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Duine, J. A., & Frank, J. (1980b) *Biochem. J. 187*, 213–219.
Duine, J. A., Frank, J., & Westerling, J. (1978) *Biochim. Biophys. Acta 524*, 277–287.
Duine, J. A., Frank, J., & Van Zeeland, J. K. (1979) *FEBS Lett. 108*, 443–446.
Duine, J. A., Frank, J., & Verwiel, P. E. J. (1980) *Eur. J. Biochem. 108*, 187–192.
Forrest, H. S., Salisbury, S. A., & Kilty, C. G. (1980) *Biochem. Biophys. Res. Commun. 97*, 248–251.
Foster, J. W., & Davis, R. H. (1966) *J. Bacteriol. 91*, 1924–1931.
Hamilton, G. A. (1973) *Prog. Bioorg. Chem. 2*, 83–157.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Martinez, A. M., Cushmac, G. E., & Rocek, J. (1975) *J. Am. Chem. Soc. 97*, 6502–6510.
Mincey, T., Tayrien, G., Mildvan, A., & Abeles, R. H. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 7099–7101.
Patel, R. N., Bose, H. R., Mandy, W. J., & Hoare, D. S. (1972) *J. Bacteriol. 110*, 570–577.
Patel, R. N., Hou, C. T., & Felix, A. (1978) *J. Bacteriol. 133*, 641–649.
Raymond, S. (1962) *Clin. Chem. (Winston-Salem, N.C.) 8*, 455–570.
Salisbury, S. A., Forrest, H. S., Cruse, W. B. T., & Kennard, O. (1979) *Nature (London) 280*, 843–844.
Westerling, J., Frank, J., & Duine, J. A. (1979) *Biochem. Biophys. Res. Commun. 87*, 719–724.

Mechanistic Studies on the Pyridoxal Phosphate Enzyme 1-Aminocyclopropane-1-carboxylate Deaminase from *Pseudomonas* sp.†

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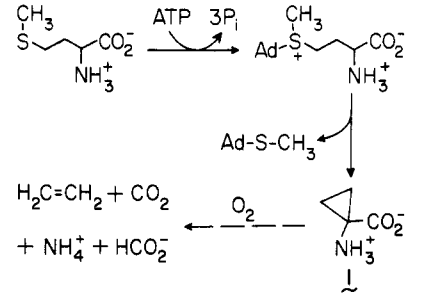
ABSTRACT: The enzyme 1-aminocyclopropane-1-carboxylate deaminase (ACPC deaminase) from a pseudomonad is a pyridoxal phosphate (PLP) linked catalyst which fragments the cyclopropane substrate to α-ketobutyrate and ammonia [Honma, M., & Shimomura, T. (1978) *Agric. Biol. Chem. 42*, 1825]. Enzymatic incubations in D_2O yield α-ketobutyrate with one deuterium at the C-4 methyl group and one deuterium at one of the C-3 prochiral methylene hydrogens. Stereochemical analysis of the location of the C-3 deuteron was accomplished by in situ enzymatic reduction to (2*S*)-2-hydroxybutyrate with L-lactate dehydrogenase and conversion to the phenacyl ester. The C-3 hydrogens of the (2*S*)-2-hydroxybutyryl moiety are fully resolved in a 250-MHz NMR spectrum. Absolute assignment of 3*S* and 3*R* loci was obtained with phenacyl (2*S*,3*S*)-2-hydroxy[3-^{2}H]butyrate generated enzymatically by D-serine dehydratase action on D-threonine. ACPC deaminase shows a stereoselective outcome with a 3*R*:3*S* deuterated product ratio of 72:28. 2-Vinyl-ACPC is also a fragmentation substrate with exclusive regiospecific cleavage to yield the straight-chain keto acid product 2-keto-5-hexenoate. The D isomer of vinylglycine is processed to α-ketobutyrate and ammonia at 8% the V_{max} of ACPC, while L-vinylglycine is not a substrate. It is likely that ACPC and D-vinylglycine yield a common intermediate—the vinylglycine–PLP–*p*-quinoid adduct—which is then protonated sequentially at C-4 and then C-3 to account for the observed deuterium incorporation. The D isomers of β-substituted alanines (fluoroalanine, chloroalanine, and *O*-acetyl-D-serine) partition between catalytic elimination and enzyme inactivation. Each shows a different partition ratio, arguing against the common aminoacrylyl-PLP as the inactivating species.

The cyclopropanoid amino acid 1-aminocyclopropane-1-carboxylic acid (ACPC)[1] (**1**) is a natural product isolated from several plant tissues (Burroughs, 1957; Vahatalo & Virtanen, 1957) including pears and apples. This cyclic amino acid is a key intermediate in the biosynthesis of ethylene, a fruit-ripening hormone in plants (Adams & Yang, 1979; Lurssen et al., 1979; Konze & Kende, 1979), and consequently there has been interest in the biotransformations to and from ACPC. A likely route for ethylene biosynthesis from methionine (Scheme I) commences with S-adenosylation to give *S*-

Scheme I



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[1] Abbreviations used: ACPC deaminase, 1-aminocyclopropane-1-carboxylate deaminase; PLP, pyridoxal 5′-phosphate; SAM, *S*-adenosylmethionine; Me_4Si, tetramethylsilane; TSP, 3-trimethylsilyl-[2,2,3,3-$^2H_4$]propionate; LDA, lithium diisopropylamide; THF, tetrahydrofuran; ORD, optical rotatory dispersion.

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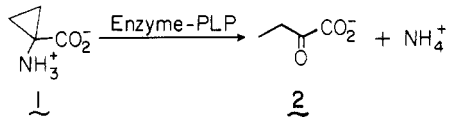
Scheme II



adenosylmethionine followed by a ring-closing displacement of methylthioadenosine to form ACPC (Adams & Yang, 1979). The cyclopropane is then oxidatively cleaved to ethylene by processes as yet poorly understood (Adams & Yang, 1979; Lurssen et al., 1979; Lieberman, 1979). The enzyme that forms ACPC from SAM may well construct the required carbon–carbon bond by an internal γ replacement, a typical pyridoxal-P-dependent enzymatic reaction. The oxidative cleavage of the cyclopropane to ethylene must be more complex.

Recently, an enzyme has been isolated from a soil bacterium (*Pseudomonas* sp.) and from a yeast (*Hansenula saturnus*) which catalyzes the cleavage of ACPC to α-ketobutyrate and ammonia (Scheme II) (Honma & Shimomura, 1978). The pure enzyme contains tightly bound PLP, and the reaction may be categorized in one sense as an intramolecular γ elimination, followed by azaallylic isomerization and hydrolysis. The conversion of SAM to ACPC in Scheme I, viewed in the reverse direction, is clearly analogous to this bacterial ACPC deaminase.

In a second sense, the ring-opening reaction of Scheme II is a C_β–C_α bond cleavage that must be initiated without obvious accessibility to a carbanionic intermediate at the α carbon, since it bears no abstractable proton and the carboxyl group is retained in the α-ketobutyrate product. Such a C_β–C_α bond cleavage is precedented in PLP-dependent enzymes only by serine transhydroxymethylase, although in that example the serine–PLP complex is set up for retro-aldol cleavage—a mechanistic impossibility for an ACPC–PLP complex.

In this paper, we report studies on cyclic and acyclic substrates and suicide substrates for the pseudomonad ACPC deaminase in an initial effort to unravel the route of cyclopropane ring cleavage by this PLP-dependent enzyme.

Materials and Methods

General. Unless otherwise stated, all solvents, organic chemicals, and inorganic chemicals were of analytical reagent grade. Deuterated buffers were prepared by dissolving appropriate quantities of buffer salts in D_2O (99.7 atom %; Merck, Sharp and Dohme), evaporating the solutions to dryness, redissolving the residue in D_2O, and making slight adjustments in pD with solutions of DCl or NaOD.

Melting points were recorded on a Mel-Temp apparatus and are uncorrected. Ultraviolet–visible spectroscopy was performed on a Perkin-Elmer 554 spectrophotometer. Proton nuclear magnetic resonance (NMR) spectra were recorded on JEOL FX90Q and Bruker WH-250 Fourier-transform spectrometers operating at 90 and 250 MHz, respectively. Chemical shifts are reported as parts per million downfield from tetramethylsilane (Me_4Si) or sodium 3-trimethylsilyl-[2,2,3,3-$^2H_4$]propionate (TSP). Deuterium NMR spectra were obtained on the latter instrument at 38.4 MHz. Gas–liquid chromatography (GLC) was carried out on a Perkin-Elmer 3920B unit equipped with a thermal conductivity detector. The columns (6 ft × $^1/_8$ in.) were packed with 8% OV-17 on Chromosorb W HP (80–100 mesh). Coupled gas–liquid chromatography–mass spectrometry (GLC–MS) was performed on a Hewlett-Packard 5992 GC/MS system, but mass spectra of solid samples were obtained by using a Varian MAT 44 spectrometer. All mass spectra were recorded by using an electron beam energy of 70 eV.

Enzymes. 1-Aminocyclopropane-1-carboxylic acid deaminase was purified as previously described (Honma & Shimomura, 1978). This enzyme (M_r 110 000) consists of three identical subunits (M_r 37 000) each bearing one molecule of pyridoxal-P (M. Honma, unpublished results). The enzyme displays a characteristic pyridoxaldimine visible absorbance at 418 nm, and homogeneous preparations typically exhibit an A_{278}/A_{416} ratio of 5.8–6.0.

