. Diethyl ether (600 mL) was added to this solution, and the white precipitate formed was filtered off. The filtrate was concentrated, and the brown residual oil was distilled under reduced pressure (ca. 10 mmHg) to give the crude product (bp 80 °C). After further purification by column chromatography on silica gel (10% diethyl ether in hexanes), the desired bromide **16** was isolated as a colorless liquid (19.8 g, 66%). ^{1}H NMR ($CDCl_3$): δ 5.92 (1H, m), 5.58 (1H, m), 4.21 (2H, m).

*Ethyl 2-Bromo-2-(tert-butyldimethylsiloxymethyl)cyclopropane-1,1-dicarboxylate (**17**).* To a solution of the bromide **15** (19.8 g, 0.15 mol), imidazole (21.8 g, 0.32 mol), and 4-(dimethylamino)pyridine (0.92 g, 7.5 mmol) in dry CH_2Cl_2 (300 mL) was added *tert*-butyldimethylsilyl chloride (24.1 g, 0.16 mol) at 0 °C. The reaction was allowed to proceed with stirring at room temperature for 24 h and then quenched with water. The aqueous layer was extracted with CH_2Cl_2, and the combined organic extracts were washed with brine (200 mL), dried over anhydrous sodium sulfate, and concentrated under reduced pressure. The residual liquid was chromatographed on silica gel (5% diethyl ether in hexanes) to give the silyl ether intermediate as a pale yellow liquid (19.8 g, 54%). ^{1}H NMR ($CDCl_3$): δ 5.95 (1H, m), 5.53 (1H, m), 4.20 (2H, m), 0.91 (9H, s), 0.11 (6H, s).

To a stirred mixture of the above silyl ether (19.8 g, 78.8 mmol) and rhodium(II) acetate dimer (0.2 g, 0.45 mmol) at 50 °C was added diethyl diazomalonate (16.3 g, 87.6 mmol) through a syringe pump at a rate of 0.15 mL/h under argon. After the addition, the resulting mixture was stirred at the same temperature until the reaction was complete. The brown liquid was cooled and loaded onto a silica gel column, which was washed first with hexanes and then with 10% ethyl acetate in hexanes to give **17** as a yellow oil (13.2 g, 41%). ^{1}H NMR ($CDCl_3$): δ 4.20 (4H, m), 4.09 (2H, s), 2.01 and 1.96 (2H, AB q, J = 6.9), 1.29 (6H, m), 0.87 (9H, s), 0.05 (6H, s). ^{13}C NMR ($CDCl_3$): δ 166.5, 166.4, 66.4, 62.3, 62.1, 42.2, 39.0, 25.9, 24.8, 18.5, 14.3, 14.2, −5.1, −5.2.

*1-Ethoxycarbonyl-2-bromo-2-(tert-butyldimethylsiloxymethyl)cyclopropane-1-carboxylic Acid (**18**).* To a solution of **17** (9.1 g, 22.2 mmol) in ethanol (45 mL) was added a solution of NaOH (0.93 g, 23.3 mmol) in water (6 mL) at 0 °C. The mixture was stirred at room temperature for 2 days. The solvent was then removed in vacuo to leave a brown gel residue, which was dissolved in water, acidified with 1 N HCl, and extracted thoroughly with ethyl acetate. The organic extracts were combined and dried over anhydrous sodium sulfate. Removal of the solvent yielded **18** as a yellowish oil (8.3 g, 98%). ^{1}H NMR ($CDCl_3$): δ 4.30 (2H, q, J = 7.1), 3.90 and 3.75 (2H, AB q, J = 11.4), 2.33 and 2.25 (2H, AB q, J = 7.3), 1.33 (3H, t, J = 7.1), 0.89 (9H, s), 0.07 (3H, s), 0.05 (3H, s).

*Ethyl 2-Bromo-1-(tert-butoxycarbonyl)amino-2-(tert-butyldimethylsiloxymethyl)cyclopropane-1-carboxylate (**19**).* To a suspension of compound **18** (8.2 g, 21.7 mmol), anhydrous potassium carbonate (6.25 g, 45.2 mmol), and dicyclohexane-18-crown-6 (84 mg, 0.23 mmol) in dry THF (50 mL) was added ethyl chloroformate (2.7 g, 24.9 mmol) at 0 °C. The resulting mixture was stirred for 2 h at room temperature, and the precipitate was removed by filtration. To the filtrate was added aqueous sodium azide (1 M, 25 mL) at 0 °C. After being stirred at the same temperature for 1 h, the reaction was quenched by the addition of ice chips (1 volume of the reaction mixture). The mixture was then extracted with diethyl ether, and the pooled reddish brown organic extracts were dried over anhydrous sodium sulfate. The solvents were removed, and the residue was purified on a silica gel column eluted with pentane to give the acyl azide intermediate, which was dissolved in toluene (20 mL) and heated to reflux under N_2 overnight. Dry *tert*-butyl alcohol was then added, and the mixture was refluxed for an additional 12 h. The solvents were removed in vacuo, and the residue was washed with ethyl acetate. The white precipitate was filtered off, and the filtrate was concentrated and purified by silica gel chromatography (10% ethyl acetate in hexanes) to give the desired product **19** as a colorless oil (2.65 g, 27% from **18**). ^{1}H NMR ($CDCl_3$): δ 5.75 (1H, br s), 4.28 and 3.81 (2H, AB q, J = 11.8), 4.24 (2H, q, J = 7.1), 2.31 (2H, m), 1.41 (9H, s), 1.28 (3H, t, J = 7.1), 0.92 (9H, s), 0.13 (3H, s), 0.10 (3H, s). ^{13}C NMR ($CDCl_3$): δ 168.7, 155.7, 80.6, 70.4, 62.0, 44.9, 41.6, 28.5, 28.1, 26.0, 18.5, 14.5, −4.9, −5.2.

*Ethyl 2-Bromo-2-bromomethyl-1-(tert-butoxycarbonyl)-aminocyclopropane-1-carboxylate (**20**).* To a solution of **19** (2.6 g, 5.7 mmol) in THF (20 mL) was added dropwise a solution of tetrabutylammonium fluoride (1 M in THF, 15 mL) at 0 °C. The resulting mixture was stirred at room temperature for 2 h. The solvent was removed, and the residue was purified by column chromatography on silica gel (20% ethyl acetate in hexanes) to give ethyl 2-bromo-2-hydroxymethyl-1-(*tert*-butoxycarbonyl)aminocyclopropane-1-carboxylate as a colorless oil. ^{1}H NMR ($CDCl_3$): δ 5.51 (1H, br s), 4.23 (2H, q, J = 7.1), 4.15 (1H, d, J = 12.9), 3.78 (1H, d, J = 12.9), 2.33 (1H, d, J = 7.5), 1.49 (1H, d, J = 7.5), 1.44 (9H, s), 1.29 (3H, t, J = 7.1). ^{13}C NMR ($CDCl_3$): δ 168.7, 156.6, 81.2, 68.1, 62.2, 44.6, 43.7, 28.3, 27.0, 14.2.

To a stirred solution of triphenylphosphine (1.8 g, 6.9 mmol) in dry methylene chloride (20 mL) was added *N*-bromosuccinimide (NBS, 1.23 g, 6.9 mmol) at −78 °C in the dark. Stirring was continued until all of the NBS had dissolved. A solution of the above cyclopropylcarbinol in dry CH_2Cl_2 (10 mL) was added dropwise, and the resulting mixture was stirred for 2 h at room temperature. Dry methanol (1 mL) and toluene (10 mL) were added, and the solvents were coevaporated in vacuo. The residue was purified by column chromatograghy on silica gel (10% ethyl acetate in hexanes) to afford the dibromide **20** as a yellow oil (2.0 g, 86%). ^{1}H NMR ($CDCl_3$): δ 5.61 (1H, br s), 4.25 (2H, m), 4.04 and 3.73 (1H, d, J = 11.4), 2.50 (1H, d, J = 7.8), 1.71 (1H, d, J = 7.8), 1.44 (9H, s), 1.30 (3H, t, J = 7.1).

