L-Vinylglycine Is an Alternative Substrate as Well as a Mechanism-Based Inhibitor of 1-Aminocyclopropane-1-carboxylate Synthase†

Liang Feng and Jack F. Kirsch*

Department of Molecular and Cell Biology, Division of Biochemistry and Molecular Biology, 229 Stanley Hall, University of California, Berkeley, California 94720-3206

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ABSTRACT: L-Vinylglycine (L-VG) has been shown to be a mechanism-based inhibitor of 1-aminocyclopropane-1-carboxylate (ACC) synthase [Satoh, S., and Yang, S. F. (1989) *Plant Physiol. 91*, 1036−1039] as well as of other pyridoxal phosphate-dependent enzymes. This report demonstrates that L-VG is primarily an alternative substrate for the enzyme. The L-VG deaminase activity of ACC synthase yields the products α-ketobutyrate and ammonia with a k_{cat} value of 1.8 s^{-1} and a K_m value of 1.4 mM. The k_{cat}/K_m of 1300 M^{-1} s^{-1} is 0.17% that of the diffusion-controlled reaction with the preferred substrate, *S*-adenosyl-L-methionine. The enzyme−L-VG complex partitions to products 500 times for every inactivation event. The catalytic mechanism proceeds through a spectrophotometrically detected quinonoid with λ_{max} of 530 nm, which must rearrange to a 2-aminocrotonate aldimine to yield final products. Alternative mechanisms for the inactivation reaction are presented, and the observed kinetics for the full reaction course are satisfactorily modeled by kinetic simulation. The inactive enzyme is an aldimine with λ_{max} of 432 nm. It is resistant to $NaBH_3CN$ but is reduced by $NaBH_4$. ACC synthase is now expressed in *Pichia pastoris* with an improved yield of 10 mg/L.

1-Aminocyclopropane-1-carboxylate synthase (*S*-adenosyl-L-methionine methylthioadenosine lyase, EC 4.4.1.14) is a pyridoxal phosphate- (PLP-)[1] dependent enzyme that catalyzes the formation of 1-aminocyclopropane-1-carboxylate (ACC) from *S*-adenosyl-L-methionine (SAM). This is the rate-determining step in the biosynthesis of the plant hormone ethylene (*1*). Ethylene has important regulatory functions in, e.g., fruit ripening, senescence, and wound healing (*2*). The ACC synthases from apple and tomato have been expressed in *Escherichia coli* and purified to homogeneity with yields ranging from 1 to 8 mg/L (*3*, *4*).

Amino acid profiling (*5*) and X-ray analysis (*6*) showed that ACC synthase is a member of the α family of PLP-dependent enzymes. The roles of several active-site residues surrounding the PLP binding pocket have been clarified by site-directed mutagenesis investigations (*3*, *7*). Random mutagenesis in vivo was also used in an attempt to identify the functional residues (*8*). The enzyme functions as a homodimer (*6*, *7*). The rate of the reaction with (*S*,*S*)-SAM is diffusion-controlled and exhibits a limiting k_{cat}/K_m value of 1.2×10^6 M^{-1} s^{-1} at pH 8.3 (*7*).

Commercial SAM exists as a 60% (*S*,*S*) to 40% (*R*,*S*) diastereomeric mixture; only the (*S*,*S*) diastereomer yields ACC (*9*, *10*). The (*R*,*S*) form inactivates the enzyme, yielding a product that is likely formed by nucleophilic attack of the active site lysine on enzyme-bound L-vinylglycine (L-VG), which in turn might arise from a β,γ-elimination of methylthioadenosine (MTA) from SAM (*11*; Scheme 1). L-VG is a suicide inhibitor of several PLP-dependent enzymes (*12*−*14*), including ACC synthase (*15*). The product was proposed to be the same adduct that arises from the reaction with (*R*,*S*)-SAM. We report here that while L-VG does inactivate ACC synthase, the primary reaction is the catalyzed conversion of L-VG to α-ketobutyrate (α-KB) and NH_3, which occurs at 500 times the rate of inactivation.

MATERIALS AND METHODS

Materials. SAM was purchased from Calbiochem and used without further purification. L-VG, L-lactate dehydrogenase (LDH, from rabbit muscle, type XI, L1254), and L-glutamate dehydrogenase (GDH, from bovine liver, type VI, G2009) were obtained from Sigma. Ni−NTA Superflow resin was from Qiagen. Other reagents were of the highest quality available commercially.

Overexpression and Purification of ACC Synthase in Pichia pastoris. C-Terminally truncated ACC synthase with a $(His)_6$ tag at its C-terminus, V435stop-H_6, was created by PCR. The PCR product was cloned with *Bam*HI/*Avr*II into the vector pPIC3 for intracellular protein expression in *Pichia pastoris* (Invitrogen, Carlsbad, CA). The recombinant plasmid pPIC3V435stop-H_6 ACS was linearized with *Stu*I for genomic integration into *P. pastoris* GS115. Transformants

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* Corresponding author.

[1] Abbreviations: AATase, aspartate aminotransferase; ACC, 1-aminocyclopropane-1-carboxylic acid; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; α-KB, α-ketobutyrate; LDH, L-lactate dehydrogenase; GDH, L-glutamate dehydrogenase; MTA, 5′-methylthioadenosine; PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate; PMSF, phenylmethanesulfonyl fluoride; SAM, *S*-adenosyl-L-methionine; TAPS, 3-[[tris(hydroxymethyl)methyl]amino]-1-propanesulfonic acid; L-VG, L-vinylglycine; WT, wild type.