D-Serine dehydratase (from *Escherichia coli*) was the generous gift of Professor Jules Shafer of the University of Michigan. L-Lactate dehydrogenase (rabbit skeletal muscle) was purchased from Boehringer-Mannheim.

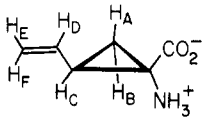
Substrates and Inhibitors. 1-Aminocyclopropane-1-carboxylic acid and 1-aminocyclopentane-1-carboxylic acid (cycloleucine) were purchased from Calbiochem. The synthesis of 1-amino-2-vinylcyclopropane-1-carboxylic acid (vinyl-ACPC) is described below.

D-Allylglycine, sodium α-ketobutyrate, sodium DL-α-hydroxybutyrate, D- and L-methionine, pyridoxal 5-phosphate, *O*-succinyl-DL-homoserine, D-serine, and DL-*threo*-β-phenylserine were all Sigma products. β-Chloro-L-alanine and β-chloro-D-alanine were purchased from Vega Fox Biochemicals. All fluoroalanine samples were gifts of Dr. J. Kollonitsch of Merck, Sharp and Dohme Research Laboratories; their preparation has been described (Kollonitsch & Barash, 1976).

O-Acetyl-D-serine was prepared according to the method of Sakami & Toennies (1942). The syntheses of vinylglycine and the 3-chloro-2-aminobutyrates (Marcotte & Walsh, 1976; Plattner et al., 1957), DL-*trans*- and DL-*cis*-2-amino-3-pentenoate (Marcotte & Walsh, 1978; Johnston et al., 1981), and D-propargylglycine and DL-[α-^{3}H]propargylglycine (Abeles & Walsh, 1973; Marcotte & Walsh, 1976; Johnston et al., 1979) have been described previously. L-[α-^{3}H]Alanine and D-[α-^{3}H]alanine, prepared by exchange of the racemic mixture in ^3H_2O and resolved by acetylation and hog kidney acylase action (Greenstein & Winitz, 1961), were gifts of Dr. Elizabeth Wang.

1-Amino-2-vinylcyclopropane-1-carboxylic Acid. Benzylideneglycine methyl ester (Stork et al., 1976) (0.02 mol, 3.54 g) and 75 mL of tetrahydrofuran (THF) were added to 1 equiv of lithium diisopropylamide (LDA) in 100 mL of dry THF. The mixture was stirred at −78 °C for 30 min, whereupon a solution of *trans*-1,4-dibromobut-2-ene (Aldrich) (0.02 mol, 4.28 g) in 100 mL of dry THF was added dropwise. The mixture, after being stirred at −70 °C for 30 min, was maintained at −20 °C for 30 min and then cooled to −78 °C. Following the addition of another equivalent of LDA, the mixture was held at −78 °C for 1 h and then warmed to room temperature. After the mixture allowed to stand overnight, the benzylidene ester isolated (workup was ice-cold aqueous NH_4Cl–ether) was then passed through 10 times its weight of acid-washed silica gel, followed by elution with ether (after removal of benzaldehyde with pentane). The α-amino ester (1.2 g, 8.5 mmol) was thus obtained in 42% yield: NMR ($CDCl_3$) δ 1.4 (m, 2 H, cyclopropyl CH_2), 1.99 (m, 1 H, cyclopropyl CH) and 2.12 (s, 2 H, NH_2) (overlapping multiplet and singlet), 3.63 (s, 3 H, CO_2CH_3), 5.0 (dd, 1 H), 5.2 (dd, 1 H), and 5.6 (m, 1 H). The amino ester (0.4 g, 2.84 mmol) was dissolved in 40 mL of 5 N HCl and refluxed for 1 h. The mixture was cooled to room temperature and then placed on a 1 × 20 cm column of Dowex 50W (H^+ form, 50X4-400; Sigma). The product amino acid was eluted in the usual manner with ammonium hydroxide (Moore & Stein, 1951). Evaporation and concentration followed by recrys-

tallization of the residue from THF–ether gave slightly yellowish crystals of 1-amino-2-vinylcyclopropane-1-carboxylic acid (**3**): mp 195–196 °C; NMR (300 MHz, $CDCl_3$–CD_3OD) δ 1.72 (dd, 1 H, J = 8.4, 6.3 Hz, H_A), 1.90 (dd, 1 H, J = 10.1, 6.3 Hz, H_B), 2.54 (ddd, 1 H, J = 10.1, 8.7, 8.4 Hz, H_C), 5.20 (dd, 1 H, J = 10.3, 1.5 Hz, H_E), 5.39 (dd, 1 H, J = 17.2, 1.5 Hz, H_F), and 5.77 (ddd, 1 H, J = 17.2, 10.3, 8.7 Hz, H_D); ^{13}C NMR (D_2O) δ 28.9, 39.6, 53.1, 129.7, 145.0, and 184.3.



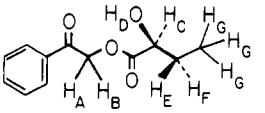
The extremely clean proton and ^{13}C NMR spectra of the synthetic vinyl-ACPC suggest that only one of two possible diastereomeric pairs is present, probably the 1*SR*,2*RS* isomer in which the vinyl and amino groups are trans (as illustrated).

Enzyme Assay. The continuous assay of keto acid formation from substrate ACPC or from substrate analogues was accomplished by the coupled reduction of the keto acid to the corresponding L-hydroxy acid in the presence of NADH and L-lactate dehydrogenase. A standard 1.0-mL assay was performed in 50 mM potassium pyrophosphate (pH 8.5) at 37 °C, containing substrate, 10–20 μg of ACPC deaminase, 300 μg of lactate dehydrogenase, and 0.3 μmol of NADH. Loss of NADH absorbance at 340 nm was monitored.

α-Hydrogen Exchanges. The rates of enzyme-catalyzed proton exchange between substrate and solvent water were determined for L-[α-^{3}H]alanine (168 μCi/mmol), D-[α-^{3}H]-alanine (255 μCi/mmol), and DL-[α-^{3}H]propargylglycine (600 μCi/mmol). A typical experiment was conducted as follows. A 1.5-mL solution was prepared containing 10–25 mM α-^{3}H-labeled amino acid and 50 mM KPP_i buffer (pH 8.5) at 37 °C. The reaction was initiated by addition of 15 μg of enzyme. Aliquots (100 μL) were withdrawn at timed intervals and loaded onto Dowex 50(H^+) columns. The columns were eluted with 2.0 mL of water, and the effluent was counted in Amersham aqueous counting scintillant.

Enzyme Inactivations. The rates of enzyme inactivation were estimated in the following way. To a 90-μL solution of 50 mM KPP_i buffer (pH 8.5) at 37 °C containing an appropriate concentration of putative inactivator was added 10 μL of a concentrated (~18 mg/mL) solution of ACPC deaminase. Measured aliquots (usually <5 μL) were withdrawn at timed intervals and assayed for remaining catalytic activity by dilution into 1.0 mL of the standard continuous assay system. The half-time for inactivation was obtained from a semilog plot of percent activity remaining vs. time.

Phenacyl α-Hydroxybutyrate. Sodium DL-α-hydroxybutyrate (300 mg) and phenacyl bromide (500 mg; Eastman) were mixed in 10 mL of 50% ethanol and heated on a steam bath for 30 min. After the mixture was cooled, the solvent was evaporated. Ether and water (20 mL of each) were added, the mixture was shaken, and the ether layer was separated, concentrated, and applied to two preparative TLC plates (silica gel 60F, 20 × 20 × 0.2 cm) which were developed with ether. The band of R_f = 0.3 was scraped off and eluted with chloroform. The chloroform was evaporated, and the residue was recrystallized from ether–hexane to yield 109 mg of phenacyl α-hydroxybutyrate, mp 57–59 °C. This material showed a single component on TLC (silica gel 1B-F; solvent, ether), R_f = 0.26, and GLC analysis (8% OV-17; 210 °C) of its trimethylsilyl ether derivative indicated a purity of 99%. The mass spectrum showed prominent ions at m/z 222 (M^+, 3%), 120 ($C_6H_5COHCH_2^+$, 27%), 105 ($C_6H_5CO^+$, 100%), 77 ($C_6H_5^+$, 40%), and 59 ($CH_3CH_2CHOH^+$, 36%). The mass spectrum of the trimethylsilyl ether derivative exhibited ions at m/z 279 ($M^+ - CH_3$, 2%), 131 [$CH_3CH_2CHOSi(CH_3)_3^+$, 52%], 119 ($C_6H_5COCH_2^+$, 22%), 105 ($C_6H_5CO^+$, 38%), 77 ($C_6H_5^+$, 33%), and 73 [$(CH_3)_3Si^+$, 100%]: NMR (250 MHz, $CDCl_3$) δ 1.08 (dd, 3 H, J = 7, 7 Hz, H_G), 1.85 (dqd, 1 H, J = 14, 7, 7 Hz, H_E), 2.01 (dqd, 1 H, J = 14, 7, 5 Hz, H_F), 2.69 (d, 1 H, J = 6 Hz, H_D; disappears on D_2O exchange), 4.37 (ddd, 1 H, J = 7, 6, 5 Hz, H_C; D_2O exchange gives dd, J = 7, 5 Hz), 5.33 and 5.58 (AB quartet, 2 H, J = 16 Hz, H_A and H_B), 7.51 (m, 2 H, aromatic H_2), 7.63 (m, 1 H, aromatic H), and 7.91 (m, 2 H, aromatic H_2). From these data, we conclude that J_{AB} = 16 Hz, J_{EF} = 14 Hz, J_{CE} = 7 Hz, J_{EG} = 7 Hz, J_{FG} = 7 Hz, J_{CD} = 6 Hz, and J_{CF} = 5 Hz. For phenacyl (2*S*)-2-hydroxybutyrate, H_F (2.01 ppm) is *pro-S* as indicated (vide infra).