*Ethyl 1-(tert-Butoxycarbonyl)amino-2-methylenecyclopropane-1-carboxylate (**21**).* To a solution of **20** (150 mg, 0.37 mmol) in a solvent mixture of diethyl ether and glacial acetic acid (20:1 v/v, 2 mL) was added zinc dust (485 mg, 7.5 mmol), and the resulting mixture was stirred at room temperature for 40 h. This acidic solution was neutralized with a saturated sodium bicarbonate solution, and the zinc dust was filtered off. The filtrate was extracted with ether (3 × 5 mL), and the combined organic layers were washed with brine and dried over anhydrous sodium sulfate. The solvent was removed, and the residue was chromatographed on silica gel eluted with 10% ethyl acetate in hexanes to give **21** as a colorless oil (81 mg, 90%). ^{1}H NMR ($CDCl_3$): δ 5.72 (1H, m), 5.56 (1H, m), 5.18 (1H, br s), 4.16 (2H, q, J = 7.1), 2.35 (1H, m), 1.77 (1H, m), 1.45 (9H, s), 1.23 (3H, t, J = 7.1). ^{13}C NMR ($CDCl_3$): δ 171.0, 155.9, 133.6, 105.7, 80.1, 61.4, 35.9, 28.3, 20.8, 14.1.

*1-Amino-2-methylenecyclopropane-1-carboxylic Acid (**9**).* To a solution of **21** (50 mg, 0.2 mmol) in methanol (5 mL) was added sodium hydroxide (10 mg, 0.25 mmol) in water (2 mL). The mixture was stirred for 2 days at room temperature. The solvents were removed in vacuo, and the residue was dissolved in water and extracted with ethyl acetate. The extracts were washed with 1 N HCl, dried over anhydrous sodium sulfate, and concentrated in vacuo. The oily residue was dissolved in 5% HCl (5 mL) and stirred for 4 h at room temperature. The solution was evaporated under reduced pressure to leave a white solid. This white solid was triturated with ether (3 × 5 mL) and dried overnight under vacuum to afford 2-methylene-ACC (**9**) as a white solid (28 mg, 95%). ^{1}H NMR (D_2O): δ 5.92 (1H, m), 5.84 (1H, m), 2.38 (1H, m), 2.09 (1H, m). ^{13}C NMR (D_2O): δ 171.3, 127.3, 109.7, 53.6, 17.3. High-resolution MS (CI): calcd for $C_5H_8NO_2$ ($M + H^+$), 114.0555; found, 114.0555.

*Synthesis of [2′-^{3}H]-1-Amino-2-methylenecyclopropane-1-carboxylic Acid ([2′-^{3}H]-**9**).* The synthesis of [2′-^{3}H]-**9** followed the protocol developed for the synthesis of unlabeled **9**. The starting material, the tritium-labeled propargyl alcohol (**22**), was prepared in two steps from unlabeled propargyl alcohol (**15**) as shown in Scheme 5. Specifically, to a 1 L three-necked flask connected to two sequential traps cooled to −40 and −78 °C, respectively, was added propargyl alcohol (40 mL, 0.69 mol) and a cold solution of sulfuric acid (4.2 M, 200 mL) at 0 °C. The pressure of the whole system was reduced to about 40 mmHg by an aspirator, and a solution of chromium oxide, which was prepared by mixing chromium oxide (70 g, 0.7 mol) in concentrated sulfuric acid (45 mL) and water (133 mL), was added dropwise to the reaction at such a rate that the internal temperature of the reaction mixture was maintained at 2−10 °C by a salt−ice bath. After the addition was completed (about 3−4 h), the salt−ice bath was removed, and the reaction was stirred at room temperature for 1 h. The propiolaldehyde product collected in the two traps was combined and poured into a separatory funnel. The lower aqueous layer was removed and discarded. The neat compound was collected and dried over anhydrous sodium sulfate. This aldehyde was used in the next step without purification.

A small ampule containing [^{3}H]$NaBH_4$ (100 mCi) was opened in a glovebag under nitrogen atmosphere. The content was suspended in THF (5 mL) and then transferred to a 250 mL flask chilled at 0 °C. The ampule was rinsed twice with a small amount of THF (ca. 2 mL), and the rinse solutions were also added to the reaction flask. A solution of the aldehyde obtained above (4.1 g, 76 mmol) in THF (20 mL) was pretreated with a trace amount of $NaBH_4$ and then added dropwise to the [^{3}H]$NaBH_4$ suspension. The resulting mixture was stirred at room temperature for 4 h, after which the reaction was cooled to 0 °C and treated with $NaBH_4$ (2.9 g, 76 mmol) in THF (50 mL) in portions. The reaction mixture was warmed to room temperature and stirred for an additional 3 h. After the reaction was complete, the mixture was acidified with 1 N acetic acid at 0 °C until the bubbling stopped. The resulting mixture was extracted with ether (3 × 50 mL), and the organic extracts were pooled, dried, and concentrated. The isolated [1-^{3}H]propargyl alcohol (**22**) had a specific activity of 0.44 mCi/mmol.

The [1-^{3}H]propargyl alcohol (**22**) was processed to [2′-^{3}H]-**9** by the method used to synthesize the unlabeled compound **9**. The specific activity of the final product was 29.2 μCi/mmol. In a separate experiment, another sample of [2′-^{3}H]-**9** with a specific activity of 3.90 mCi/mmol was prepared by a more efficient synthetic sequence (*24*). Both samples were used in the labeling experiments reported in this paper as indicated.

*Inhibition of ACC Deaminase by 2-Methylene-ACC (**9**).* The effect of **9** on the activity of ACC deaminase was examined by incubating the enzyme (ca. 90 μg) with various concentrations of **9** (0, 0.1, 0.3, 0.5, 0.8, and 1.0 mM) in 50 mM potassium phosphate buffer (pH 7.5). Each 1 mL assay mixture also contained NADH (50 μM), L-lactate dehydrogenase (50 units), and various amounts of ACC (**1**) (2.0, 3.0, and 4.0 mM). After each sample was incubated for 5 min, the reaction was initiated by the addition of ACC deaminase. The decrease of absorption at 340 nm was monitored. A plot of the reciprocal of the reaction rate versus the concentration of **9** was used to determine the K_I.

*Time-Dependent Inactivation of ACC Deaminase by 2-Methylene-ACC (**9**).* Various amounts of **9** (0−10 mM) were incubated with ACC deaminase (2.45 μM) in 0.5 mL of 100 mM potassium phosphate buffer (pH 7.5) at 25 °C. At various time intervals, aliquots of the incubation mixture (75 μL) were removed and assayed for remaining activity as described above. A control was run in parallel in which the same volume of buffer was added instead of the inhibitor solution.

*Examination of the Reversibility of the Inactivation of ACC Deaminase by 2-Methylene-ACC (**9**).* A mixture containing 5 mM **9** and 4.1 μM ACC deaminase was incubated in 600 μL of 100 mM potassium phosphate buffer (pH 7.5) at 25 °C for 12 h to ensure complete inactivation of the enzyme. The incubation mixture was dialyzed against 600 mL of the same buffer for 72 h at 4 °C with six buffer changes. The residual enzyme activity was determined before and after dialysis. A control experiment was performed under identical conditions without the inhibitor.

*Determination of the Partition Ratio for the Inactivation of ACC Deaminase by 2-Methylene-ACC (**9**).* A series of samples were prepared by mixing the appropriate amount of **9** with ACC deaminase in 100 mM potassium phosphate buffer (pH 7.5) to make the concentration ratios between **9** and the enzyme as 0, 1000, 2500, 3250, 5000 and 6000, respectively. These mixtures were incubated at room tem-

perature for 12 h. An aliquot of 100 μL was withdrawn from each reaction and diluted with 400 μL of the same buffer, and the residual enzyme activity was determined. The activity was plotted against the concentration ratio between **9** and ACC deaminase. The partition ratio was determined by extrapolating the linear part of the curve to the abscissa where it intercepted.

*Identification of the Turnover Product (**11**).* A large-scale reaction was carried out in which 44 mM 2-methylene-ACC (**9**) was incubated with 7.2 μM ACC deaminase in 1.5 mL of 100 mM potassium phosphate buffer (pH 7.5) at 25 °C for 96 h. The mixture was passed through a Dowex-50W (H^+) column (0.7 × 5 cm). The effluent was collected, and its pH was adjusted to 6.0 with 1 N sodium hydroxide. After lyophilization, the white solid was collected and subjected to NMR analysis. ^{1}H NMR (D_2O): δ 6.14 (1H, s), 5.97 (1H, s), 1.75 (3H, s). ^{13}C NMR (D_2O): δ 199.7, 173.7, 140.3, 133.4, 15.0.

*Incubation of 3-Methyl-2-oxobutenoic Acid (**11**) with ACC Deaminase.* The effect of **11** on ACC deaminase was determined by incubating this compound (80 mM) with the deaminase (32 μM) in 100 μL of 100 mM potassium phosphate buffer (pH 8.0) at 25 °C. Aliquots (10 μL) of the incubation mixture were withdrawn at different time intervals (0, 10, 20, 35, and 60 min) and assayed for the remaining activity. In a separate experiment, **11** (120 mM) was incubated with the deaminase (16 μM) in the same buffer for 12 h, and the residual enzyme activity was determined. The controls were performed under the identical conditions without **11**.