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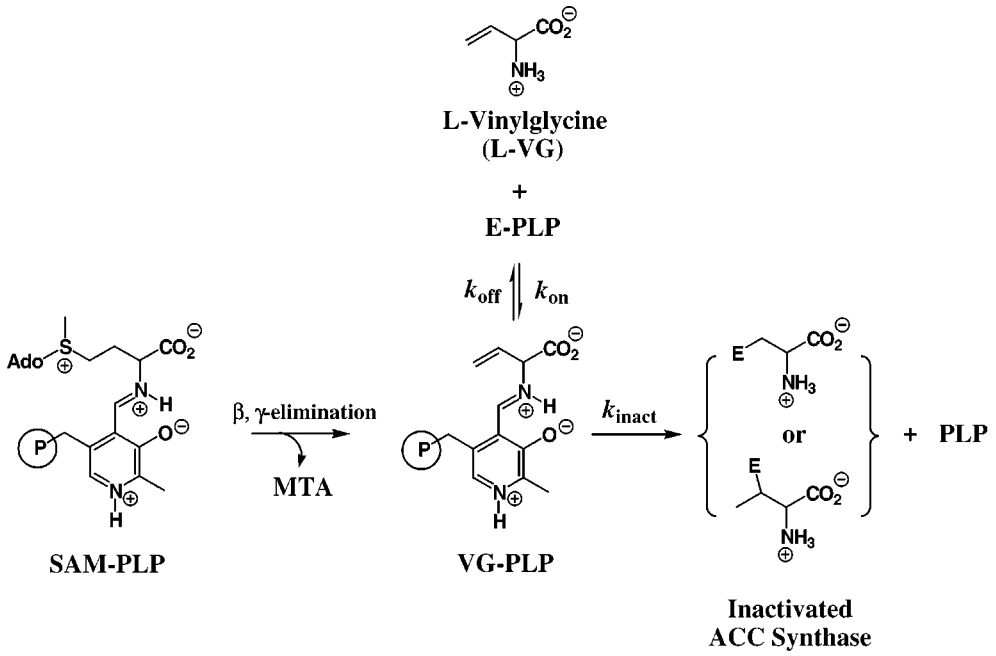
Scheme 1: Proposed Model for the Inactivation of ACC Synthase by *S*-Adenosyl-L-methionine and L-Vinylglycine[a]



[a] Adapted from refs *11* and *15*.

were selected by their ability to grow in medium lacking histidine. Colonies with the highest level of expression judged by activity were selected to express the protein in large scale. Typically, 25 mL of BMGY medium (1% yeast extract, 2% peptone, 1.3% yeast nitrogen base, 1% glycerol, 0.4 mg/L biotin, and 100 mM potassium phosphate, pH 6.0) were inoculated with a single colony and grown at 30 °C to A_{600} of 2−6. This overnight culture was diluted 40-fold in BMGY medium and grown to A_{600} of 2−6. The cells were harvested, and suspended in 1 L of BMMY medium (1% yeast extract, 2% peptone, 1.3% yeast nitrogen base, 0.5% methanol, 0.4 mg/L biotin, and 100 mM potassium phosphate, pH 6.0) to induce protein expression. Methanol was added to the medium to 0.5% every 24 h. Cells were harvested by centrifugation after 84 h of induction at 30 °C. The cell paste was suspended in 100 mL of lysis buffer (50 mM potassium phosphate, pH 8.0, 1 mM EDTA, 0.2 mM DTT, 20 μM PLP, 10% glycerol, and 1 mM PMSF), and mixed with an equal volume of 0.5 mm glass beads. The suspension was subjected to high-speed vortexing in a Bead-Beater (BioSpec, Bartlesville, Oklahoma) to lyse the cells. Eight 30-s runs were conducted with alternating 1 min pauses to cool the chamber. The lysate was centrifuged at 40000*g* for 40 min at 4 °C. The pH of the lysate was adjusted to 8.0 with 0.5 M TAPS (pH 13) and centrifuged at 40000*g* for 20 min at 4 °C to remove precipitated material. The supernatant was loaded onto a Ni−NTA Superflow column equilibrated with buffer A (50 mM potassium phosphate, pH 8.0, 20 μM PLP, 10 mM imidazole, 0.3 M KCl, and 15% glycerol). Nonspecific binding proteins were removed with about 2 column volumes of wash buffer (50 mM potassium phosphate, pH 8.0, 20 μM PLP, 20 mM imidazole, 0.3 M KCl, and 15% glycerol). ACC synthase was eluted with a 10−250 mM imidazole gradient in buffer A. Fractions exhibiting ACC synthase activity were pooled and dialyzed against l L of dialysis buffer (50 mM potassium phosphate, pH 8.0, 1 mM EDTA, 0.2 mM DTT, 20 μM PLP, and 15% glycerol) overnight to remove the imidazole. The protein was concentrated in a Centiprep 30 (Millipore, Bedford, MA) and stored at 4 °C. V435stop-H_6 ACC synthase was found to be >95% pure by SDS−PAGE. This truncated protein has the same activity as that purified from *E. coli* (*3*) and therefore was used in all subsequent experiments. It is referred to as WT ACC synthase for simplification.

Identification of α*-Ketobutyrate by HPLC*. L-VG (16 mM) and ACC synthase (10 μM) were incubated at 25 °C in buffer containing 50 mM potassium phosphate, pH 8.4, 1 mM EDTA, 0.2 mM DTT, and 15% glycerol. Ten percent of the reaction mixture was quenched after 3 h by addition of HCl to a final concentration of 0.5 N and centrifuged to remove protein. Twenty percent of the quenched reaction mixture containing ∼0.1 mM products was reacted with 2 mM phenylhydrazine in 0.2 M Tris-HCl buffer (pH 8.0) at 25 °C for about 1 h. A standard was prepared similarly from an authentic sample of α-KB. Samples were mixed with an equal volume of HPLC buffer (0.14 M NaH_2PO_4, pH 3.1, 30% CH_3CN, and 8 mM octanesulfonic acid) before injection onto the HPLC column. Both the reaction product and the α-KB standard eluted identically at 14.8 min (identified at 210 and 330 nm) from a Beckman ODS C-18 column (25 cm × 4.6 mm) run isocratically in the same buffer. Co-injection of the derivatized α-KB and the reaction mixture produced a single peak with the same retention time.

NMR Sample Preparation and Measurement. L-VG (1 mM) was incubated with ACC synthase (9 μM) in 50 mM potassium phosphate (pH 8.2) for 2 h. The 1 mL reaction mixture was lyophilized, and the salts were resuspended in 500 μL of D_2O. A one-dimensional ^1H NMR spectrum was measured with a Bruker DRX-500 MHz spectrometer equipped with a 5 mm broadband probe, and data were processed with Bruker Xwin-nmr software. The spectrum was referenced to the water signal (4.81 ppm vs tetramethylsilane). The spectrum of the product [δ 0.98 (t, C_β), 2.67 (q, C_γ)] matched that of authentic α-KB.