Isolation and Characterization of Enzymatically Produced (2S)-2-Hydroxybutyrate as Its Phenacyl Ester. After completion of an enzymatic incubation, the reaction mixture [containing ca. 50 μmol of (2*S*)-2-hydroxybutyrate, 70 μmol of pyridine nucleotides, 400 μmol of phosphate buffer, and 3 mg of total protein] was passed through an AG 50W-X4(H^+) column (0.7 × 4 cm). The effluent was concentrated to about 5 mL, if necessary, and the pH was adjusted to 7 with 1 N NaOH. Phenacyl bromide (300 mg) was added in 5 mL of ethanol, and the resulting mixture was heated on a steam bath for 30 min. Trisodium citrate (500 mg) was then added, and heating was continued for 15 min to consume the excess phenacyl bromide and render it water soluble. After the mixture was cooled, the solvent was evaporated, and ether and water (30 mL of each) were added. After vigorous shaking, the ether layer was separated, dried, concentrated, and applied to a prepatative TLC plate (silica gel 60F, 20 × 20 × 0.2 cm) which was developed in ether. The band of R_f = 0.3 was eluted with chloroform. The chloroform was evaporated, and the residue was recrystallized from ether–hexane to yield the desired phenacyl ester. This crystalline material contained varying amounts of α-hydroxyacetophenone (0–60%); however, this contaminant in no way interfered with the NMR or GLC–MS analyses, since none of its proton NMR signals (except those of the aromatic ring) overlap those of phenacyl (2*S*)-2-hydroxybutyrate, and the GLC mobilities of the Me_4Si ethers of the two compounds are vastly different.

For analysis by proton NMR, 5 mg of the ester was dissolved in 0.5 mL of $CDCl_3$, and ~1 μL of Me_4Si was added. The solution was shaken with 0.1 mL of D_2O, and the spectrum was recorded after separation of the phases. For analysis by GLC–MS, 0.5 mg of the ester was treated with 50 μL of Tri-Sil (Pierce Chemical Co.). The resulting solution of trimethylsilyl ether derivatives was used directly.

Results

Conversion of 1-Aminocyclopropane-1-carboxylic Acid (ACPC) to α-Ketobutyrate in Deuterated Buffer. As an initial step in the analysis of the pathway for the unique cleavage of the cyclopropane ring of ACPC in an internal redox reaction to α-ketobutyrate and ammonia by ACPC deaminase, enzymatic incubations were conducted in 99% D_2O to evaluate the number and location of the solvent-derived deuterium atoms incorporated into the keto acid product. One prochiral methylene carbon in ACPC ends up as the C-4 methyl group of α-ketobutyrate while the other prochiral methylene of

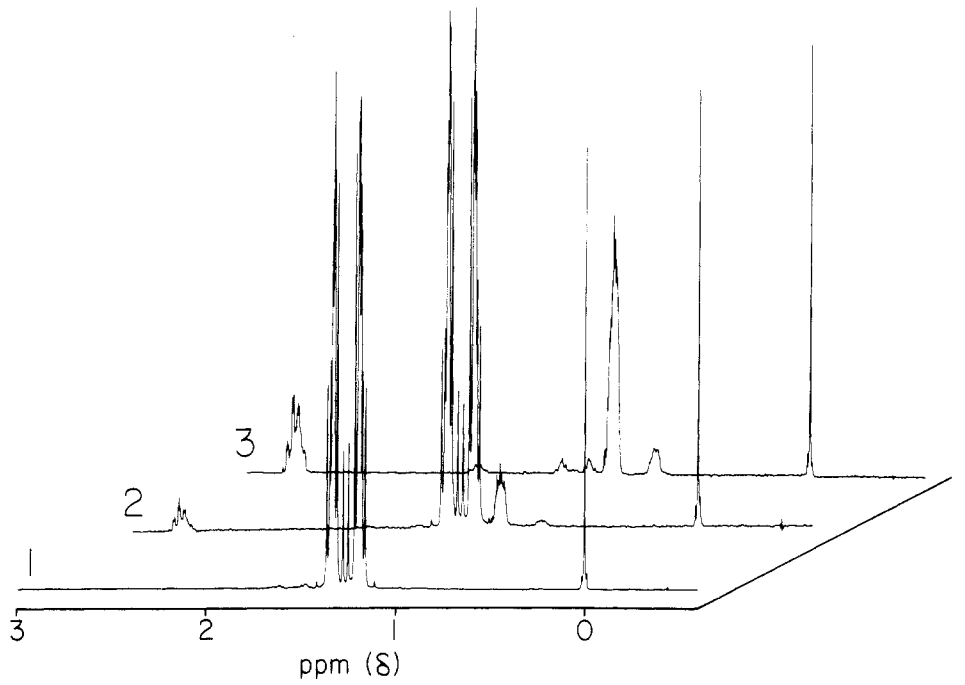


FIGURE 1: Conversion of ACPC to α-ketobutyrate by ACPC deaminase in deuterated buffer monitored by 250-MHz proton NMR. (The reaction conditions are described in the text.) Spectrum 1 was recorded before the addition of ACPC deaminase; spectra 2 and 3 were recorded 1 and 7 h, respectively, after addition of the enzyme. The AA'BB' multiplet centered at δ 1.26 is due to the four ACPC methylene protons. The signals appearing at δ 1.07 and δ 2.76 are due to the methyl and methylene protons, respectively, of α-ketobutyrate, and the smaller signals appearing at δ 0.85 and δ 1.78 are similarly due to the hydrated form of α-ketobutyrate [$CH_3CH_2C(OH)_2CO_2^-$].

Table I: Decrease in the Total Number of Carbon-Bonded Protons during Conversion of ACPC to α-Ketobutyrate by ACPC Deaminase in Deuterated Buffer[a]

time of reaction (h)	% ACPC remaining[b]	total integral[c]
0	100	4.00
1.0	81	3.78
3.0	48	3.38
5.1	17	2.63
7.0	4	2.54
24.7	0	1.92

[a] Reaction conditions are described in the text. The course of the reaction was monitored by 250-MHz proton NMR. The signal from an internal sample of TSP was employed as a chemical shift reference (δ 0.00) and as an integration standard. [b] Determined by integration of the multiplet centered at δ 1.26 (see Figure 1). [c] Determined by integration of the signals at δ 0.85, 1.07, 1.26, 1.78, and 2.76 (see Figure 1), which represent all of the carbon-bonded protons in the reaction mixture (except for the inert TSP standard).

Table II: Relative Numbers of Methyl and Methylene Protons in α-Ketobutyrate Formed from ACPC by ACPC Deaminase in Deuterated Buffer[a]

time of reaction (h)	methyl integral[b]	methylene integral[c]
1.0	2.00	1.00
3.0	2.00	0.88
5.1	2.00	0.80
7.0	2.00	0.76
24.7	2.00	0.29

[a] Reaction conditions are described in the text. The course of the reaction was monitored by 250-MHz proton NMR (see Figure 1). [b] The signal at δ 1.07 was integrated and assigned a value of 2.00 protons at each time point. [c] Determined by integration of the signal at δ 2.76. This figure represents the number of protons in the methylene of α-ketobutyrate, assuming that the methyl group contains 2.00 protons.

ACPC is nominally converted to the C-3 methylene of the product. Stoichiometric considerations mandate that at least one solvent hydrogen must be incorporated, but greater incorporation might be expected for a number of potential mechanisms for ACPC cleavage.

ACPC (5 mg) and TSP (~0.2 mg) were dissolved in deuterated potassium phosphate buffer (100 mM, pD 8.8, 0.5 mL). The proton NMR spectrum was recorded. ACPC deaminase (112 μg) was added, and spectra were recorded at timed intervals over a 25-h period of incubation at 37 °C. Figure 1 illustrates the spectra obtained at the start and after 1 and 7 h of incubation. The signal from TSP was used both as a chemical shift reference (0 ppm) and as an integration standard.

Table I summarizes the kinetic course of this reaction. The cyclopropane substrate has four nonexchangeable C-H protons, so the "total integral" value in the 250-MHz spectrum (Figure 1, spectrum 1) was set to 4.00 at $t = 0$, prior to addition of the enzyme. At 24.7 h, after complete conversion of the substrate, the value of 1.92 for the total integral indicates that only two of these protons remain in the product ketobutyrate. The appearance of the methyl and methylene resonances of the accumulating ketobutyrate (Figure 1, spectra 2 and 3) allows integration of those signals to determine the ratio of protons in each. These values are summarized in Table II. It is clear that at early reaction times (1 h, 19% substrate conversion) the methyl/methylene proton ratio was 2.00/1.00. As the reaction progressed, the nonenzymatic exchange of the other acidic C-3 methylene proton in ketobutyrate commenced. A separate control study established that the half-time for such non-enzymatic enolization and exchange with deuterated solvent was 13.8 h under these conditions. As expected, there was no adventitious exchange of the C-4 methyl protons from the enzymatic product ketobutyrate. It is clear from these aggregate data that enzymatic turnover introduces one solvent deuterium at the C-4 methyl and one at the C-3 methylene of the α-ketobutyrate product and that the second methylene proton is exchanged nonenzymatically.