Thiol Titrations. The free sulfhydryl groups in the wild type and mutants of ACC deaminase were titrated with 5,5′-dithiobis(2-nitrobenzoate) (DTNB) following a known procedure (*25*). In a typical experiment, the deaminase was mixed with 4.0 M N_2-saturated guanidine hydrochloride (pH 7.5) in a final volume of 900 μL for 4 min at room temperature. The exposed thiols in the denatured deaminase were titrated by mixing with 100 μL of 10 mM DTNB in 0.1 M N_2-saturated potassium phosphate buffer (pH 7.5) and measuring the change in absorbance at 412 nm ($\epsilon_{412} = 13700$ $M^{-1}\cdot cm^{-1}$). The same procedure was also used to quantify the free thiols in proteins that had been treated with 2-methylene-ACC (**9**, 70 mM) or 3-methyl-2-oxobutenoic acid (**11**, 60 mM) for 12 h at room temperature. The protein concentration was determined by the Bradford method (*16*).

*Determination of Stoichiometry of Incorporation of 2-Methylene-ACC (**9**) into ACC Deaminase.* The wild-type ACC deaminase (280 μg, 8.1 nmol) was incubated with [2′-^{3}H]-**9** (10.3 μmol, specific activity 3.90 mCi/mmol) (*24*) in 400 μL of 100 mM potassium phosphate buffer (pH 7.5) for 12 h at room temperature. The incubation mixture was then loaded onto a HiTrap desalting column (Amersham) pre-equilibrated with 25 mM Tris-HCl buffer (pH 7.5). The column was eluted with the same buffer, and fractions containing the deaminase were pooled and dialyzed against 25 mM potassium phosphate buffer (2 × 1 L, pH 7.5) for 15 h at room temperature. The dialyzed solution was subjected to scintillation counting to determine the amount of radioactivity and to the Bradford assay (*16*) to determine the protein concentration. The ratio between the specific radioactivity of the inactivated protein and that of [2′-^{3}H]-**9** indicated the stoichiometric amount of inactivator per enzyme subunit. For the S78A and S78C mutants, 300 μg of protein and 11.3 μmol of [2′-^{3}H]-**9** were used in the otherwise identical procedure.

*Inhibition of the S78A Mutant by 2-Methylene-ACC (**9**).* The effect of **9** on the activity of the S78A mutant was examined by incubating the mutant enzyme (38 μM) with **9** (38 mM) in 50 mM potassium phosphate buffer (pH 7.5). Aliquots (10 μL) of the incubation mixture were withdrawn at different time intervals (0, 10, 20, 60, and 120 min) and assayed for the remaining activity. The controls were performed under the identical conditions without **9**.

Isolation and Sequence Analysis of Labeled Peptides. ACC deaminase (0.83 mg) was incubated with 2-methylene-ACC (**9**, 7.6 mg) and [2′-^{3}H]-**9** (3.7 mg, specific activity 29.2 μCi/mol) in 100 mM potassium phosphate buffer (pH 7.5) at room temperature for 24 h. The excessive inhibitor was removed by repeated cycles of concentration/dilution using a Microcon filter until the radioactivity of the filtrate matched that of the background. The enzyme solution was recovered and diluted to 500 μL with the same phosphate buffer and treated with TPCK-treated trypsin (20 μg, type IX from porcine pancreas) for 12 h at 24 °C. The digestion mixture was resolved by HPLC on a Vydac C_{18} reverse-phase column (4.6 mm × 25 cm). The elution profile was as follows: 0−10 min, 100% buffer A (0.1% TFA in H_2O); 10−100 min, 0−50% linear gradient of buffer B/A (buffer B, 0.1% TFA in acetonitrile), followed by 10 min wash with 90% B/A and 10 min reequilibration with 100% buffer A. The flow rate was 1 mL/min, and the UV monitor was set at 216 nm. Fractions (1 mL) were collected, and an aliquot (100 μL) of each fraction was used to determine the amount of radioactivity. The major radioactive fraction eluted at 30 min was then repurified under the same HPLC conditions to give a clean single peak, which was collected and concentrated using a speedvac. The radioactive sample was reduced with tricarboxyethylphosphine (TCEP) and alkylated with iodoacetamide to convert the cysteine residues to *S*-(carboxaminomethyl)cysteines. Sequencing of this tryptic fragment sample was carried out at the Protein Core Facility in the Mayo Clinic.

Using a similar approach, inactivation of the C41A mutant was performed by incubating the protein (0.78 mg) with **9** (17 mg) and [2′-^{3}H]-**9** (10.0 mg, specific activity 3.90 mCi/mmol) at room temperature for 12 h. The incubation mixture was then dialyzed against 50 mM potassium phosphate buffer (3 × 1 L) for 20 h at 4 °C and digested with 12 μg of TPCK-treated trypsin for 23 h at 24 °C. The resulting mixture was purified by HPLC as described above. The radiolabeled tryptic fragments were isolated and submitted for sequence analysis.

Analysis of Peptide Samples by Mass Spectrometry. Proteolytic digestion of the native or the inactivated ACC deaminase was performed by mixing the enzyme (200 μg) with 20 μL of TPCK-treated trypsin (1 mg/mL) in 100 mM potassium phosphate buffer (pH 7.5, final volume 200 μL). The digestion was conducted at room temperature for 12 h, after which the reaction mixture was frozen in liquid N_2 and shipped to the University of British Columbia for MS analysis. In a separate experiment, the protein sample was digested by pepsin from porcine stomach mucosa (20 μg) in 200 μL of 100 mM sodium phosphate (pH 2.0) at 37 °C for 1.5 h. The sample was processed as described above and

subjected to MS analysis. When the inactivated ACC deaminase was subjected to digestion, the enzyme (200 μg) was first incubated with **9** (6.8 mg) in 100 mM potassium phosphate buffer (pH 8.0) at room temperature for 12 h, followed by dialysis in the same buffer (pH 7.5) at 4 °C for 12 h. Control samples in which an appropriate volume of buffer was incubated with the enzyme in lieu of **9** were also prepared and subjected to the identical treatment.

Mass spectra were recorded at the University of British Columbia on a PE-Sciex API 300 triple quadrupole mass spectrometer (Sciex, Thornhill, Ontario, Canada) equipped with an Ionspray ion source. Peptides were separated by reverse-phase HPLC on an Ultrafast microprotein analyzer (Michrom BioResources Inc., Pleasanton, CA) directly interfaced with the mass spectrometer through a postcolumn split tee with the split ratio of 9 to 1. In each of the MS experiments, the proteolytic digest was loaded onto a C_{18} column (Waters, 3.9 × 150 mm), which was then eluted with a gradient of 0−60% solvent B over 60 min followed by 100% solvent B over 2 min at a flow rate of 700 μL/min (solvent A, 0.05% trifluoroacetic acid and 2% acetonitrile in water; solvent B, 0.045% trifluoroacetic acid and 80% acetonitrile in water). The eluant from the column was divided such that 10% was introduced into the mass spectrometer, and 90% was collected manually for further MS/MS experiments. Mass spectra were obtained in either the single-quadrupole scan mode (LC/MS) or the tandem MS product ion scan mode (MS/MS).

Examination of D*-Vinylglycine as an Alternative Substrate for ACC Deaminase and Mutants.* D-Vinylglycine (**3**) was examined as a substrate for the wild type as well as mutants of ACC deaminase in the standard enzyme assay. An overnight incubation was also performed in an NMR tube by mixing **3** (20 mM) with the wild type (0.35 mg), S78A mutant (0.13 mg), or S78C mutant (0.68 mg) in 600 μL of 100 mM potassium phosphate buffer (pD 8.0) prepared with deuterated water. After 14 h, these samples were subjected to NMR analysis to determine whether product had formed.

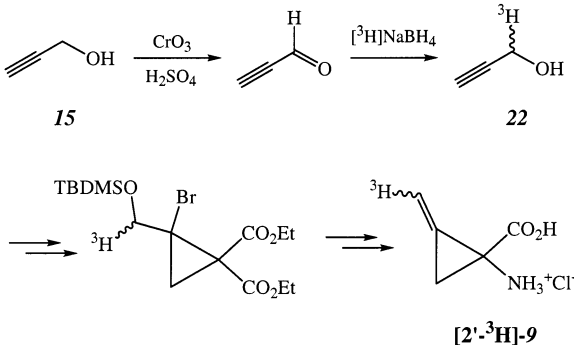
Electronic Absorption of ACC Deaminase Complexed with D*-Alanine and* D*-Vinylglycine.* The wild-type enzyme (24.8 μM) and S78A mutant (18.9 μM) were each mixed with D-alanine (380 mM) or D-vinylglycine (430 mM) in 100 mM potassium phosphate buffer (pH 8.5). The electronic spectra of these samples were recorded using only the corresponding amino acid solution in the same buffer as blanks.