Table 1: Purification of WT ACC Synthase Expressed in *Pichia pastoris*

sample	total activity (μM s^{-1})	total protein (mg)	specific activity (μM s^{-1}/mg)	recovery %	purification (~fold)
crude extract	1460	~1000[a]	1.5	100	1
Ni−NTA fractions	1150	10	115	79	~75

[a] Estimated according to ref *3*. The purification protocol is given in Materials and Methods.

Enzymatic Assays of α-Ketobutyrate and Ammonia. The rate of α-KB or ammonia formation was monitored by NADH or NADPH oxidation in LDH- or GDH-coupled assays. The optimal concentration of dehydrogenases needed was determined by titration. At the highest L-VG concentration employed (8 mM), 43 μM LDH and 45 μM GDH were required for sufficient coupling. The assay conditions for determining the kinetic parameters are described in Figure 3. All data were collected in 0.1 mL microcuvettes with a Perkin-Elmer Lambda 4B spectrophotometer.

Spectrophotometric Measurements and Enzymatic Assays. UV−visible spectra of ACC synthase and its variants were recorded on an HP 8453 single-beam spectrophotometer equipped with a diode-array detector. Time-resolved spectra were used to detect the intermediates on the reaction pathway of L-VG and ACC synthase. The kinetics of the reaction of ACC synthase with SAM were followed spectrophotometrically by the adenosine deaminase coupled assay described previously (*3*).

Preparation and Reduction of L-Vinylglycine-Inactivated ACC Synthase. ACC synthase (10 μM) and L-VG (6.4 mM) were incubated at 25 °C in 50 mM potassium phosphate (pH 8.0). The enzyme was completely inactivated after 2 h of incubation. The reaction mixture was diafiltered with 50 mM potassium phosphate (pH 8.0) in a Microcon 30 (Millipore, Bedford, Massachusetts) to remove small molecules. The protein retentate was collected and stored at 4 °C. For reduction of the inactivated ACC synthase, 10 μM retained protein was incubated with either 0.1 M $NaBH_3CN$ for 40 min or 0.1 M $NaBH_4$ for about 10 min at 25 °C in 50 mM potassium phosphate, pH 8.0.

Data Processing. The inactivation time course of ACC synthase by L-VG was simulated in the Matlab application (The Math Works Inc., Natick, MA). The following model (eq 1) was used in the numerical simulation to calculate k_1, k_2, k_{cat}, and k_{inact}:

$$\mathrm{E} + \mathrm{I} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathrm{EI} \xrightarrow{k_{cat}} \mathrm{E} + \mathrm{P} \qquad \mathrm{EI} \xrightarrow{k_{inact}} \mathrm{E\text{-}I'} \tag{1}$$

Four sets of data with the enzyme and inhibitor concentrations given in Figure 7 were fitted simultaneously without restriction on the values of the fitted parameters. The inactivation time course was also fitted to a single-exponential decay in KaleidaGraph, and the residual plot was calculated with the SAS program (SAS Institute, Cary, NC). All other data were processed in KaleidaGraph by nonlinear regression fitting to the corresponding equations.

RESULTS

Expression of WT ACC Synthase in Pichia pastoris. The low levels of ACC synthases extant in natural sources virtually mandate the use of recombinant enzyme in order to give sufficient material for physical characterization. Quantities of 1−2 mg/L WT apple ACC synthase have been obtained from expression in *E. coli* (*3*), and recently Zhou et al. (*4*) reported a yield of 8 mg/L for the tomato enzyme. The yields of many mutant forms, however, are often much lower for reasons that are not understood (*3*; L. Feng and J. F. Kirsch, unpublished data). The levels of enzyme resulting from expression in *P. pastoris* are typically 10 mg/L for WT and the few mutant forms expressed to date. A typical purification result is summarized in Table 1.

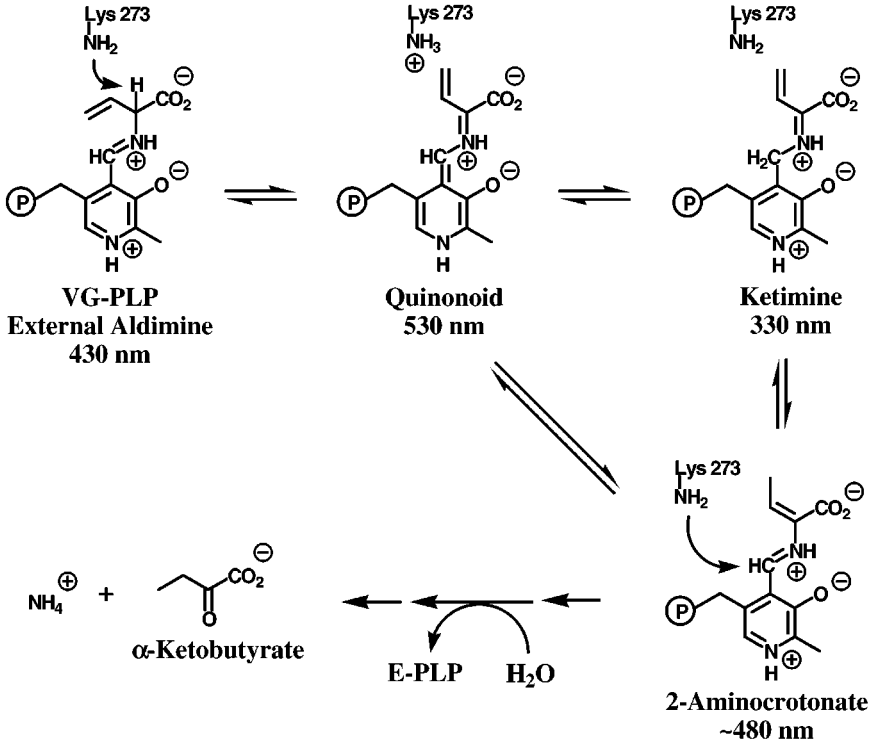
The C-terminally truncated ACC synthase runs as a single band on SDS−PAGE with the correct predicted subunit mass of 50 kDa (data not shown). This contrasts with the observation that full-length ACC synthase often runs as multiple bands on SDS−PAGE due to truncation of its C-terminal tail at various positions (*3*, *16*). The truncated protein, V435stop-H_6 ACC synthase, is stable at 4 °C for at least 6 months; therefore, the previously observed truncation of apple ACC synthase must occur at residues following Val435. Li and Mattoo (*16*) reported that some of the C-terminally truncated variants are more active than the full-length enzyme. The V435stop-H_6 ACC synthase has similar activity to the full-length enzyme (474 residues for apple) purified from *E. coli* (data not shown).