Deuterium NMR Analysis of α-Ketobutyrate Produced by ACPC Deaminase in Deuterated Buffer. ACPC (20 mg) and ACPC deaminase (93 μg) were incubated in deuterated potassium phosphate buffer (100 mM, pD 8.8, 1.0 mL) for 3 days at 37 °C. The resulting solution was lyophilized, water was added, it was added, it was lyophilized again, water was added, and the solution was left at room temperature for 2 days to exchange the deuteriums out of C-3 of the ketobutyrate. The solution was passed through a small Dowex 50(H^+) column; 5 μL of acetonitrile-d_3 was added, and the sample was submitted for deuterium NMR. The spectrum was recorded at 38.4 MHz with broad-band ^1H decoupling. Chemical shifts are reported relative to deuterioacetonitrile at 1.93 ppm. Three signals were observed—4.84 (HDO), 1.93 (CD_3CN), and 0.85 ppm. Since ^2H NMR chemical shifts are comparable to those for ^1H NMR, and since the C-3 methylene deuterons had been exchanged away, the latter resonance must reflect the incorporation of deuterium into the terminal methyl of α-ketobutyrate.

Characterization of Phenacyl (2S)-2-Hydroxybutyrate Derived from Enzymatic Incubations in Deuterated Buffer. To validate the locations of solvent-derived deuterium and to analyze the stereochemical outcome of enzyme-mediated incorporation of a solvent proton at the prochiral C-3 methylene center of α-ketobutyrate, we performed incubations with in situ reduction of α-ketobutyrate to (2S)-2-hydroxybutyrate by NADH and lactate dehydrogenase. The coupled reduction vitiates the nonenzymatic exchange and/or racemization at the C-3 methylene group. The resulting (2S)-2-hydroxy acids were isolated as their phenacyl esters as described under Materials and Methods. This crystalline derivative was easily handled and characterized by NMR and mass spectrometric techniques. Spectrum 1 of Figure 2 displays the partial 250-MHz NMR spectrum of phenacyl (2S)-2-hydroxybutyrate isolated from an incubation of ACPC with ACPC deaminase in a protic buffer. This spectrum is identical with that of phenacyl α-hydroxybutyrate prepared by chemical synthesis. Of particular interest are the resonances of the now diastereotopic C-3 methylene protons, differing in chemical shift, which are observed at 2.01 and 1.85 ppm. The distinct resonance frequencies for the C-3 protons allow analysis of the stereospecificity of enzymatic action in D_2O by integration of the remaining hydrogen signals at 2.01 and 1.85 ppm from an enzymatic sample of phenacyl (2S)-2-hydroxy[3,4-$^2H_2$]-butyrate. Absolute assignment of chirality requires a phenacyl (2S)-2-hydroxy[3-^2H]butyrate of known configuration. For this, we turned to the D-serine dehydratase of *E. coli*, a PLP-dependent enzyme reported to convert D-threonine to α-(3S)-3-keto[3-^2H]butyrate (Yang et al., 1975). In the reaction coupled to lactate dehydrogenase, (2S,3S)-2-hydroxy-[3-^2H]butyrate would be the expected product. Therefore, the following incubations were performed.

D-Threonine (6 mg), D-serine dehydratase (15 μg), NADH (50 mg), lactate dehydrogenase (3 mg), and deuterated sodium phosphate buffer (50 mM, pD 7.9, 3.5 mL) were incubated for 24 h at 37 °C. The (2S)-2-hydroxy[3-^2H]butyrate formed was isolated as the phenacyl ester. The results of NMR and mass spectrometric analyses are listed in Table III. The mass spectral data revealed the incorporation of a single deuterium into each hydroxybutyrate ester. From the 250-MHz NMR spectrum (Figure 2, spectrum 2), it can be seen that the methylene resonance at about 1.98 ppm is preferentially diminished; therefore, this must be the 3S-proton signal. Furthermore, the incorporation of a single deuterium at C-3 and only at C-3 is reflected in the collapse of the methine (4.37 ppm) and methyl (1.05 ppm) proton resonances to doublets without a diminution of intensity.

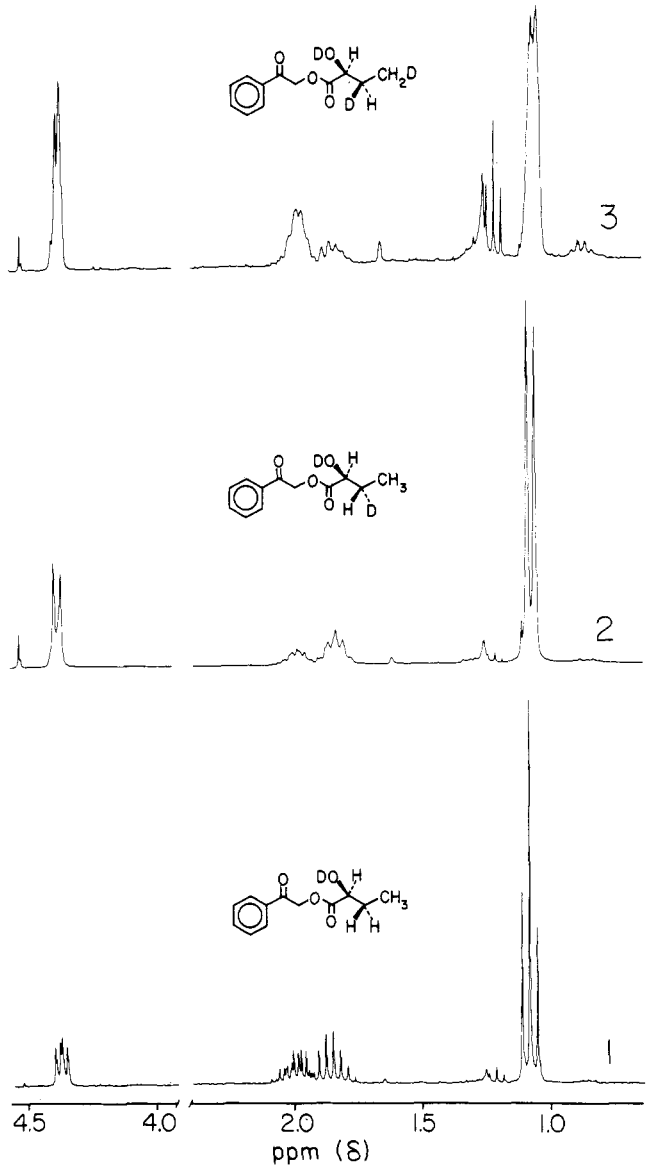


FIGURE 2: Partial 250-MHz proton NMR spectra of phenacyl (2S)-2-hydroxybutyrates derived from incubations of ACPC with ACPC deaminase in protic buffer (spectrum 1), D-threonine with D-serine dehydratase in deuterated buffer (spectrum 2), and ACPC with ACPC deaminase in deuterated buffer (spectrum 3). (The reaction conditions and isolation methods are described in the text.)

ACPC (5 mg), ACPC deaminase (93 μg), NADH (50 mg), lactate dehydrogenase (3 mg), and deuterated potassium phosphate buffer (100 mM, pD 8.8, 3.0 mL) were incubated for 24 h at 37 °C. The (2S)-2-hydroxy[3,4-$^2H_2$]butyrate formed was isolated as its phenacyl ester. Mass spectrometric analysis (Table III) indicated that two solvent deuterons had been incorporated, as expected. The NMR spectrum (Figure 2, spectrum 3) shows a preferential reduction of the resonance at 1.85 ppm; therefore, ACPC deaminase must have introduced a solvent deuterium at the 3R position of α-ketobutyrate. Integration of the methyl, methylene, and methine proton signals (see Table III) reveals that the C-4 methyl group retains only two protons, confirming that ACPC deaminase introduced the second deuteron at this location.

Neither ACPC deaminase nor D-serine dehydratase exhibited complete stereospecificity with regard to the introduction of deuterium at carbon 3. In order to determine if nonenzymatic racemization occurred at C-3 during the derivatization

Table III: Proton NMR and Mass Spectral Data for Phenacyl (2*S*)-2-Hydroxybutyrates Isolated from Various Enzymatic Incubations

substrate	enzyme	buffer solvent	GLC–MS[a]		proton NMR[b] (δ)				C-3 chirality[c] (*R*:*S*)
			$M^+ - CH_3$[d]	$CH_3CH_2CHOSi(CH_3)_3^+$[d]	C-2 H	C-3 H_S	C-3 H_R	C-4 H_3	
ACPC	ACPC deaminase	H_2O	279	131	4.37 (dd, 1 H)	2.01 (dqd, 1 H)	1.85 (dqd, 1 H)	1.08 (dd, 3 H)	
D-threonine	D-serine dehydratase	D_2O	280	132	4.37 (d, 1 H)	1.98 (m, 0.38 H)	1.85 (m, 0.71 H)	1.07 (d, 3.0 H)	32:68
ACPC	ACPC deaminase	D_2O	281	133	4.37 (d, 1 H)	1.98 (m, 0.76 H)	1.85 (m, 0.34 H)	1.05 (m, 2.18 H)	73:27
ACPC	ACPC deaminase	D_2O	281	133	4.37 (d, 1 H)	1.98 (m, 0.73 H)	1.85 (m, 0.29 H)	1.05 (m, 2.02 H)	72:28

[a] The trimethylsilyl ether derivative was employed for GLC–MS analyses. The spectra were obtained at 70 eV. [b] Spectra were recorded at 250 MHz in chloroform solutions with tetramethylsilane as an internal reference. [c] The *R*:*S* ratio is corrected for the presence of molecules lacking a deuterium at C-3. Thus, for the D-threonine/D-serine dehydratase experiment, the sum of the integrations of the C-3 H_S and C-3 H_R protons is 1.09 H, so 9% of the molecules contain no deuterium at C-3. By subtracting 0.09 H each from the C-3 H_S and C-3 H_R integrations, we obtain a C-3 H_S:C-3 H_R ratio of 0.29 H:0.62 H or 32:68. Therefore, of the deuterated chiral molecules, 68% have the *S* configuration at C-3, and 32% have the *R* configuration. [d] The deuterium content of the phenacyl (2*S*)-2-hydroxybutyrate Me_4Si ether derivatives could not be *precisely* determined because of the presence of smaller, but significant, ions adjacent to the $M^+ - CH_3$ and $CH_3CH_2CHOSi(CH_3)_3^+$ ions, e.g., ions formed by the gain or loss of a proton from the principal ion. Nevertheless, the predominant state of deuteration was evident from the principal ion in these clusters.