RESULTS

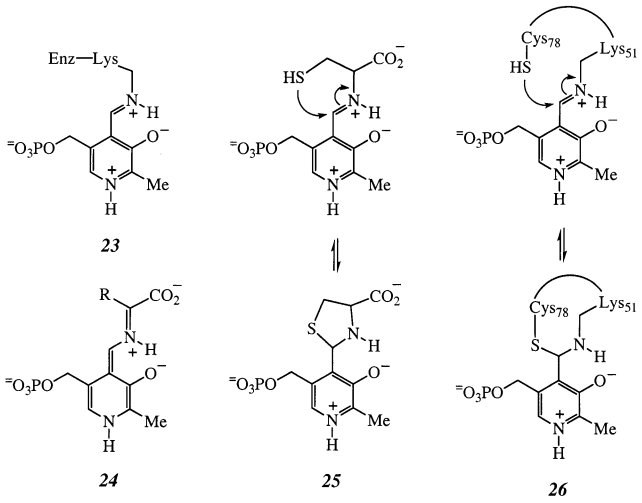
Gene Cloning, Protein Purification, and Biochemical Characterization of the Wild Type and Mutants of ACC Deaminase. The *Pseudomonas* ACC deaminase gene was amplified by PCR and subcloned into the pET17b(+) vector to give a new construct, pQNst23, which was then used to transform *E. coli* BL21(DE3)pLysS to overproduce ACC deaminase upon IPTG induction. The C41A, C41S, S78A, and S78C mutants were individually constructed using commercial mutagenesis kits according to the manufacturer's instructions. The overexpressed wild-type and mutant proteins were purified to near homogeneity by ammonium sulfate fractionation and DEAE-Sepharose and Sephacryl S-200 chromatography. On the average, each run gave more than 30 mg of protein/L of culture. The purified wild-type, C41A, C41S, and S78C proteins are stable toward repeated freeze−thaw cycles. However, the S78A protein is prone to precipitation at high concentration or elevated temperature (>4 °C). The UV−vis spectra of the wild-type enzyme and the C41A, C41S, and S78A mutants are typical for PLP-dependent enzymes ($A_{328}/A_{418} \sim 0.41$ and $A_{280}/A_{418} \sim 5.5$), with the absorption maxima indicative of a protonated internal PLP−aldimine (**23**) in the active site (Scheme 6). Interestingly, the electronic spectrum of the S78C mutant exhibits a much enhanced absorption band at 328 nm but is devoid of absorbance at 418 nm (data not shown). These spectral features resemble those of a thiazolidine-substituted pyridinium species (**25**) (*26*), which may result from coupling between PLP−aldimine and the sulfhydryl group of Cys-78 in the mutant protein (**26**; see Scheme 6). Such an intramolecular coenzyme modification is feasible because Ser-78 has been shown to be in close proximity to the bound PLP in the crystal structure of the *Hansenula saturnus* enzyme (*27*).

Kinetic Characterization of ACC Deaminase and Its Mutants. The K_m and k_{cat} for the recombinant wild-type ACC deaminase were determined to be 4.0 mM and 165.6 min^{-1}, respectively, while those previously reported for the wild-type enzyme isolated from the natural source were 9.2 mM and 290 min^{-1}, respectively (*8a*). However, the overall catalytic efficiency (k_{cat}/K_m) is comparable for both natural and recombinant enzyme. A significant change in the kinetic behavior was observed for the S78A mutant, whose K_m and k_{cat} were determined to be 9.6 mM and 9.2 min^{-1}, respectively. This translates to a 43-fold decrease in the catalytic

Scheme 5



Scheme 6



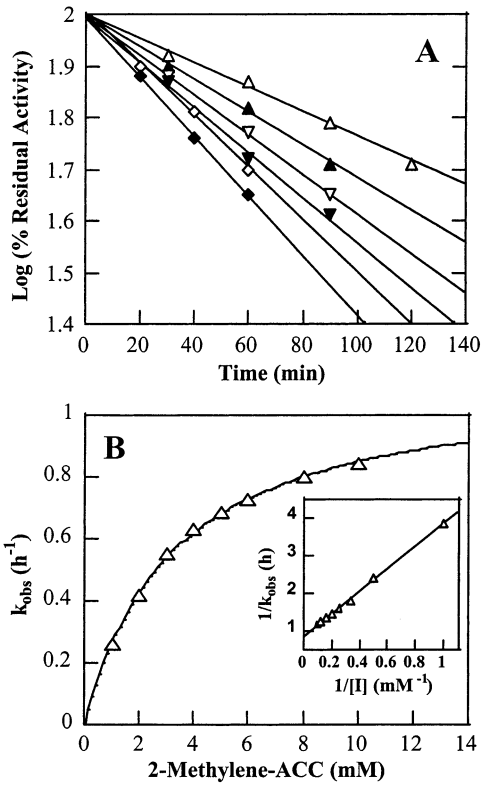


FIGURE 1: (A) Time- and concentration-dependent inactivation of ACC deaminase by 2-methylene-ACC (**9**). ACC deaminase (2.45 μM) was incubated with the indicated amounts of **9** (△, 1.0 mM; ▲, 2 mM; ▽, 3 mM; ▼, 4 mM; ◇, 6 mM; ◆, 10 mM) in a reaction volume of 500 μL. At the indicated times, aliquots of the reaction mixture (75 μL) were removed and assayed. The values of k_{obs} were determined from the slopes of the linear fit for the inactivation data obtained with the respective concentrations of 2-methylene-ACC. (B) Plot of k_{obs} as a function of 2-methylene-ACC concentration. The inset shows the double-reciprocal plot of k_{obs} versus 2-methylene-ACC concentration. The data from plot B were used to calculate k_{inact} and K_I, which are reported in the text.

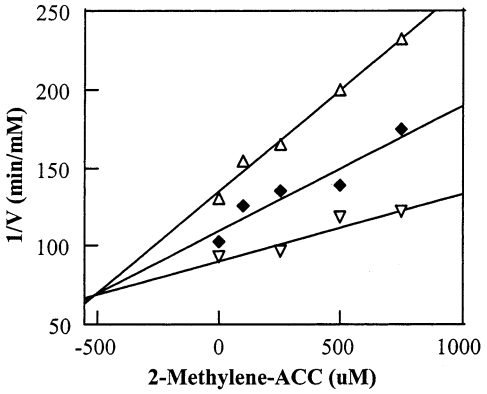


FIGURE 2: Plot of the reciprocal of the reaction velocity observed with ACC deaminase (v) as a function of 2-methylene-ACC (**9**) in the presence of 2 mM (△), 3 mM (◆), and 4 mM (▽) ACC (**1**).

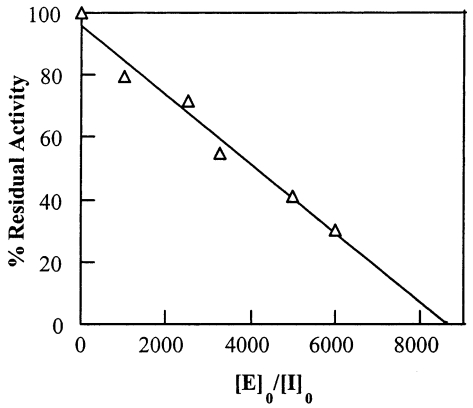


FIGURE 3: Effect of 2-methylene-ACC (**9**) on the catalytic activity of ACC deaminase. These figures show the percentage of residual activity versus the ratio of **9** to enzyme. See Experimental Procedures for details.

efficiency as compared to the wild-type enzyme. An even greater reduction (>99%) of activity was observed for the S78C mutant, likely due to the inaccessibility of the PLP coenzyme (see **26** in Scheme 6) as a result of its modification. Because the proposed PLP modification is in principle an equilibrium process, the very low activity associated with the S78C mutant may be ascribed to the presence of a small portion of the unmodified PLP−Schiff base available at equilibrium. Since the specific activity of the S78C mutant was only 0.8% of that of the wild-type enzyme, no kinetic analysis of this protein was performed. Unlike the Ser-78 mutants, the C41A and C41S mutants behave comparably with the wild-type enzyme, both retaining more than 95% of the wild-type activity.