Inactivation of ACC Synthase by L-Vinylglycine. Yip et al. (*11*) showed that the 2-aminobutyrate portion of SAM becomes covalently linked to the enzyme as a consequence of suicide inactivation of ACC synthase by its substrate, SAM. The proposed mechanism shown in Scheme 1 involves irreversible attack of an enzyme nucleophile, the active-site lysine, at the β- or γ-carbon of enzyme-bound L-VG. L-VG may arise by β,γ-elimination from enzyme-bound SAM (*11*). High concentrations of L-VG were shown to inactivate the enzyme irreversibly (*15*).

The kinetics of inactivation were reported to be first order in L-VG (*15*). The data in Figure 1 confirm that L-VG inactivates the enzyme. The rates of decay, however, do not obey first-order kinetics, as shown by the systematic deviations of the data from the least-squares exponential fitting. About 60% of the activity was lost rapidly and the remainder slowly. This biphasic inactivation could be explained by the simple mechanism illustrated in Scheme 1 only if the equilibrium denoted by k_{off}/k_{on} were established with a k_{obs} that is comparable to k_{inact}. However, we observe that this L-VG binding step reaches equilibrium rapidly (see Discussion).

L-Vinylglycine Is Converted to α-Ketobutyrate and Ammonia by ACC Synthase. The data of Figure 2 confirm that a high molar ratio of L-VG/E (over 500) is necessary for complete inactivation (*15*). These observations raise the possibility that the major products (99.8%) of the reaction of ACC synthase with L-VG are not associated with enzyme inactivation. Likely products are α-KB and ammonia produced by the mechanism of Scheme 2. α-KB was identified by HPLC analysis of its phenylhydrazine derivative, which

Scheme 2: A Mechanism for the ACC Synthase-Catalyzed Conversion of L-Vinylglycine to α-Ketobutyrate and Ammonia[a]



[a] The C_α proton is abstracted from the external aldimine (VG-PLP) by the active-site lysine 273 to generate the quinonoid species, which may proceed to the 2-aminocrotonate directly by protonation at the γ-carbon or indirectly through the ketimine. Hydrolysis of the aminocrotonate aldimine gives rise to α-ketobutyrate and ammonia.

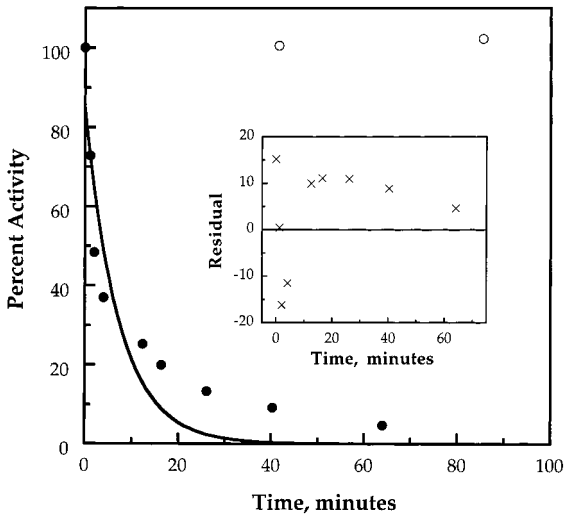


FIGURE 1: Loss of activity of ACC synthase in the presence of L-vinylglycine. (●) ACC synthase (0.9 μM) incubated with 1.6 mM L-VG in 50 mM potassium phosphate (pH 8.0) at 25 °C; (○) same but with L-VG omitted. Aliquots of the reaction mixture were taken at time intervals and assayed for activity by a 200-fold dilution into buffer containing 50 mM TAPS (pH 8.4), 5 μM PLP, and 100 μM SAM. The least-squares fit of the data to a single-exponential decay is shown by the solid line. Inset: Residual plot of percent activity (observed − calculated) vs time.

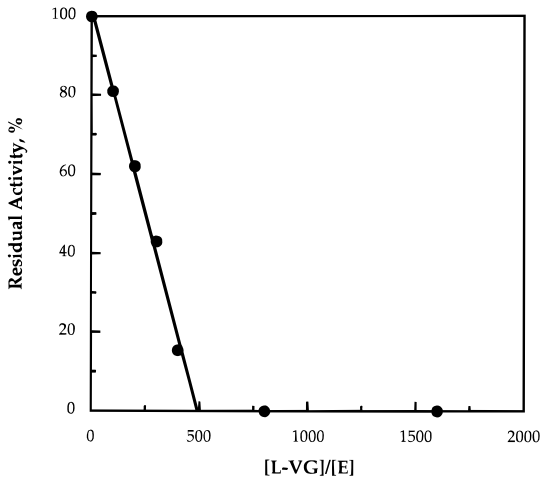


FIGURE 2: Residual activity of ACC synthase as a function of the molar ratio of L-vinylglycine to enzyme. ACC synthase (2 μM) was incubated with 0, 0.2, 0.4, 0.6, 0.8, 1.6, and 3.2 mM L-VG, respectively, in 40 mM potassium phosphate (pH 8.0) and 5 μM PLP at 25 °C. The final residual activity was determined after 5 h (3 h for [L-VG] = 1.6 and 3.2 mM), when no significant further loss of activity was observed as compared to the control. The data points with nonzero values were fitted with a straight line. The intercept gives the L-VG partition ratio of [product]/[inactive enzyme].

eluted identically with a sample prepared from authentic α-KB. The identity of the keto acid was further confirmed by NMR (data not shown). The time course of enzymatic production of α-KB was monitored by coupling with LDH. Ammonia formation was quantitated by GDH-coupled assay in the presence of NADPH. About 90% of the starting material was converted to α-KB and ammonia in a 1:1 ratio. In a typical experiment, 1.5 mM α-KB and 1.4 mM ammonia were recovered after complete reaction of 8 μM enzyme and 1.6 mM L-VG.