Scheme III

process, synthetic phenacyl α-hydroxybutyrate was heated on a steam bath for 45 min in the presence of 0.2 M trisodium citrate in 1:1 ethanol:D_2O (v:v; such a solution is 86 atom % D in exchangeable hydrogens) at pD 7. NMR and mass spectral analysis detected no deuterium in the hydroxybutyrate portion of the molecule, so nonenzymatic racemization is unlikely. (The methylene protons of the phenacyl portion of the molcule, however, were 70% exchanged for deuterium under these conditions.)

The observed stereoselectivity in the D-threonine/D-serine dehydratase experiment (68:32 *S*:*R*, see Table III) is in good agreement with previous literature reports. Yang et al. (1975) reported that α-ketobutyrate formed by the action of D-serine dehydratase on D-threonine in D_2O yielded, upon H_2O_2 oxidation, (2*S*)-2-deuteriopropionate with a specific rotation "80% of that reported by Krongelb et al. (1968) for synthetic sodium (2*R*)-2-deuteriopropionate". The sample of Krongelb et al. (1968) exhibited a specific rotation of −6.8° at 257 nm. However, LaRoche et al. (1971) later obtained (2*R*)-2-deuteriopropionate via a fermentation process with a specific rotation of −21.0° at 257 nm. With the assumption that this latter material contained 100% of the 2*R* isomer, then the sample of Yang et al. must have contained a 26% [(0.80 × 6.8°)/21.0° × 100] enantiomeric excess of (2*S*)-2-deuteriopropionate, implying a 63:37 *S*:*R* stereochemical preference.

In two separate experiments, ACPC deaminase exhibited a stereochemical preference of approximately 72:28 *R*:*S* with regard to the incorporation of deuterium at C-3 of α-ketobutyrate. The overall reaction catalyzed by ACPC deaminase in deuterated buffer is illustrated in Scheme III.

Reaction of 2-Vinyl-ACPC with ACPC Deaminase. The vinyl analogue of ACPC, 1-amino-2-vinylcyclopropane-1-carboxylic acid (vinyl-ACPC), was prepared, as a synthetic mixture of four stereoisomers, to test several mechanistic points in enzyme catalysis, including regiospecificity of cyclopropane bond fragmentation, detection of intermediates, determination of kinetic site of protonation, and analysis for any mechanism-based inactivation during turnover.

Our previous studies proved that of the four possible stereoisomers of 2-ethyl-ACPC, also known as coronamic acid, only the 1*S*,2*S* isomer, *d*-coronamic acid, was utilized with exclusive regiospecific enzymatic cleavage to yield the straight-chain 2-ketohexanoate product (Honma et al., 1979). In the present study, vinyl-ACPC was found to be efficiently processed by ACPC deaminase to an α-keto acid product (Table IV), with V_{max} and K_m values (Table V) determined by a coupled LDH/NADH assay.

The identity of the keto acid was established by proton NMR analysis of the enzymatic products formed in protic and deuterated buffers. Vinyl-ACPC (4 mg) and ACPC deaminase (93 μg) were incubated in 1.5 mL of 200 mM KP_i buffer (pH 8.3) for 3 days at 37 °C. The reaction mixture was passed through a 0.7 × 4 cm column of Dowex AG 50W-X4(H^+), the pH of the effluent was adjusted to about 6 with 1 N NaOH, the solvent was evaporated, and the residual keto acid and phosphate buffer salts were dissolved in D_2O for NMR analysis. A similar incubation was carried out in deuterated KP_i buffer (100 mM, pD 8.8). The 250-MHz NMR spectrum of the product of the protic incubation displayed the following signals: δ 2.28 (m, 2 H, H_X), 2.80 (t,

Table IV: Substrate Specificity for ACPC Deaminase Catalyzed Keto Acid Formation

substrate	concn (mM)	% rate of 20 mM ACPC
vinyl-ACPC	20	110
1-aminocyclopentane-1-carboxylic acid	20	0
D-β-fluoroalanine	20	22
L-β-fluoroalanine	20	0
D-β,β-difluoroalanine	20	35
D-β-chloroalanine	40	7
L-β-chloroalanine	40	0
DL-β,β-dichloroalanine	20	11
D-serine	20	2
L-serine	20	0
O-acetyl-D-serine	20	12
O-acetyl-L-serine	20	0
O-succinyl-L-homoserine	20	0
O-succinyl-DL-homoserine	20	0
D-methionine	20	0
L-methionine	20	0
D-vinylglycine	20	8
L-vinylglycine	20	0
D-allylglycine	20	0
D-propargylglycine	40	0
DL-*trans*-2-amino-3-pentenoate	40	0
DL-*cis*-2-amino-3-pentenoate	40	0
D-*erythro*-2-amino-3-chlorobutyrate	20	3
L-*erythro*-2-amino-3-chlorobutyrate	20	0
L-*threo*-2-amino-3-chlorobutyrate	20	0
DL-*threo*-2-amino-3-fluorobutyrate	17	4
DL-*erythro*-2-amino-3-fluorobutyrate	22	10

Table V: Kinetic Parameters[a] for the Reactions of ACPC Deaminase with Various Substrates

compound	turnover K_M (mM)	turnover k_{cat} (min^{-1})	inactivation K_I (mM)	inactivation k_{inact} (min^{-1})	partition ratio, k_{cat}/k_{inact}
ACPC	9.2	290		0.0	
vinyl-ACPC	4.0	310		0.0	
β-chloro-D-alanine	5.4	22	9.6	0.33	68
β-fluoro-D-alanine	1.1	34	0.7	0.18	190
O-acetyl-D-serine	56	30	4.5	0.10	300
D-vinylglycine	97	36		0.0	

[a] The kinetic parameters listed in this table are defined by the equation $v = V_{max}S/(K_M + S)$ for the process

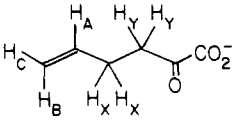
$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} E + P$$

where v = initial velocity, $K_M = (k_{cat} + k_{-1})/k_1$, and $k_{cat} = V_{max}/[E]$. Enzyme inactivation kinetics conform to the process

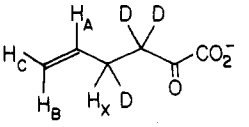
$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EI \xrightarrow{k_{inact}} E\text{-}I$$

where the binding constant K_I is given by k_{-1}/k_1 and k_{inact} describes the apparent pseudo-first-order rate constant for inactivation extrapolated to infinite inhibitor concentration.

1.2 H, J = 7 Hz, H_Y; after 3 weeks of storage at −20 °C, this signal virtually disappeared), 4.96 (dtd, 1 H, J = 10, 1.5, 1.5 Hz, H_C), 5.01 (dtd, 1 H, J = 17, 2, 1.5 Hz, H_B), and 5.82 (ddt, 1 H, J = 17, 10, 6.5 Hz, H_A). A slightly different spectrum



was obtained from the deuterated incubation product: δ 2.27 (m, 1 H, H_X), 4.97 (ddd, 1 H, J = 10, 1.5, 1.5 Hz, H_C), 5.02 (ddd, 1 H, J = 17, 2, 1.5 Hz, H_B), and 5.82 (ddd, 1 H, J = 17, 10, 6.5 Hz, H_A). The olefinic proton regions of these



spectra are illustrated and analyzed in Figure 3. That the products of the enzymatic reactions are the illustrated 2-keto-5-hexenoates is indicated by the absence of any methyl resonance, the presence of three mutually coupled vinyl proton resonances, and the extremely facile exchange of the C-3 protons (H_Y) of the protic incubation product. The reduction of the H_X signal from 2 H to 1 H and the collapse of the H_A resonance from a doublet of doublet of triplets to a doublet of doublet of doublets in the deuterated incubation product (see Figure 3) leave no doubt that one solvent deuteron has been enzymatically introduced at C-4 of the product.