*Inactivation of ACC Deaminase by 2-Methylene-ACC (**9**).* As illustrated in Figure 1A, when ACC deaminase was treated with **9**, a time-dependent inactivation was observed. Plots of the logarithm of the residual activity versus time were linear, indicating that the rate of inactivation follows pseudo-first-order kinetics. The inactivation rate also showed dependence on the concentration of **9**. Despite the sluggish inactivation rate, ACC deaminase could be completely inactivated by prolonged incubation (~12 h) with excess **9**. The inactivation is active site directed and irreversible, as the rate of inactivation significantly decreases in the presence of excess substrate, and the enzyme activity cannot be regenerated after extensive dialysis (*14*).

The limiting rate of inactivation (k_{inact}) and the apparent dissociation constant for the reversibly formed complex (K_I) were determined to be 1.13 ± 0.01 h^{-1} and 3.26 ± 0.10 mM, respectively (Figure 1). The value of K_I is comparable to that of K_m for ACC (**1**), suggesting that both 2-methylene-ACC and ACC may have similar binding affinity toward ACC deaminase. Because **9** is a slow irreversible inhibitor, it also functions as a competitive inhibitor when the activity of ACC deaminase is monitored in the presence of both **1** and **9**. Assuming that the initial encounter between **9** and ACC deaminase is fast and reversible, a Dixon plot of the reciprocal enzyme activity as a function of inhibitor concentration at different substrate concentrations gave a K_I value of 0.48 mM (Figure 2).

*Product Identification and Kinetic Characterization of Enzymatic Turnover of 2-Methylene-ACC (**9**).* While 2-methylene-ACC (**9**) is an irreversible inactivator of ACC deaminase, an excess amount of **9** is required to achieve full enzyme inactivation. As shown in Figure 3, a plot of the residual activity versus total equivalents of **9** added led to a partition ratio of approximately 8250. Since the partition ratio defines the number of latent inhibitor molecules converted to product relative to each turnover leading to enzyme inactivation (*28*), the large ratio deduced from this experiment clearly indicates that **9** is not an efficient inactivator. The product was isolated from a large-scale incubation and

determined to be 3-methyl-2-oxobutenoic acid (**11**), whose identity was confirmed by comparison with a synthetic standard (*20*). The k_{cat} and K_m values for the conversion of **9** to **11** were found to be 46.8 ± 1.8 min^{-1} and 220 ± 21 μM, respectively.

*Incubation of 3-Methyl-2-oxobutenoic Acid (**11**) with ACC Deaminase.* While compound **11** is expected to be a good electrophilic reagent capable of reacting with protein nucleophiles, no activity loss was observed when ACC deaminase was treated with **11** (80 mM) in potassium phosphate buffer (pH 8.0) for 1 h. Prolonged incubation (12 h) of ACC deaminase with excess of **11** (120 mM) at room temperature resulted in a reduction of only 13% of the catalytic activity. Apparently, **11**, which accumulates in the incubation of **9** with ACC deaminase, does not play a significant role in the inactivation of this enzyme.

Thiol Titration. Titration of the wild-type enzyme with Ellman's reagent (DTNB) under denaturation conditions revealed the presence of 6.1 ± 0.1 free sulfhydryl groups in ACC deaminase, which is consistent with the presence of six cysteine residues in the amino acid sequence. Under the same conditions, a total of 6.4 ± 0.1 equiv of thiol groups was titratable in the S78C mutant. These results show that a significant portion of this newly introduced cysteine residue at position 78 is not accessible to DTNB and is consistent with its proposed involvement in a thiazolidine-type adduct with the PLP−aldimine complex (**26**, Scheme 6). Interestingly, the number of accessible sulfhydryl groups (3.6 ± 0.1) was reduced by 40% when the titration was performed on the wild-type enzyme that had been treated with 2-methylene-ACC (**9**) for 12 h at room temperature. Thus, at least two cysteine residues were modified by **9** in the inactivated protein. In contrast, the number of titratable thiols remained nearly unchanged (5.7 ± 0.1) in the wild-type enzyme that had been treated with excess **11**. This finding is consistent with the above notion that compound **11** does not participate in the inactivation of ACC deaminase.

*Stoichiometry of Modification of the Wild Type and S78A and S78C Mutants of ACC Deaminase by 2-Methylene-ACC (**9**).* The results of the thiol titration experiments described above revealed that inactivation of ACC deaminase by **9** is a much more complicated event than previously thought and involves multisite modifications. Indeed, inactivation of wild-type ACC by [2′-^{3}H]-**9** showed that 5.5 ± 0.3 equiv of inhibitor was incorporated into the inactivated enzyme. When the same labeling experiment was performed on the S78A mutant, the stoichiometry was estimated to be 4.5 ± 0.5. These data provide direct evidence showing that covalent modification of ACC deaminase by **9** is not limited to a single site, and Ser-78 is one of the residues labeled during inactivation. Surprisingly, incubation of **9** with the catalytically incompetent S78C mutant led to a stoichiometry of 5.7 ± 0.4, which is comparable to the ratio obtained for the wild-type enzyme, indicating that **9** can react directly with reactive enzyme nucleophiles at multiple sites without enzymatic activation. The above result also showed that while the newly introduced sulfhydryl group at position 78 is not accessible to Ellman's reagent, perhaps due to its steric hindrance, it appears to be susceptible to **9**. Since formation of the thiazolidine adduct **26** in S78C mutant is expected to be reversible, giving this mutant a small but detectable activity (0.8% of wild-type activity), prolonged treatment of this mutant with **9** may eventually trap the sulfhydryl group at position 78 (or perhaps via **10**, Scheme 3, after enzymatic processing).

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  1 MNLQRFPRYP LTFGPTPIQP LARLSKHLGG KVHLYAKRED CNSGLAFGGN
 51 KTRKLEYLIP EALAQGCDTL VSIGGIQSNQ TRQVAAVAAH LGMKCVLVQE
101 NWVNYSDAVY DRVGNIQMSR ILGADVRLVP DGFDIGFRRS WEDALESVRA
151 AGGKPYAIPA GCSDHPLGGL GFVGFAEEVR AQEAELGFKF DYVVVCSVTG
201 STQAGMVVGF AADGRADRVI GVDASAKPAQ TREQITRIAR QTAEKVGLER
251 DIMRADVVLD ERFAGPEYGL PNEGTLEAIR LCARTEGMLT DPVYEGKSMH
301 GMIEMVRNGE FPEGSRVLYA HLGGVPALNG YSFIFRDG
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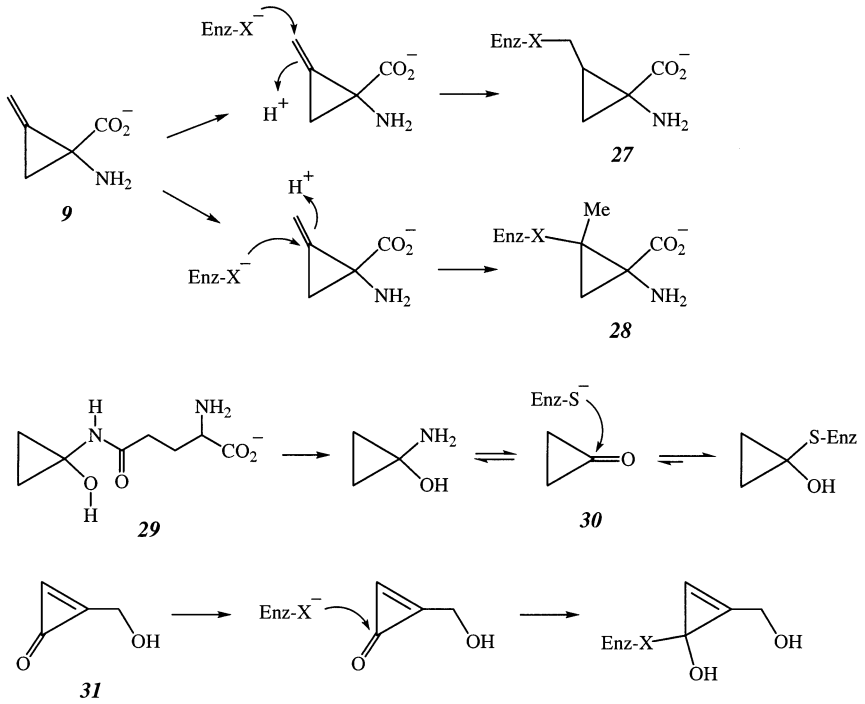
FIGURE 4: Amino acid sequence of ACC deaminase. Highlighted are fragments labeled by 2-methylene-ACC (**9**). Peptides in bold were separated and identified by ESI MS/MS analysis. Peptides in bold and underlined were isolated and identified by Edman sequencing. The amino acid residues being modified, or proposed to be modified, are marked with an asterisk (*).