Kinetics of L-Vinylglycine Turnover. Initial velocities of α-KB or ammonia formation were used to determine the rate constants of L-VG reaction with ACC synthase in LDH- or GDH-coupled assays. The kinetics are well approximated by the Michaelis−Menten equation because the relative rate of inactivation is negligible (see above). The data in Figure

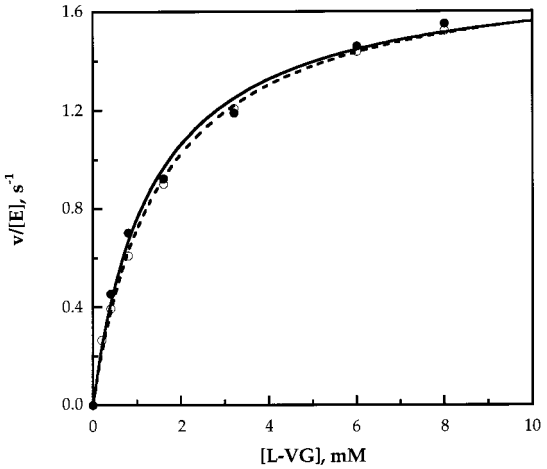


FIGURE 3: v/[E] for the conversion of L-vinylglycine to α-ketobutyrate and ammonia. (●) α-Ketobutyrate formation measured by LDH-coupled assay in buffer containing 40 mM potassium phosphate (pH 8.1), 1 μM PLP, 150 μM NADH, 0.1 μM ACC synthase, 43 μM LDH, and 0.4−8 mM L-VG at 25 °C. (○) NH_3 formation measured by GDH-coupled assay in buffer containing 40 mM potassium phosphate (pH 8.1), 1 μM PLP, 150 μM NADPH, 10 mM α-ketoglutarate, 0.1 μM ACC synthase, 45 μM GDH, and 0.2−8 mM L-VG at 25 °C. The lines represent fits to the Michaelis−Menten equation.

3 yield a k_{cat} value of 1.8 s^{-1} and K_m value of 1.4 mM for the conversion of L-VG to α-KB and ammonia catalyzed by ACC synthase.

Spectral Evidence for Quinonoid and Ketimine Species. WT ACC synthase displays a low pH absorption maximum at 422 nm (Figures 4A and *7*) that is characteristic of the protonated Schiff base formed between the cofactor PLP and the active-site lysine in vitamin B_6-dependent enzymes. About 15% of the absorbance at 422 nm disappears and a transient species absorbing at 530 nm is observed shortly after mixing ACC synthase with L-VG (Figure 4A). The position and shape of this absorption band are consistent with the highly conjugated quinonoid shown in Scheme 2. These species are generally characterized by molar extinction coefficients between 20 000 and 50 000 M^{-1} cm^{-1} (*17*−*19*). The concentration of the VG-quinonoid can be approximated by the absorbance decrease at 422 nm at the early stage of the experiment where the external aldimine decay is mainly due to the formation of the quinonoid species. Therefore, at t = 11 s, [VG-quinonoid] ≈ $[E_0]\Delta A_{422}^{t_0-t_{11}}/A_{422}^{t_0}$ = 13.7 μM(0.111 − 0.094)/0.111 = 2.0 μM (Figure 4A). The estimated extinction coefficient of the VG-quinonoid ($\epsilon^{VG-quinonoid}$), ∼26 000 M^{-1} cm^{-1} ≈ A_{530}/[VG-quinonoid] = 0.051/2.0 μM, is within the above range (Figure 4A). It appears that the external aldimine and quinonoid are at equilibrium, because they both decay with a common isosbestic point at 360 nm to a 330-nm-absorbing entity (Figure 4B). The apparent rate constant for the 420/530 absorbance decay is about the same as that calculated for substrate depletion under these conditions (i.e., $k_{cat}[E_0]/K_m$ = 0.02 s^{-1}, where $[E_0]$ = 13.7 μM and $[S_0] < K_m$). At longer time periods the 330 nm absorbance is converted back to 420 nm with a k_{app} of ∼0.01 s^{-1}. About 12% of the enzyme is inactivated at the end of the experiment.

Characterization of the L-Vinylglycine-Inactivated ACC Synthase. L-VG-inactivated ACC synthase exhibits a similar absorption spectrum to that of WT enzyme, except that the

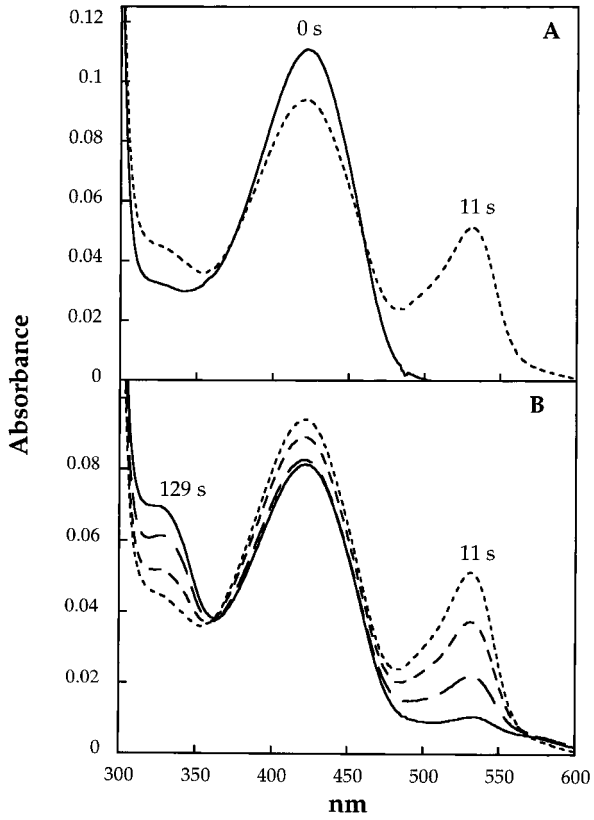


FIGURE 4: Time-resolved spectra following the addition of L-vinylglycine to ACC synthase. L-VG and ACC synthase were mixed to give final concentrations of 0.8 mM and 13.7 μM, respectively, at pH 8.0, 50 mM potassium phosphate, 25 °C. (A) Formation of the L-VG quinonoid at 530 nm. Time, 0 s (—) and 11 s (···). (B) Decay of the L-VG quinonoid and external aldimine to ketimine at 330 nm. Time, 11 s (···), 24 s (---), 54 s (− − −), and 129 s (—).