A likely intermediate in the conversion of vinyl-ACPC to

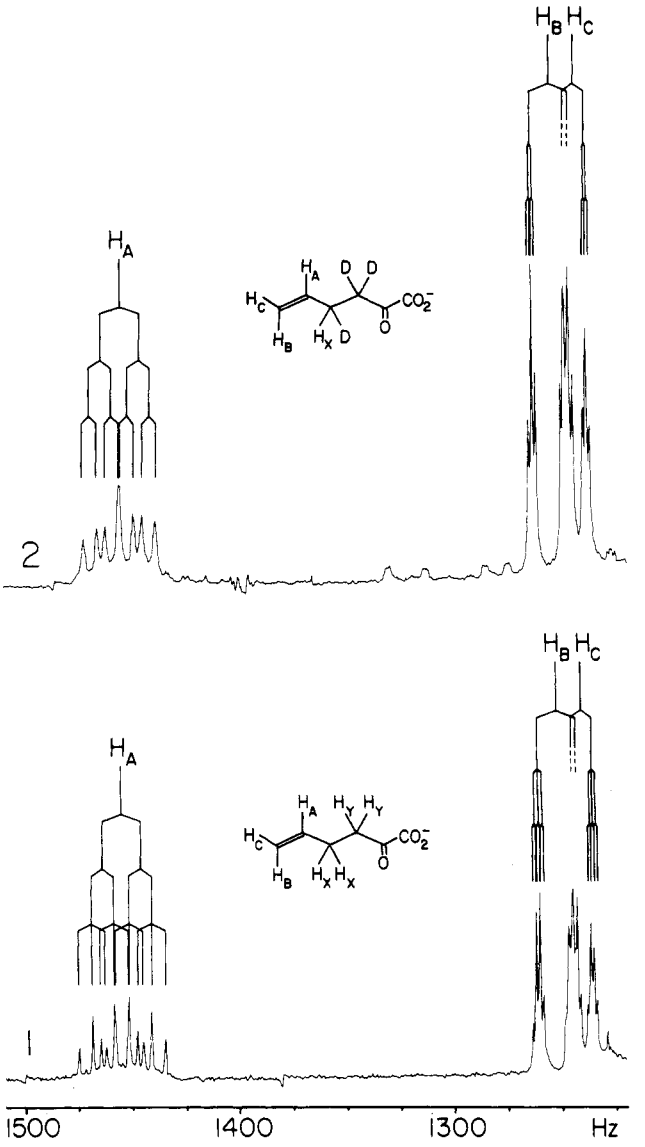
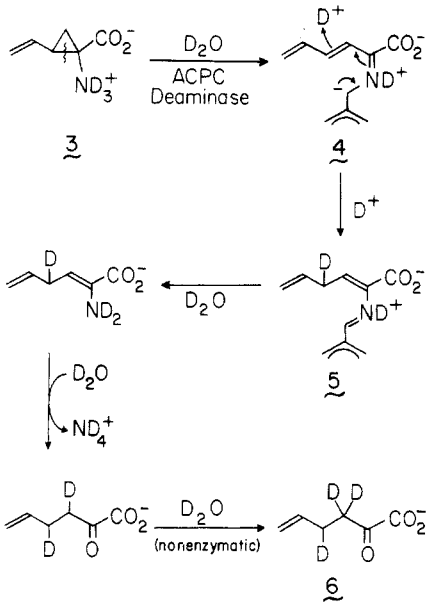


FIGURE 3: Olefinic proton regions of the 250-MHz proton NMR spectra of the 2-keto-5-hexenoates isolated from incubations of vinyl-ACPC with ACPC deaminase in protic buffer (spectrum 1) and in deuterated buffer (spectrum 2). (The reaction conditions and isolation procedures are described in the text.) The magnitudes of the observed coupling constants are $^3J_{AB}$ = 17 Hz, $^3J_{AC}$ = 10 Hz, $^3J_{AX}$ = 6.5 Hz, $^2J_{BC}$ = 1.5 Hz, $^4J_{BX}$ = 2 Hz, and $^4J_{CX}$ = 1.5 Hz.

Scheme IV

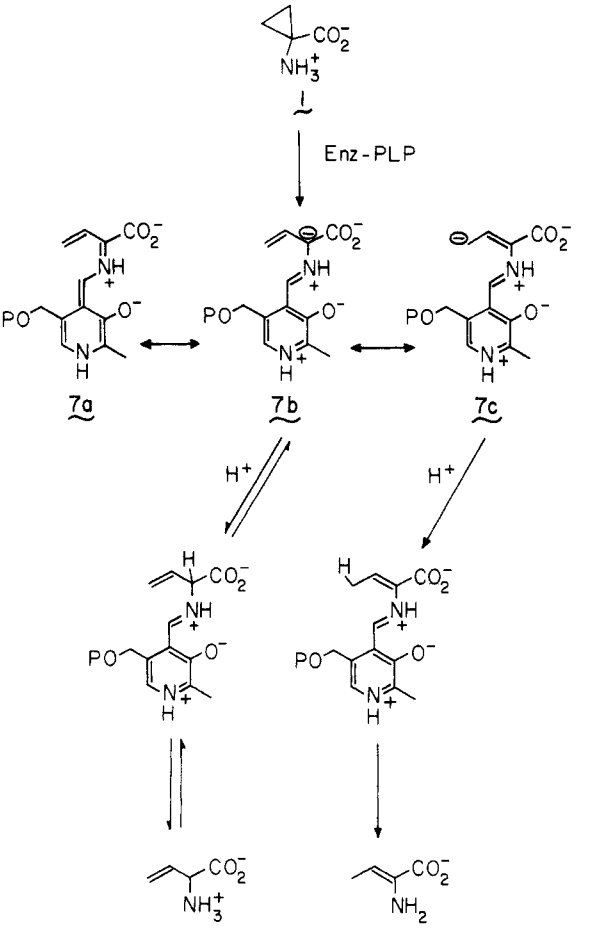


2-keto-5-hexenoate is then the enamine **5** (Scheme IV) arising from fragmentation at the indicated bond via the fully conjugated β,γ–δ,ϵ-stabilized α-carbanion–PLP complex **4**. Some indication that this species may be detectable obtains from the buildup of a small amount of enzyme during steady-state turnover as a species with λ_{max} at 550 nm, a long-wavelength absorbance consistent with conjugated PLP–substrate *p*-quinoidal intermediates. Despite apparent formation of the extended conjugated system, there is no evidence that vinyl-ACPC causes suicidal inactivation of enzyme during turnover, a contrast to the α-haloamino acids addressed in a subsequent section.

D-*Vinylglycine Turnover to α-Ketobutyrate.* The deuterium incorporation data with ACPC as substrate suggest, as noted below under Discussion, that a key intermediate in cyclopropane processing should be species **7a**, the β,γ-olefinic PLP–*p*-quinoid, a resonance hybrid of **7b** (Scheme V), the structural contributor that is the α anion of vinylglycine in aldimine linkage with PLP. This analysis indicated that vinylglycine ought to be a substrate for ACPC deaminase provided the enzyme had the capacity for abstraction of an α hydrogen of an α-amino acid. ACPC, of course, has no such α hydrogen. It was thus disappointing to observe that the enzyme made no detectable keto acid product when offered L-vinylglycine as a possible substrate. However, the chemical logic was salvaged by the finding that the D isomer of vinylglycine was processed at 8% the V_{max} of ACPC (Table V), albeit with a high K_m, 97 mM (vs. 9.2 mM for ACPC), to the identical α-ketobutyrate product. This observation validates the possibility of the mechanistic proposal and proves this enzyme can remove the α proton of the D isomer and catalyze a net 1,3-allylic isomerization. The rate-determining step must be distinct from that with ACPC, and it must be at a point prior to mechanistic convergence, i.e., α-hydrogen abstraction (not yet tested).

At this juncture, the unusual specificity for D-amino acids was probed further, first with D- and L-alanine. Exposure of each of the enantiomers of alanine to the enzyme in D_2O resulted in complete exchange of the α proton of the D isomer, by NMR analysis, and no exchange with the L-alanine. This qualitative observation was quantitated by using D-[α-^{3}H]- and L-[α-^{3}H]alanine. ACPC deaminase reversibly removed the α tritium of D-alanine (k_{cat} = 14 min^{-1}) and exchanged it into

Scheme V

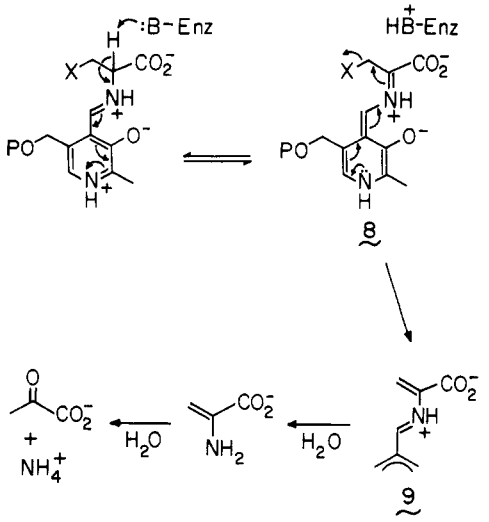


solvent, but no removal of the α tritium of L-alanine was detected.

Behavior of β- and γ-Substituted Amino Acids. One mechanistic hypothesis to explain the ACPC deaminase mediated cleavage of cyclopropane rings invokes the addition of an enzymatic nucleophile to the cyclopropane with fragmentation to yield an enzyme–γ-substituted α carbanion. From this intermediate, β H abstraction and γ elimination of the added enzyme nucleophile would yield the vinylglycine–PLP α anion (**7b**) (see Discussion). To test the possibility that the enzyme could catalyze elimination reactions, we examined the capacity for α,β-HX and α,γ-HY eliminations, well precedented with other PLP enzymes (Walsh, 1979; Davis & Metzler, 1972).