*Inactivation of the S78A Mutant by 2-Methylene-ACC (**9**).* To assess whether the observed inactivation of the wild-type enzyme by **9** is due primarily to the modification of Ser-78 or to multisite labeling, the effect of **9** on the S78A mutant was determined. Since this mutant retains 2.3% of the wild-type activity but no longer has a reactive function group at position 78, no loss of activity is expected if the inactivation solely depends on the modification of Ser-78. Interestingly, upon treatment with **9** for 2 h, a reduction of nearly 80% of the original activity of the S78A mutant was observed. Clearly, covalent modification at loci other than Ser-78 can also be detrimental.

Identification of Modified Residues in Inactivated ACC Deaminase by Peptide Sequencing. As described in Experimental Procedures, tryptic digestion of ACC deaminase labeled with [2′-^{3}H]-**9** gave a mixture of peptides. Since the specific activity of [2′-^{3}H]-**9** used in this experiment was low (29.2 μCi/mmol), only one peptide with detectable radioactivity could be isolated. Analysis by standard Edman degradation revealed the sequence of this peptide as ^{38}RED**X**NSGLAFGGNK^{51}, where **X** is the covalently modified Cys-41 (see Figure 4). Interestingly, several labeled peptides could be isolated from the incubation of the C41A mutant with a sample of [2′-^{3}H]-**9** having a higher specific radioactivity (3.90 mCi/mmol). Two of these peptides were identified as ^{24}LS**X**HLGGK^{31} and ^{241}QTAE**X**VGLER^{250}. In both cases, residue **X** at positions 26 and 245 is a lysine (see Figure 4). Thus, the labeling and sequencing data allowed the identification of Lys-26, Cys-41, and Lys-245 as three of the six modified residues implicated by the stoichiometry study.

Identification of Modified Residues in Inactivated ACC Deaminase by LC/MS. The above experiments on the wild-type enzyme were repeated using LC/ESI-MS to characterize the inactivated protein. A molecular mass of 36674 Da per monomer was readily determined for the wild-type enzyme (calculated 36671 Da). Unfortunately, attempts to determine the molecular mass of the inactivated enzyme were unsuccessful. Nevertheless, two sets of modified peptides were identified by comparative peptide mass mapping of the tryptic-digested inactivated enzyme. The peak with a mass of 3233 Da was assigned to peptide 54−82, which, in its native form, has a molecular mass of 3119 Da. The increase

Scheme 7



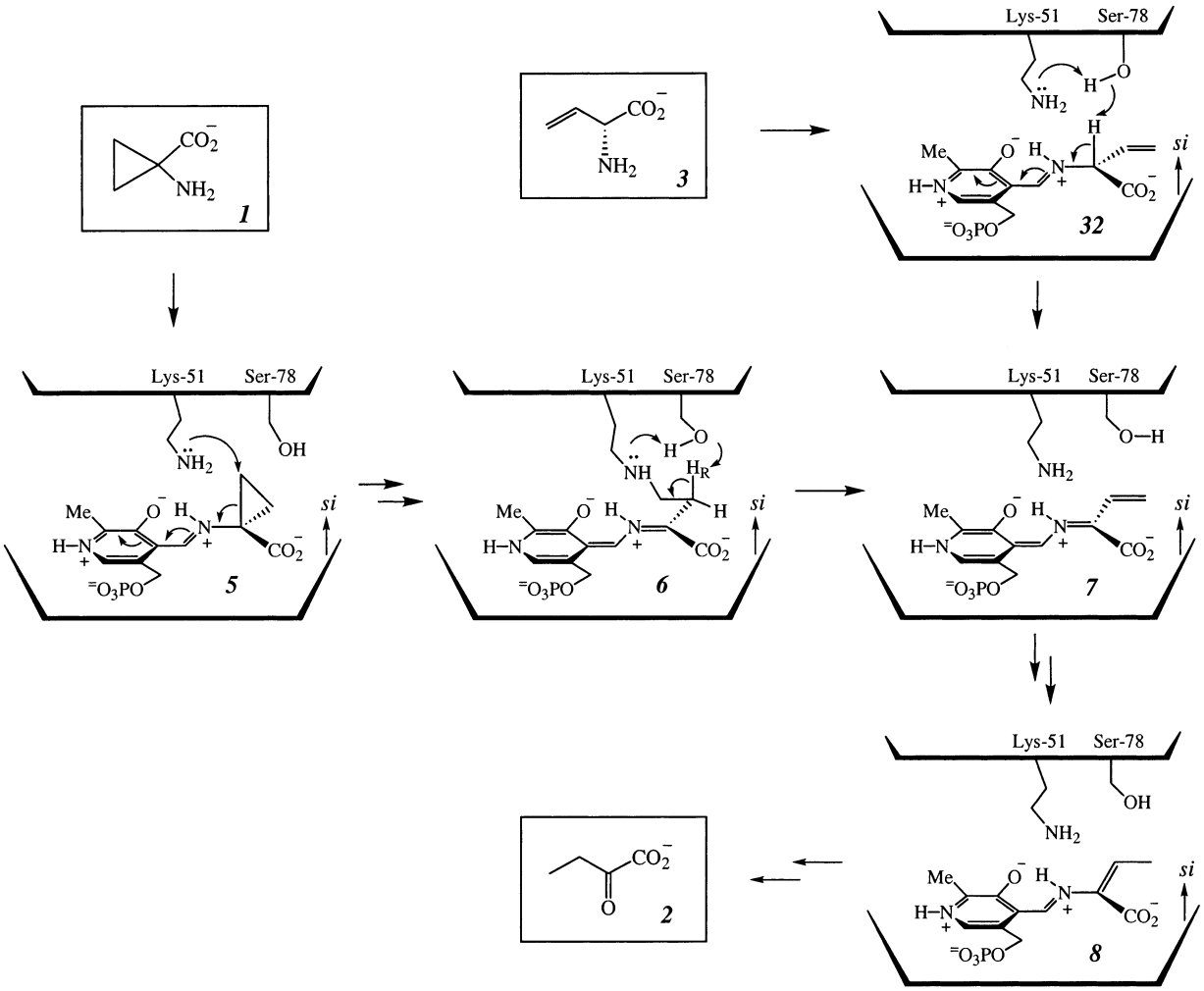
in mass of 114 Da of this modified peptide is indicative of the attachment of one molecule of **9** (or its equivalent). The site of modification is likely Ser-78, which has been established as a modified residue on the basis of the results of our stoichiometry experiments with the S78A mutant. Two other new species with a mass of 3159 and 3273 Da, respectively, were found to be derivatives of the same peptide 150−180 that has a native molecular mass of 3045 Da. The increase in mass of these modified peptide fragments revealed the covalent attachment of 1 or 2 equiv of **9** (or its equivalent) to the parent peptide 150−180. One of the labeled residues may be Cys-162, which has been shown to be reactive toward sulfhydryl-directed reagents (*32b*). Unfortunately, attempts to sequence these labeled peptides to pinpoint the modified residue(s) by tandem MS were not successful. Complications were also encountered in analyzing the fragments of inactivated enzyme digested with pepsin. Only one labeled peptide, ^{25}SKHLGGKVHL^{34}, could be unambiguously identified. This fragment is modified by 1 equiv of **9** (or its equivalent), since it has a molecular mass of 1190 Da, which is 114 Da greater than that of the parent peptide (1076 Da). As mentioned earlier, this peptide has been sequenced by Edman degradation from which Lys-26 is identified as the site of covalent attachment. Overall, Lys-26, Ser-78, Cys-162, and one more amino acid residue within the sequence of 150−180 have been implicated as the sites of covalent modification according to the LC/ESI-MS results.

Chemical Competence of D*-Vinylglycine in the ACC Deaminase- and Mutant-Catalyzed Reactions.* It has been previously shown that ACC deaminase converts D-vinylglycine (**3**) to 2-ketobutyric acid (**2**) (*8a*). A specific activity of 69.1 μmol·mg^{-1}·min^{-1} was determined using **3** as the substrate with the wild-type enzyme, indicating that **3** is about 53-fold less effective than the normal substrate ACC (**1**). However, when D-vinylglycine was incubated with S78A and S78C mutants, no turnover was discernible under the assay conditions. Prolonged incubation carried out in an NMR tube with the S78A and S78C mutants also failed to detect any new product. In contrast, a control using the wild-type enzyme revealed complete conversion of **3** to **2** under the same conditions. Interestingly, the recovered D-vinylglycine in these samples showed no exchange at the α-position with solvent deuteriums. Clearly, these two mutant enzymes have lost the ability to abstract the α-H of D-vinylglycine and thus are unable to initiate the turnover of this compound. It should be noted that an absorption band at 510 nm (data not shown), characteristic of the formation of the quinoidal form of PLP−aldimine (**24**; see Scheme 6), was observed when the wild-type enzyme was incubated with excess D-alanine. In the presence of a high concentration of D-vinylglycine (**3**), the band associated with the quinoidal species was red shifted to 545 nm due to the extension of the conjugated π-system (such as in **7**). Not surprisingly, since the S78A mutant lacks the capability to abstract the α-H of D-alanine and D-vinylglycine, no such absorption at the long wavelength region was observed under identical incubation conditions.