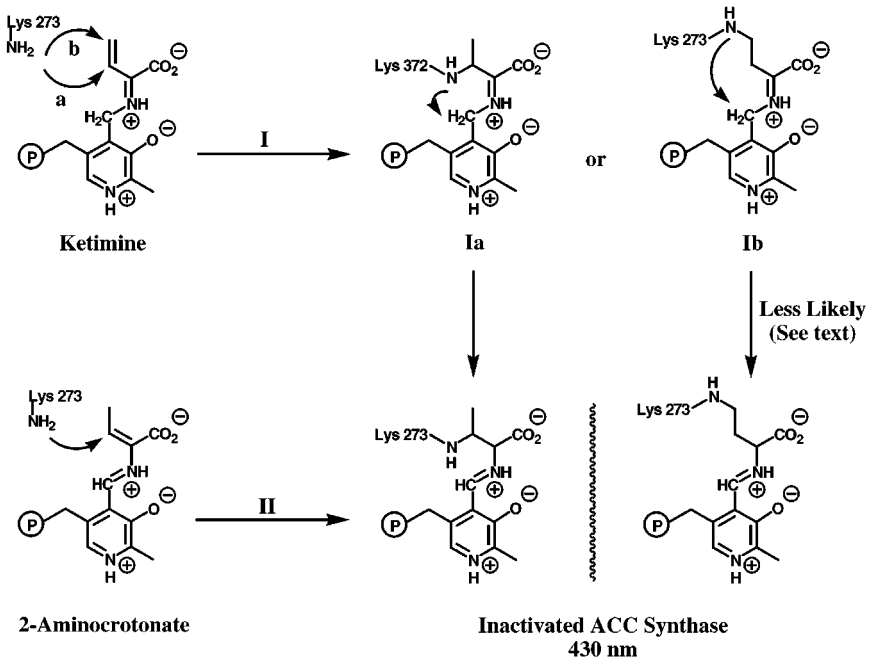
λ_{max} is red-shifted by 10 nm to 432 nm, and the molar extinction coefficient is reduced by ∼2000 units to ∼7000 M^{-1} cm^{-1} (Figure 5, dashed line). The chromophore remains tightly bound to the enzyme, surviving extensive dialysis. These observations are consistent with the proposed covalent structure for the L-VG-inactivated ACC synthase shown in Scheme 3. The Schiff base linkage in WT ACC synthase, in common with those of other PLP-dependent enzymes, is readily reduced to a secondary amine by $NaBH_3CN$ with characteristic maximal absorbance at 325 nm, but the aldimine present in the inactivated enzyme is refractory to this reagent (Figure 5). However, $NaBH_4$, a more potent reducing agent, does convert the imine to a species resembling the reduced WT enzyme.

DISCUSSION

Expression in Pichia pastoris. The overall yield of WT and mutant enzymes has been improved to about 10 mg/L by a combination of His-tagged affinity purification and expression in *P. pastoris*. The effects of the changes introduced separately were not evaluated. The C-terminal $(His)_6$ appendage does not adversely affect activity, as the specific activity of the protein is experimentally indistinguishable from that expressed in *E. coli* without the affinity tag (*3*).

Kinetics and Mechanism of ACC Synthase-Catalyzed Conversion of L-Vinylglycine to α-Ketobutyrate and Am-

Scheme 3: The Inactive Form of ACC Synthase Resulting from Reaction with L-Vinylglycine Is an Aldimine Adduct of the Active-Site Lysine ϵ-NH_2 Group with PLP-Bound L-VG[a]



[a] See text. This product can arise from either the ketimine or 2-aminocrotonate intermediates shown in Scheme 2. The mechanistic alternatives are described in the Discussion section.

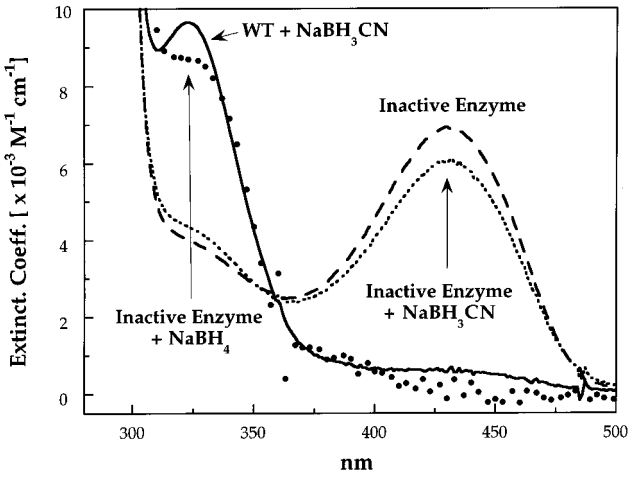


FIGURE 5: Reduction of WT and L-vinylglycine-inactivated ACC synthases by $NaBH_3CN$ and $NaBH_4$. WT ACC synthase (10 μM) with a 422 nm absorbance maximum (not shown) was reacted with 100 mM $NaBH_3CN$ in 50 mM potassium phosphate (pH 8.0) at 25 °C for 40 min (—). L-VG-inactivated ACC synthase prepared as described under Materials and Methods exhibits the spectrum shown by the dashed line (---) with a peak at 432 nm. This peak decreases only slightly upon treatment with 100 mM $NaBH_3CN$ for 40 min (···); however, 100 mM $NaBH_4$ does reduce the imine (●).

monia. Enzymatic deamination of L-VG to α-KB and NH_3 has been previously demonstrated for several PLP-dependent enzymes such as threonine deaminase and tryptophan synthase of the β-family (*20*, *21*) and methionine γ-lyase and cystathionine γ-synthase of the γ-family (*22*, *23*). The present experiments are the first to establish that ACC synthase, a member of the α-family of PLP-dependent enzymes, also catalyzes the conversion of L-VG to α-KB and NH_3. The k_{cat} and K_m values given in Table 2 show that this alternate substrate has a k_{cat}/K_m 600-fold lower than that for the reaction of the enzyme with SAM. The latter reaction is diffusion controlled (*7*). The major difference in the kinetic

Table 2: Kinetic Parameters for the Reactions of *S*-Adenosyl-L-methionine and L-Vinylglycine with ACC Synthase

substrate	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (M^{-1} s^{-1})	k_{inact} (s^{-1})
SAM	9.2[a] (0.3)	0.012[a] (0.002)	770 000[a] (100 000)	ND
L-VG	1.8 (0.1)	1.4 (0.2)	1300 (200)	0.004[b] (0.001)

[a] From ref *3*. Commercial SAM contains ~40% (*R*,*S*) and ~60% (*S*,*S*) diastereomers. The physiological form is (*S*,*S*)-SAM. Assay conditions for L-VG are described in Figure 3. [b] Calculated by dividing k_{cat} with 500, the partition ratio of L-VG.

constants for the two substrates, ca. 100-fold, is reflected in K_m. This is probably a measure of the importance of the sulfonium ion and adenosine in anchoring SAM to the enzyme. K_m is not a dissociation constant for the diffusion-controlled reaction with SAM but is a K_d value for the much slower L-VG reaction (see below.).