Initial studies with β-substituted D-amino acids revealed the anticipated capacity to generate pyruvate but also turned up time-dependent inactivation of the catalyst, as summarized in Table V. For example, D-β-fluoroalanine inactivates with a partition frequency of 190 turnovers to pyruvate per inactivation event. The L isomer was inert as both substrate and inactivator. An interesting comparison with D-β-chloroalanine and *O*-acetyl-D-serine reveals that the partition frequency of each inactivator is distinct; the turnovers per inactivator vary with the nature of the leaving group. This observation contravenes the idea that the inactivating species is an aminoacrylate–PLP complex (**9** in Scheme VI) because that species would be common to D-Fluoroalanine, D-chloroalanine, and *O*-acetyl-D-serine and *must yield an identical partition ratio.* Therefore, one must focus on the β-substituted *p*-quinoidal PLP complex (**8**) as the killing species. As a β-haloimine, the β carbon is activated for S_N2 attack by an active-site nucleophile in a low-energy process. In this event, the nature

Scheme VI



of the leaving group (F^-, Cl^-, AcO^-) clearly conditions the rate and so affects the partition ratio. In passing, we note that this distinct partition ratio is different from the cognate inactivations of *E. coli* alanine racemase (Wang & Walsh, 1978) and liver serine transhydroxymethylase (Wang et al., 1981) where the symmetric, common aminoacrylate–PLP is the killer molecule. Finally, the ACPC deaminase results strongly mandate a nonconcerted elimination process where the β-substituted α-amino-PLP species (**8**) has a finite, long-lived existence, a unique demonstrate of this fact in PLP–enzymatic β-elimination sequences.

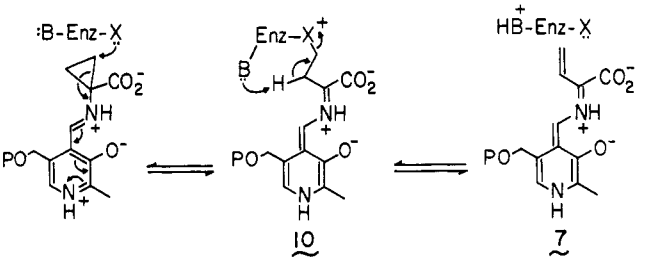
To date, the stoichiometry of labeling of ACPC deaminase with a radioactive inactivator remains to be undertaken but will form the subject of a future study. It is emphasized here that neither D-vinylglycine nor either cyclopropane substrate shows any suicide substrate tendencies.

To probe the catalytic capacity of ACPC deaminase to carry out γ-elimination reactions, which require sequential α- and then β-carbanion equivalents in other PLP enzymes, we analyzed the ability of the enzyme to convert *O*-succinyl-DL-homoserine or D-methionine to α-ketobutyrate. No reaction was detected nor would the enzyme isomerize D-allylglycine to a detectable keto acid product, a known capacity of the γ-elimination enzyme L-methionine-γ-lyase (Johnston et al., 1981). We have previously used L-propargylglycine as a suicide substrate for γ-elimination and γ-replacement enzymes where α- and then β-carbanion formation followed by protonation of the latter anion at the δ locus completes a propargylic rearrangement to a conjugated allenic *p*-quinoidal PLP, a very potent suicide reagent (Abeles & Walsh, 1973; Marcotte & Walsh, 1975; Johnston et al., 1979). D-Propargylglycine did not inactivate ACPC deaminase, although the enzyme did exchange tritium out of the α locus of DL-[α-^{3}H]propargylglycine (78 nmol min^{-1} mg^{-1}). Thus, although ACPC deaminase can clearly produce substrate–PLP α-anion equivalents, there is no evidence with any of the above substrates that a β-anion equivalent can be generated. Further, although the α protons of D-alanine with enzyme in D_2O are exchanged out, the β-methyl protons are not, casting doubt on the ability of the enzyme to remove substrate β protons (now acidic) from an α-anionic substrate–PLP complex.

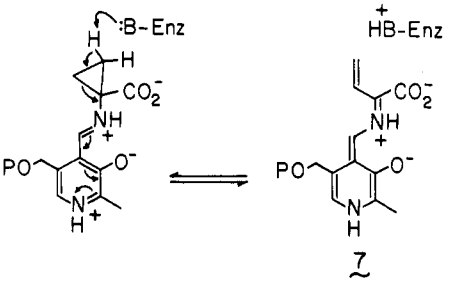
Discussion

A key mechanistic question in analysis of the unusual cyclopropane fragmentation sequence catalyzed by aminocyclopropanecarboxylate deaminase is the ring-opening process

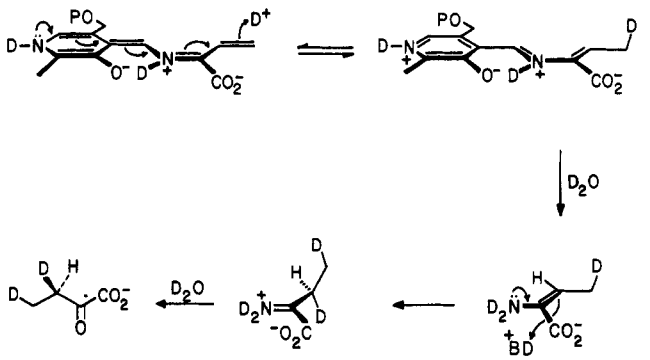
Scheme VII



Scheme VIII

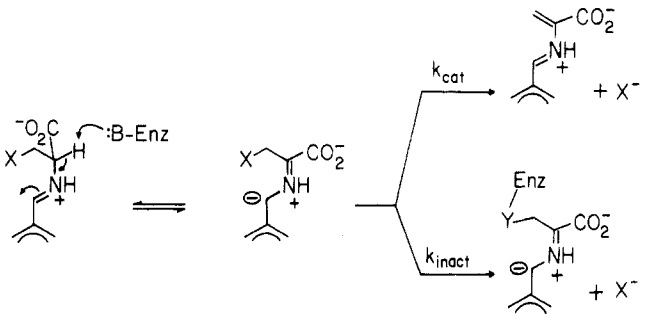


Scheme IX



itself. Two limiting alternatives consistent with the accumulated data can be termed (1) a nucleophilic addition/elimination route (Scheme VII) and (2) a β-proton abstraction fragmentation route (Scheme VIII). In the first alternative, fragmentation is achieved by attack of an active-site nucleophile at a methylene carbon with generation of a stabilized α anion, shown as the *p*-quinoidal PLP contributor. This species (**10**) will now have acidic diastereotopic β hydrogens, and if the enzyme can abstract one, then a formal β,γ-elimination sequence, well precedented in γ-elimination and γ-replacement PLP enzymes (Walsh, 1979), could occur to expel the initially added enzymatic group and yield the fully conjugated vinylglycine–PLP α anion, independently suggested as an intermediate from the D-vinylgycine turnover studies. The idea of nucleophilic addition to cyclopropanes is well buttressed by model chemistry precedents. The other route (Scheme VIII) involves general base catalysis by the deaminase to begin catalysis by a β-methylene proton abstraction, initiating the cyclopropane fragmentation in a probably concerted fashion to yield **7** directly. The acidity of the cyclopropyl hydrogens is not very impressive ($pK_a > 40$), although similar acid-catalyzed fragmentations are known in organic chemistry. There is no clear-cut evidence to distinguish either route yet, but the first route bears closest analogy to the reverse reaction of the plant enzymatic synthesis of ACPC from SAM. From the vinylglycine α-anion PLP intermediate onward, ACPC deaminase behaves like a typical PLP γ-elimination enzyme on the way to α-ketobutyrate. It is the sequential γ- then β-reprotonation steps that incorporate one solvent deuterium at each of carbons 4 and 3 of α-ketobutyrate. The re-

Scheme X



protonation at C-3 probably occurs on free aminocrotonate still in the active-site region, explaining the stereopreferential 7:3 3*R*:3*S* outcome (Scheme IX). The opposite stereochemical sense is seen with D-serine dehydratase action on D-threonine [or on chiral (2*R*)-2-[3-^{2}H]serines (Cheung & Walsh, 1976)] to yield predominantly the 3*S*-^{2}H product. This reverse stereoselectivity occurs despite the fact that each of these PLP enzymes uses D-amino acids. Further stereochemical studies with [4-^{2}H]vinylglycine isomers should elucidate these stereochemical features more fully.

A point worth mentioning is the determination of C-3 chirality by 250-MHz proton NMR on phenacyl (2*S*)-2-hydroxy[3-^{2}H]butyrate samples. This method has clear advantages over the classical ORD methods which require oxidative decarboxylation of the ketobutyrate to [2-^{2}H]-propionate, a process requiring more material and susceptible to nonenzymatic racemization while ketobutyrate accumulates. The assay here permits immediate reduction of nascent ketobutyrate and allows direct observation by NMR of both diastereotopic C-3 protons for side-by-side integration.

While neither cyclopropane substrate nor D-vinylglycine causes detectable inactivation during processing despite the generation of electrophilic conjugated intermediates, the D isomers of three-carbon α-amino acids with good leaving groups as β substituents are suicide substrates. This adds ACPC deaminase to bacterial alanine racemase (Wang & Walsh, 1978), bacterial D-amino acid transaminase (Soper et al., 1977), and liver serine transhydroxymethylase (Wang et al., 1981) as PLP enzymes susceptible to these D isomers. The killing of ACPC deaminase shows the first-order, saturable kinetic criteria anticipate for a suicide substrate (Walsh et al., 1979). The partition ratios for catalytic HX elimination to pyruvate per enzyme inactivation event were determined for D-fluoroalanine, D-chloroalanine, and *O*-acetyl-D-serine. In contrast to the common partition ratio seen with alanine racemase and serine transhydroxymethylase for their suicide substrates, the ACPC deaminase yields distinct killing partition ratios. This is strong evidence that the killing species bound at the active site *does not have a structure common for all substrates* and effectively rules out the aminoacrylate–PLP intermediate in product formation as the inactivator by Michael addition. In fact, it points to a stabilized α anion as a finite intermediate preceding β elimination and suggests that this is the inactivator in an S_N2 displacement process. The nature of the departing X group will affect the partitioning between k_{inact} and k_{cat} (Scheme X). These data imply a nonconcerted pathway for this α,β-elimination mechanism and possibly for β replacement and β elimination. Stoichiometry of labeling by these inactivators remains to be done to establish the chemical specificity criterion.