DISCUSSION

PLP-dependent enzymes have been classified into four groups on the basis of sequence comparison and are known as the α-, β-, D-alanine aminotransferase, and alanine racemase families (*29*). ACC deaminase is a member of the β-family with its PLP-binding lysine (Lys-51) located close to the N-terminus of its polypeptide chain. Although it was first isolated from a soil bacterium *Pseudomonas* sp. ACP in the 1960s (*1*), dozens of its homologues have now been found in a variety of living organisms including bacteria, fungi, and even mammalian cells (*30*). The enzyme catalyzes the conversion of ACC (**1**) to α-ketobutyric acid (**2**), initiated by Schiff base formation between the active-site bound PLP and the amino group of ACC. The cyclopropane ring of ACC, already electrophilic due to the C-1 carboxylate group, becomes more so due to the activation by the newly formed PLP−aldimine group (as shown in Scheme 2, **5**) (*31*). A

Scheme 8



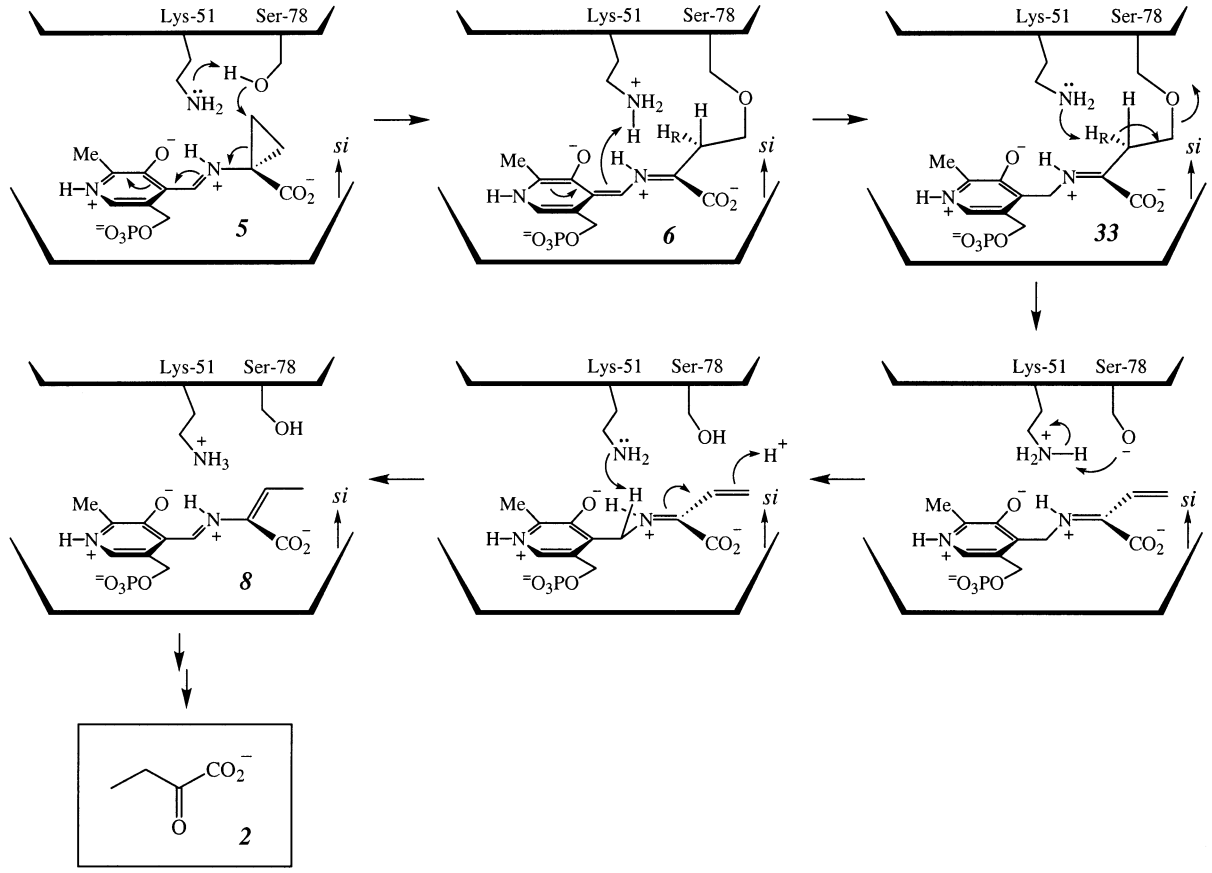
ring opening induced by the attack of an active site nucleophile has been proposed as the mechanism of this unusual enzymatic transformation (Scheme 2) (*8*−*11*, *14*, *15*).

2-Methylene-ACC (**9**) was designed and synthesized as a mechanistic probe of ACC deaminase. The exocyclic methylene group renders this compound more electrophilic than ACC, making it possible to trap active site nucleophiles (see Scheme 3). Indeed, compound **9** was found to be a time-dependent irreversible inactivator as well as a substrate, resulting in the formation of 3-methyl-2-oxobutenoic acid (**11**) (*14*). The large partition ratio (8250) estimated for this inactivation indicated that **9** is a relatively poor inhibitor. Interestingly, compound **9** is a better substrate than ACC (**1**), having a catalytic efficiency (k_{cat}/K_m) that is 5.1-fold greater than that of **1**. The enhanced reactivity of **9** as a substrate for ACC deaminase collaborates well with the proposed catalytic mechanism.

To determine the modified active site residue, [2′-^{3}H]-**9** was prepared and used to label ACC deaminase. To our surprise, more than 5 equiv (5.5 ± 0.3) of [2′-^{3}H]-**9** was found to be incorporated into the inactivated enzyme. This observation was initially puzzling in view of the fact that the inactivation was thought to be active site directed so that only a single residue would be modified (*14*). Further experiments to determine the sites of covalent attachment led to several labeled peptides after proteolytic digestion of the inactivated protein. The sequence analysis results of these peptides are summarized in Figure 4. Three of the modified residues, Lys-26, Cys-41, and Lys-245, were readily identified on the basis of peptide sequencing data. Attempts to locate the other three modified residues using the MS/MS sequencing technique met with little success since these residues were located on two long peptides, 54−82 and 150−180. However, one of the three remaining residues is likely to be Cys-162, which has previously been identified as the site of modification by an iodoacetamide derivative (*13*). Since this residue is reactive, it may likewise react with the electrophilic 2-methylene-ACC (**9**). The involvement of two reactive cysteine residues (Cys-41 and Cys-162) in the inactivation by **9** is further implicated by our thiol titration results, which show that a minimum of two cysteines were no longer susceptible to DTNB after prolonged treatment with **9**. However, since the activities of the C41A/S and C162A (*32b*) mutants are comparable to that of the wild-type enzyme, these two cysteine residues, which are accessible and reactive toward **9**, do not directly participate in the catalysis of ACC deaminase.

The crystal structure of native ACC deaminase from *H. saturnus* without a ligand has recently been determined to 2.0 Å resolution (*27*). Since the amino acid sequence of this yeast enzyme is 60% identical to that of the *Pseudomonas* enzyme, the structures of these two proteins are likely to be similar. Docking ACC (**1**) in the active site of ACC

Scheme 9



deaminase to model the enzyme−substrate binary complex revealed that Ser-78 is close to the substrate−PLP−aldimine adduct (**5** in Scheme 2) and thus may be important for catalysis as well as the inactivation of ACC deaminase by **9**. The fact that peptide 54−82, which contains Ser-78 (Figure 4), is labeled upon incubation with **9** further implicated Ser-78. As expected, the S78C mutant is barely active (0.8% wild-type activity). However, this result bears little mechanistic relevance, since the loss of activity is likely due to the formation of a cyclic adduct (**26**) between Cys-78 and the PLP−aldimine complex in the active site. In contrast, the experimental results on the S78A mutant were more revealing. When this mutant was subjected to inactivation by **9**, 1 less equiv of **9** (5.5 ± 0.3 versus 4.5 ± 0.5) was found to be incorporated into the inactivated enzyme. Thus, it can be concluded that Ser-78 is a reactive residue being modified by **9** during inactivation, and such a modification accounts for the labeling of peptide 54−82. Kinetic analysis of the S78A mutant showed that its Michaelis constant (K_m) for **1** increases by more than 2-fold and the turnover rate (k_{cat}) decreases by 18-fold as compared to the wild-type enzyme, resulting in greater than 40-fold reduction of the overall catalytic efficiency. The sum of the observations indicates that Ser-78 is an important catalytic residue.