The likely mechanism leading to the formation of α-KB and NH_3 from L-VG is illustrated in Scheme 2. The first step of the reaction is the formation of the external aldimine between L-VG and enzyme-bound PLP. The active-site base Lys273, released after the transaldimination, abstracts the C_α proton from the L-VG−PLP intermediate, giving rise to the highly conjugated quinonoid species. The formation and accumulation of this intermediate is confirmed by the transient appearance of the 530-nm-absorbing chromophore (Figure 4). This species is often not detected in other PLP-dependent enzymes because its rate of decay is comparable to or greater than its rate of formation. The route to the 2-aminocrotonate along the pathway leading from the quinonoid to α-KB and NH_3 is uncertain. It could arise directly by protonation at the γ-carbon of the substrate (diagonal arrow) or indirectly via the ketimine. The latter species is a defined intermediate in the classical aminotransferase mechanism (*24*). The ketimine in aminotransferase chemistry is hydrolyzed to the PMP form of the enzyme and

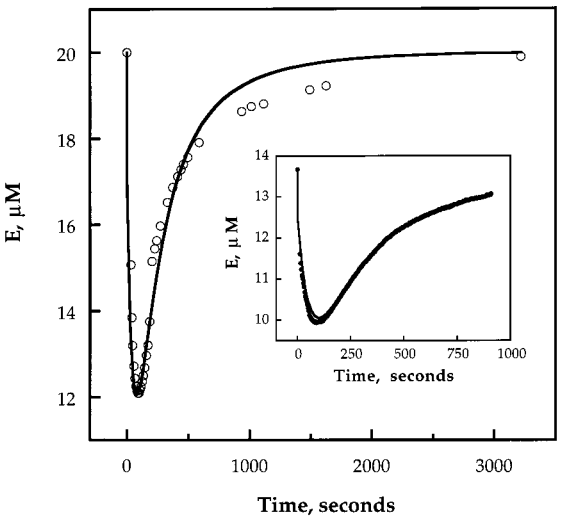
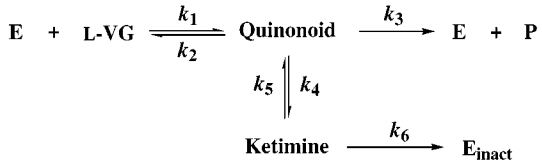


FIGURE 6: Absorbance at 420 nm following the addition of 1.6 mM L-vinylglycine to 20 μM ACC synthase (○) or 0.8 mM L-vinylglycine to 13.7 μM enzyme (inset, ●). The conditions are those of Figure 4. The data were fit to Scheme 4 by the Matlab application (solid lines). The fitted values of the rate constants are in the legend to Scheme 4.

Scheme 4: A Possible Reaction Pathway to Account for both the Turnover and Inactivation Processes during the Reaction of ACC Synthase with L-Vinylglycine[a]



[a] A 330-nm absorbing ketimine intermediate is included on the path to the inactivated enzyme. The fitted rate constants have the following values: $k_1 = 5.2 \times 10^3$ M^{-1} s^{-1}, $k_2 = 45$ s^{-1}, $k_3 = 2.2$ s^{-1}, $k_4 = 0.076$ s^{-1}, $k_5 = 0.0072$ s^{-1}, and $k_6 = 0.0067$ s^{-1}.

the α-keto acid, while that formed from L-VG in the ACC synthase reaction would rearrange to the 2-aminocrotonate aldimine. Transaldimination of the Schiff base gives α-iminobutyric acid, whose spontaneous hydrolysis produces α-KB and NH_3.

The approximate rate constant for the decay of 420/530-nm-absorbing species (Figure 4B) to a 330-nm-absorbing moiety of 0.02 s^{-1} and that for the subsequent conversion of the latter back to the 420 nm species (~0.01 s^{-1}) are both much less than $k_{cat} = 1.8$ s^{-1}. These observations appear to raise the question of the kinetic competency of these entities, but the low values of the rate constants are a simple result of the L-VG-mediated inactivation. The experimental data (Figure 6) are reasonably well accommodated by the set of rate constants given in the Scheme 4 legend. They were obtained by numerical integration. The simulated value of k_3 (2.2 s^{-1}) is very close to the steady-state value of k_{cat}, while the inactivation rate constant calculated from the fitted values of k_4, k_5, and k_6:

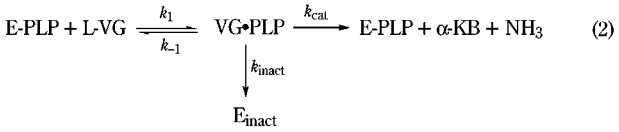
$$k_{inact}\,(\text{from Q}) = \frac{k_4 k_6}{k_4 + k_5 + k_6}$$

is 0.0057 s^{-1}, the ratio of 2.2/0.0057 = 390 is reasonably close to the independently determined partition ratio of 500.

L-Vinylglycine as a Mechanism-Based Inhibitor of PLP-Dependent Enzymes. Rando (*12*) demonstrated that aspartate aminotransferase (AATase) is inactivated by L-VG (10 mM L-VG, 2 μM enzyme). The final product absorbs maximally at 330 nm, and the structure was suggested to be the product of nucleophilic attack at the γ-carbon of the β,γ-unsaturated ketimine. No other products were reported. The nucleophile that is covalently linked to the cofactor was later identified as the active-site residue Lys258 in AATase (*13*). Soper et al. (*14*) showed that D-vinylglycine is a mechanism-based inactivator for D-amino acid aminotransferase. The inactivation requires catalytic turnover and added α-ketoglutarate. Only about one in 800 catalytic events results in inactivation. The product of the catalytic reaction was suggested to be 2-keto-3-butenoate, but this entity was not identified.