Given the ability of serine transhydroxymethylase to cleave the C_β–C_α linkage of L-serine and derivatives, and its ability to abstract the α proton of D-amino acids and thereby to kill itself with D-fluoroalanine (Wang et al., 1981), one might speculate with regard to its catalytic similarity to ACPC deaminase. The deaminase shows no tendency to cleave DL-β-phenylserine, but other potential aldol cleavage substrates have yet to be examined. On the other hand, serine transhydroxymethylase does not catalyze a 1,3-allylic isomerization of D-vinylglycine (Wang, 1980).

Although ACPC has two chemically equivalent enantiotopic methylene groups, the ethyl and vinyl analogues are processed regiospecifically to the straight-chain keto acid products. Studies with these and other analogues should help unravel the mechanistic and stereochemical paths of cyclopropane fragmentation and also shed light on its mechanism of formation in the fruiting stage of plants.

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References

Abeles, R. H., & Walsh, C. (1973) *J. Am. Chem. Soc. 95*, 6124.

Adams, D. O., & Yang, S. F. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 170.

Burroughs, L. F. (1957) *Nature (London) 179*, 360.

Cheung, Y., & Walsh, C. (1976) *J. Am. Chem. Soc. 98*, 3397.

Davis, L., & Metzler, D. (1972) *Enzymes, 3rd Ed. 7*, 33.

Greenstein, J. P., & Winitz, M. (1961) *Chemistry of the Amino Acids*, Vol. 3, p 1834, Wiley, New York.

Honma, M., & Shimomura, T. (1978) *Agric. Biol. Chem. 42*, 1825.

Honma, M., Shimomura, T., Shiraishi, K., Ichihara, A., & Sakamura, S. (1979) *Agric. Biol. Chem. 43*, 1677.

Johnston, M., Jankowski, D., Marcotte, P., Tanaka, H., Esaki, N., Soda, K., & Walsh, C. (1979) *Biochemistry 18*, 4690.

Johnston, M., Raines, R., Chang, M., Esaki, N., Soda, K., & Walsh, C. (1981) *Biochemistry 20*, 4325–4333.

Kollonitsch, J., & Barash, L. (1976) *J. Am. Chem. Soc. 98*, 5591.

Konze, J. R., & Kende, H. (1979) *Planta 146*, 293.

Krongelb, M., Smith, T. A., & Abeles, R. H. (1968) *Biochim. Biophys. Acta 167*, 473.

LaRoche, H.-J., Simon, H., Kellner, M., & Gunther, H. (1971) *Z. Naturforsch., B: Anorg. Chem., Org. Chem., Biochem., Biophys., Biol. 26B*, 389.

Liberman, M. (1979) *Annu. Rev. Plant Physiol. 30*, 533.

Lurssen, K., Naumann, K., & Schroder, R. (1979) *Z. Pflanzenphysiol. 92*, 285.

Marcotte, P., & Walsh, C. (1975) *Biochem. Biophys. Res. Commun. 62*, 677.

Marcotte, P., & Walsh, C. (1976) *Biochemistry 15*, 3070.

Marcotte, P., & Walsh, C. (1978) *Biochemistry 17*, 5613.

Moore, S., & Stein, W. H. (1951) *J. Biol. Chem. 192*, 663.

Plattner, Pl. A., Boller, A., Frick, H., Furst, A., Hegedus, B., Kirchensteiner, H., Majnoni, St., Schlapfer, R., & Spegelberg, H. (1957) *Helv. Chim. Acta 40*, 1531.

Sakami, W., & Toennies, G. (1942) *J. Biol. Chem. 144*, 203.

Soper, T. S., Manning, J. M., Marcotte, P. A., & Walsh, C. (1977) *J. Biol. Chem. 252*, 1571.

Stork, G., Leong, A. Y. W., & Touzin, A. M. (1976) *J. Org. Chem. 41*, 3491.

Vahatalo, M. L., & Virtanen, A. I. (1957) *Acta Chem. Scand. 11*, 741.
Walsh, C. (1979) *Enzymatic Reaction Mechanisms*, pp 777–827, W. H. Freeman, San Francisco, CA.
Walsh, C., Shannon, P., & Wang, E. (1979) in *Drug Action and Design: Mechanism-Based Inactivators* (Kalman, T., Ed.) p 93, Elsevier/North-Holland, Amsterdam.
Wang, E. (1980) Ph.D. Thesis, Massachusetts Institute of Technology.
Wang, E., & Walsh, C. (1978) *Biochemistry 17*, 1313.
Wang, E., Kallen, R., & Walsh, C. (1981) *J. Biol. Chem. 256*, 6917.
Yang, J. Y., Huang, Y. Z., & Snell, E. E. (1975) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 34*, 496.

Modification of the Allosteric Activator Site of *Escherichia coli* ADP-glucose Synthetase by Trinitrobenzenesulfonate†

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ABSTRACT: Limited modification of *Escherichia coli* B ADP-glucose synthetase (EC 2.7.7.27) by trinitrobenzenesulfonate (TNBS) appeared to affect primarily the allosteric properties of the enzyme. There was little loss of the catalytic activity assayed in the absence of activator. However, the abilities of fructose 1,6-bisphosphate or hexanediol 1,6-bisphosphate to activate the enzyme, or of 5′-adenylate to inhibit the enzyme, were rapidly lost upon trinitrophenylation. Modification progressively decreased the affinity for activator, decreased the V_{max} at saturating concentrations of activator, and decreased the cooperativity among activator binding sites. These effects could be completely prevented by the presence of allosteric effectors during reaction with TNBS, although a low amount of trinitrophenylation still occurred. Substrates partially protected the enzyme from reaction with TNBS. The lysyl ε-amino side chain was modified by trinitrophenylation, but the target was not primarily the same residue which could form a Schiff base with pyridoxal phosphate, another activator of the enzyme. A large peptide containing most of the trinitrophenyl residue was isolated after cleavage of the enzyme and was identified as part of the N-terminal amino acid sequence. The migration of the enzyme on polyacrylamide gel electrophoresis or on agarose column chromatography was unchanged by modification. However, the ability of fructose-1,6-P_2 to induce the oligomerization of a mutant form of the enzyme was completely prevented by trinitrophenylation. This effect could be protected against by the presence of activator or inhibitor during reaction with TNBS.

ADP-glucose synthetase (EC 2.7.7.27) catalyzes the rate-limiting step in bacterial glycogen biosynthesis, the formation of a glucosyl donor (Preiss, 1969, 1973, 1978). Recent studies on the purified enzyme from *Escherichia coli* B strain AC70R1 have indicated that it is a tetramer with a molecular weight of about 2×10^5 and is composed of identical subunits (Haugen et al., 1976a). The enzyme activity is allosterically regulated by glycolytic intermediates notably fructose-P_2 (Preiss et al., 1966), and by the overall energy charge (Shen & Atkinson, 1970). A survey of activators suggested that the region of the *E. coli* enzyme around their common binding site must contain at least two residues with cationic side chains (Preiss, 1972). Pyridoxal-P was recently shown to react with a lysine in the vicinity of the allosteric activator binding site (Haugen et al., 1976b; Parsons & Preiss, 1978a), and a peptide containing pyridoxylated lysine was isolated and partially sequenced (Parsons & Preiss, 1978b). Chemical modification studies were also initiated with 2,4,6-trinitrobenzenesulfonate (TNBS),[1] another reagent specific for amino groups. This report demonstrates the presence of additional lysine residue(s) essential to allosteric regulation which preferentially react with TNBS. The major TNP-lysine-containing peptide was isolated after cleavage of the modified enzyme with cyanogen bromide, and its identification is described.

Experimental Procedures

Reagents. TNBS, purchased from Sigma, was recrystallized from dilute HCl. α-Dinitrophenylvaline was from Sigma. α-TNP-valine and ε-TNP-lysine (Okuyama & Satake, 1960; Kotaki & Satake, 1964), *S*-TNP-cysteine (Hollenberg et al., 1971), [^{3}H]pyridoxal-5′-P (Haugen & Preiss, 1979; Stock et al., 1966), and hexanediol-P_2 (Hartman & Barker, 1965) were synthesized as described. Spectrapor 3 dialysis tubing was a product of Spectrum Medical Industries. Fluorescamine was purchased as Fluram from Roche Diagnostics. Trypsin treated with 1-(tosylamido)-2-phenylethyl chloromethyl ketone was from Worthington. All other reagents were of the highest possible commercial grade.

ADP-glucose Synthetase Assays (Synthesis Direction). (*a*) *Activated Conditions.* Assay A-1 for measuring the synthesis of ADP-[^{14}C]glucose from [^{14}C]glucose-1-P and ATP was routinely used as described (Ghosh & Preiss, 1966). The reaction mixture (0.20 mL) contained 20 μmol of Hepes, pH 7.0, 100 μg of bovine serum albumin, 0.1 μmol of [^{14}C]-

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[1] Abbreviations used: TNBS, 2,4,6-trinitrobenzenesulfonate; TNP, 2,4,6-trinitrophenyl; [^{3}H]pyridoxal-P, [4′-^{3}H]pyridoxal 5′-phosphate; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Mops, 3-(*N*-morpholino)propanesulfonic acid; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); $NaDodSO_4$, sodium dodecyl sulfate; FDNB, 1-fluoro-2,4-dinitrobenzene.

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