Having assigned all (Lys-26, Cys-41, Ser-78, Cys-162, Lys-245) but one of the modified residues in the inactivated enzyme, we turned our attention to the identification of the reactive species responsible for these modifications. One possible candidate is the turnover product **11**, which accumulates in the incubation mixture. This compound is expected to be a good Michael acceptor capable of trapping enzyme nucleophiles as illustrated in Scheme 3. However, no cysteine residues (or other nucleophiles) of ACC deaminase were modified by **11** even after prolonged incubation. More significantly, **11** exhibits little effect on the catalytic efficiency of ACC deaminase. Thus, **11** is not likely to be the reactive species responsible for the multisite modifications of ACC deaminase.[2] Interestingly, when [2′-^{3}H]-**9** was used to inactivate ACC deaminase, both the wild type and the S78C mutant incorporated identical equivalents of inhibitor per enzyme monomer. Since the S78C mutant is catalytically impaired, the observed multisite modifications by **9** cannot be enzyme catalyzed. Thus, 2-methylene-ACC (**9**) itself appears to be reactive enough to trap accessible enzyme nucleophiles directly.

As illustrated in Scheme 7, the high reactivity of **9** toward nucleophilic addition may be explained by the energetically favored conversion of the exocyclic carbon from an sp^2 to an sp^3 configuration (see **27** and **28**) to alleviate the ring strain (*33*). A similar tendency for cyclopropanone (**30**) to accumulate as the stabilized tetrahedral adduct has been proposed to account for the toxicity of coprine (**29**) (*34*) which, after hydrolysis by glutaminase, can inactivate aldehyde dehydrogenase known to have a kinetically reactive and essential cysteinyl thiolate side chain in its active site.

[2] It is possible that some reactive species generated during turnover, such as **10**, may be responsible for the modification of non-active site enzyme nucleophiles. Attempts to trap these putative reactive species with excess thiol reagents (12.5-fold more than **9**), such as DTT, resulted in the reduction of the inactivation rate by 29%. However, it is difficult to distinguish whether the effect is due to the eradication of the reactive species or the decomposition of compound **9**, which is sensitive to DTT.

Also, penitricin (**31**) (*35*), a cyclopropenone derivative, may execute its antimicrobial activity using a similar mechanism. While the high electrophilicity of **9** has been implicated by its enhanced activity (>5-fold than that of **1**) as a substrate for ACC deaminase, the methylenecyclopropane group is clearly less electrophilic than a cyclopropanone or a cyclopropenone group. Thus, a full account for the capability of **9** to trap enzyme nucleophiles without apparent preactivation must await more experimental data. It should be noted that although covalent modification of Ser-78 in the active site during turnover will certainly inactivate the enzyme, the fact that the S78A mutant, which still retains 2.3% of the wild-type activity, can be inactivated by the treatment with **9** indicates that modifications at sites other than Ser-78 also contribute to enzyme inactivation.

Further characterization of the S78A mutant showed that it has lost its ability to catalyze the turnover of D-vinylglycine (**3**), which is a good substrate for the wild-type enzyme. The key step for the conversion of **3** to **2** (Scheme 1) involves the abstraction of the α-proton to yield a β,γ-olefinic PLP−quinoid α-anion intermediate (**32** → **7**, Scheme 8). This highly conjugated π-system (**7**) exhibits an absorption maximum at 545 nm (*36*) and is also the common point of entry of ACC into the reaction flux (**5** → **6** → **7**). Beyond this point, a series of tautomerizations including two consecutive protonations at γ- and β-carbons take place to generate the ketoacid product **2** (see Scheme 2). Since the S78A mutant retains partial activity (2.3%) toward ACC, its capability to catalyze the final tautomerization steps (**7** → **8** → **2**; Schemes 2 and 8) must remain intact. Thus, the failure to turn over **3** by this mutant must be associated with the α-deprotonation step. Indeed, no conjugated quinoid intermediate was formed upon incubation of the S78A mutant with **3** as indicated by the lack of absorption above 500 nm. Hence, Ser-78 can be assigned as the active site base responsible for the removal of α-H from D-vinylglycine. It is well documented that serine can serve as a base in enzyme catalysis. For example, in the oxidation of dihydroorotate to orotate catalyzed by *E. coli* dihydroorotate dehydrogenase in de novo pyrimidine biosynthesis (*37*), Ser-175 has been implicated as the base involved in the abstraction of the *pro-S* hydrogen at C-5 of dihydroorotate to facilitate the subsequent hydride transfer from C-6 to the flavin coenzyme (*38*).

The current evidence regarding the catalysis of ACC deaminase can be accommodated by the scenarios shown in Schemes 8 and 9. As mentioned earlier, the proposed mechanism for the conversion of **1** to **2** requires the participation of at least two basic groups, one serving as the nucleophile to attack the cyclopropane ring, thereby initiating ring cleavage, and the other serving as the base to remove the β-H (Scheme 2). For PLP-dependent enzymes, the PLP-binding lysine in many cases assumes the function of the base to abstract protons (*39*) and has often been the nucleophile trapped by an electrophilic inhibitor (*40*). Hence, the conserved Lys-51, which binds PLP in ACC deaminase, is expected to play a significant role in ACC turnover. As predicted, the K51A mutant is inactive (*13*). Thus, a mechanism in which Ser-78 acts as the nucleophile to attack the ring in **5** and in which Lys-51 serves as the base to abstract the β-H in **33** as shown in Scheme 9 is consistent with the acid−base role commonly found for the conserved lysine in PLP-dependent enzymes and the nucleophilic role assumed by the active site serine in many enzyme catalyses. However, it was found that although the K51A mutant could still bind 30% of the original content of PLP and showed capacity to form the PLP−aldimine complex with ACC, no turnover to **2** was observed (*13*). This finding is more compatible with a mechanism in which Lys-51 is the nucleophilic residue directly involved in cyclopropane ring fragmentation (see Scheme 8). Since Ser-78 is the likely base that abstracts the α-H of **3**, it might also be the residue responsible for β-H abstraction in the catalysis of **1**. Such an assignment is supported by the fact that the S78A mutant retains appreciable activity (∼25%) toward **9**, whose turnover to **11** requires no β-H abstraction (see Scheme 3), but the capability of the S78A mutant to convert **1** to **2**, in which β-H abstraction is requisite (**6** → **7**; Scheme 2), is lowered by more than 40-fold. The residual activity associated with the S78A mutant in the turnover of **1** to **2** may be ascribed to another nearby basic residue partially fulfilling the function of Ser-78. Thus, while Ser-78 may not be considered as an absolutely essential residue in ACC turnover, it is crucial for the conversion of **3** to **2** since mutation of this serine to an alanine resulted in the loss of catalytic activity.

In summary, our results reveal that 2-methylene-ACC (**9**) is a Janus-faced substrate for ACC deaminase, being a better substrate than the natural substrate but also a time-dependent, irreversible inhibitor. The reactivity of **9** is associated with its methylenecyclopropyl ring skeleton whose electrophilic nature facilitates the nucleophilic attack needed to induce the ring opening, a critical step for both the catalysis and inhibition. Although the inactivation shows saturation kinetics and involves trapping of an active site residue, Ser-78, the fact that more than 5 equiv of inhibitor is incorporated into the inactivated enzyme after prolonged incubation has complicated our assessment of whether the inhibition is truly mechanism based. However, our data show that Ser-78 is likely the active site base which abstracts the α-H from D-vinylglycine (**3**) and removes the β-H in the catalysis of ACC (**1**). Lysine-51 is the likely active site nucleophile that attacks the cyclopropane ring and initiates the fragmentation of ACC. In view of the specificity imposed by the PLP cofactor, which ensures that the orientation of the scissile bond is perpendicular to the cofactor−substrate complex (*41*), the C_α−H bond in the D-vinylglycine−PLP−aldimine adduct and the bond to be cleaved between C_α and *pro-S* C_β in the case of ACC are expected to align in the same direction as illustrated in Schemes 8 and 9. Consequently, both Ser-78 and Lys-51 must be located on the same side (*si* face) of the substrate−PLP−aldimine complex in order to execute their assigned roles in catalysis (*42*). Whether our proposed roles for Ser-78 and Lys-51 and their juxtapositions in the active site will hold true awaits the results of experiments currently in progress.

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