Yip et al. (*11*) isolated the peptide SLSXDLGLPGFR from SAM-inactivated apple ACC synthase, where X is a modified amino acid, coincident with the position of the active-site Lys273. X has a calculated mass of 229 from the *m*/*z* value of the dodecapepetide (1390.6). This figure is consistent with the mass of the alkylation product resulting from the nucleophilic attack of Lys273 at either the β- or γ-position of L-VG. These investigators proposed that the inactivation of ACC synthase is a result of nucleophilic attack on the residual enzyme-bound L-VG originating from β,γ-elimination of MTA from the internal SAM aldimine (Scheme 1). According to this model, L-VG should be an irreversible inhibitor of ACC synthase.

The data of Figure 1 establish that the rate of L-VG-mediated inactivation is biphasic. This is a result of the majority of the L-VG being converted to product other than inactive enzyme. If $k_{cat} \ll k_{inact}$ (eq 2), then the inactivation pathway would obey simple first-order kinetics unless $k_1[\text{L-VG}] + k_{-1} \approx k_{inact}$ (*25*):



(2)

This equality does not hold. The value of k_{cat}/K_m (1300 M^{-1} s^{-1}) is too low for the L-VG reaction to be diffusion-controlled; therefore $k_{-1} \gg k_{cat} = 1.8$ s^{-1}, which is $\gg$0.004 s^{-1} (k_{inact}). Thus the deviation from first-order kinetics (Figure 1) must be due to the fact that the majority of the L-VG is diverted from the inactivation pathway. The experimental data for the simultaneous inactivation of the enzyme and conversion of L-VG to products are well accommodated by the simulation described in Figure 7. The computed values of k_{cat} (1.7 s^{-1}) and k_{inact} (0.004 s^{-1}) are very close to the experimentally determined ones of 1.8 s^{-1} and 1.8/500 = 0.004 s^{-1}, respectively.

Inactivation Mechanism. There are a number of possible routes to the inactive covalent adduct of Lys273 with the L-VG external aldimine (Scheme 3). The nucleophile could attack either the β- or γ-carbon of the ketimine. Azallylic rearrangement of the β-adduct yields product. The bound ϵ-amino group of the γ-adduct would have to abstract the C_4' proton via a seven-member ring transition state to yield an aldimine. This is less likely but not forbidden. The β-adduct might also arise from direct nucleophilic attack on the 2-aminocrotonate aldimine.

Evolutionary Implications. ACC synthase and aminotransferases catalyze completely different reactions and share

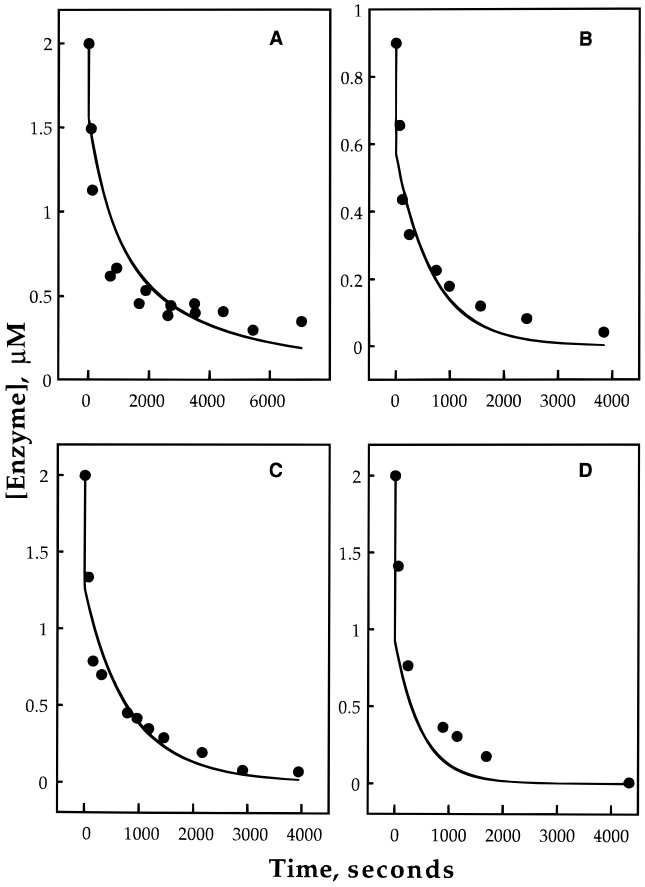


FIGURE 7: Data and simulation for the time courses of inactivation of ACC synthase by L-vinylglycine. Four sets of data are plotted. (A) L-VG = 0.8 mM and E = 2 μM; (B) L-VG = 1.6 mM and E = 0.9 μM; (C) L-VG = 1.6 mM and E = 2 μM; (D) L-VG = 3.2 mM and E = 2 μM. The four curves are calculated for the model of eq 1 with the following globally minimized rate constants: $k_1 = 1 \times 10^4$ M^{-1} s^{-1}, $k_2 = 26$ s^{-1}, $k_{cat} = 1.7$ s^{-1}, and $k_{inact} = 0.004$ s^{-1}.

about 15% sequence similarity. Both classes of enzymes belong to the α-family subgroup of PLP-dependent enzymes (*5*). While aminotransferases are universally distributed among all organisms, ACC synthases have been found only in higher plants (*26*), where they have a regulatory role, and in the fungus *Penicillium citrinum* (*27*). It might therefore be suggested that ACC synthases are derived from ancestral aminotransferases that existed before the emergence of seed plants (*28*). The point of mechanistic divergence is the fate of the C_α carbanion. The electron flow in the aminotransferases is to the C_4' position of the cofactor, while it is directed to the γ-carbon of SAM in ACC synthase. The electrons can apparently go to both sites when ACC synthase is presented with the alternative substrate, L-VG (Scheme 2). The k_{cat}/K_m of the L-VG reaction catalyzed by ACC synthase is only reduced by ~600-fold compared to that of its natural substrate SAM. These results suggest that ACC synthase might have true aminotransferase activity for an amino acid substrate lacking a functional side chain, because the C_α carbanion could only accept a proton productively at the C_4' position. This expectation is realized in the observations that low levels of alanine and arginine aminotransferase as well as threonine deaminase activities are found in ACC synthase (L. Feng, A. Eliot, and J. F. Kirsch, unpublished results).

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