Aminotransferase Activity and Bioinformatic Analysis of 1-Aminocyclopropane-1-carboxylate Synthase†

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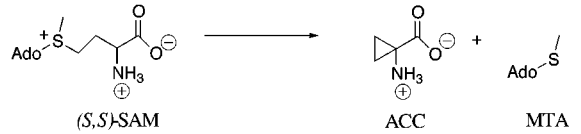
ABSTRACT: The mechanistic fate of pyridoxal phosphate (PLP)-dependent enzymes diverges after the quinonoid intermediate. 1-Aminocyclopropane-1-carboxylate (ACC) synthase, a member of the α family of PLP-dependent enzymes, is optimized to direct electrons from the quinonoid intermediate to the γ-carbon of its substrate, *S*-adenosyl-L-methionine (SAM), to yield ACC and 5′-methylthioadenosine. The data presented show that this quinonoid may also accept a proton at C_4' of the cofactor to yield α-keto acids and the pyridoxamine phosphate (PMP) form of the enzyme when other amino acids are presented as alternative substrates. Addition of excess pyruvate converts the PMP form of the enzyme back to the PLP form. C_α-deprotonation from L-Ala is shown by NMR-monitored solvent exchange to be reversible with a rate that is less than 25-fold slower than that of deprotonation of SAM. The rate-determining step for transamination follows the formation of the quinonoid intermediate. The rate-determining step for α,γ-elimination from enzyme-bound SAM is likewise shown to occur after C_α-deprotonation, and the quinonoid intermediate accumulates during this reaction. BLAST searches, sequence alignments, and structural comparisons indicate that ACC synthases are evolutionarily related to the aminotransferases. In agreement with previously published reports, an absence of homology was found between the α and β families of the PLP-dependent enzyme superfamily.

Members of the superfamily of PLP[1]-dependent enzymes catalyze a wide range of reactions in amino acid metabolism. Catalytic promiscuity has been reported for several superfamily members. For example, aspartate aminotransferase (AATase) catalyzes racemization, elimination, and decarboxylation reactions at low levels (*1*−*4*), and both L-Ser and L-Thr deaminase activities have been reported for threonine synthase (*5*, *6*).

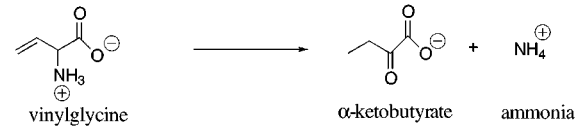
1-Aminocyclopropane-1-carboxylate (ACC) synthase, a superfamily member responsible for fruit ripening and plant senescence (*7*), catalyzes the rate-determining step in ethylene biosynthesis: the conversion of *S*-adenosyl-L-methionine (SAM) to ACC (*8*, *9*) (Scheme 1). We have shown previously that this enzyme exhibits substantial L-vinylglycine (L-VG) deamination activity (*10*), and report here that ACC synthase also has aminotransferase activity which is characterized kinetically.

Scheme 1: Reactions Catalyzed by ACC Synthase

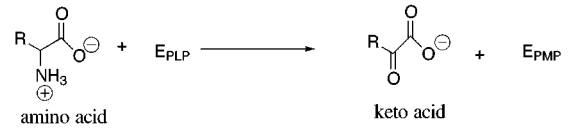
1. α,γ-Elimination of MTA from SAM



2. Deamination of Vinylglycine



3. Transamination Half-reaction



There are two classification systems for the PLP-dependent enzyme superfamily. The first (*11*) is based on the results of profile analysis and has four primary divisions: (1) the α family (AATase is the prototype, and ACC synthase is included); (2) the β family (tryptophan synthase β is the prototype, and threonine deaminase is included); (3) the D-alanine aminotransferase family; and (4) the alanine racemase family. A second classification divides the superfamily into 5 fold types based on three-dimensional structures. Aminotransferases are similarly split into two groups (*12*). The ACC synthase structure was unavailable when this

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[1] Abbreviations: AATase, aspartate aminotransferase; ACC, 1-aminocyclopropane-1-carboxylate; α-KB, α-ketobutyrate; CE, combinatorial extension of the optimal path; DALI, distance alignment matrix; MTA, 5′-methylthioadenosine; NRES, number of equivalent pairs of C_α atoms superimposed between two structures; PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate; RMSD, root-mean-square superposition residual, in angstroms; SAM, *S*-adenosyl-L-methionine; TAPS, 3-[[tris(hydroxymethyl)methyl]amino]-1-propanesulfonic acid; TATase, tyrosine aminotransferase; VAST, vector alignment search tool; L-VG, L-vinylglycine; WT, wild type.

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second classification system was proposed. In the view of Christen and co-workers, proteins within a given family/fold are homologous but the families/folds are unrelated to each other (*11*, *13*, *14*). This issue is reexamined in this paper specifically for the case of ACC synthase in order to probe the evolutionary implications of the catalytic promiscuity of this plant enzyme.

MATERIALS AND METHODS

Materials. C-terminally truncated apple ACC synthase with a His_6 tag at its C-terminus and a mass of ~46 kDa was purified from *Pichia pastoris* (*10*) and used in all experiments. It has the same specific activity with *(S,S)*-SAM as the recombinant untagged, full-length ACC synthase purified from *E. coli*, and is referred to as ACC synthase for simplicity. LDH (from rabbit muscle, Type XI, L1254) and GDH (from bovine liver, Type VI, G2009) were purchased from Sigma. Amino acids were from Sigma or Aldrich.

Aminotransferase Activity of ACC Synthase. UV−visible spectroscopy was used to monitor the spectral changes associated with the transamination reaction catalyzed by ACC synthase as described in the figure legends. The reverse reaction was demonstrated by concentrating the products of the reaction with amino acids approximately 4-fold with a Microcon 30 (Millipore, Bedford, MA), diluting the retentates 2-fold with an equal volume of 0.1 M potassium phosphate (pH 8.4), and reacting the resulting mixtures with pyruvate to give final concentrations of ~20 μM ACC synthase and 100 mM pyruvate.

Single-Turnover Assay of Aminotransferase Activity of ACC Synthase. ACC synthase (5 μM) was reacted with 10−200 mM L-Ala or L-Arg in 200 mM TAPS, pH 8.4, 5 μM PLP at 25 °C. Absorbances at 435 or 320 nm, taken as a function of time, were fit to eq 1a or 1b, respectively:

$$A_{435} = A_{min} + \Delta A\, e^{-k_{obs}t} \quad (1a)$$

$$A_{320} = A_{max} - \Delta A\, e^{-k_{obs}t} \quad (1b)$$

The corresponding k_{cat} and K_m values for transamination were obtained by nonlinear regression fitting of the *pseudo*-first-order rate constants to eq 2:

$$k_{obs} = \frac{k_{cat}[S]}{K_m + [S]} \quad (2)$$

Measurement of C_α-Proton Exchange by NMR. ACC synthase (10 μM) was incubated with 600 mM [α-^{2}H]-D,L-alanine in 50 mM potassium phosphate buffer (pH 8.5) at 25 °C in a total volume of 500 μL. D_2O was added to 5% as a lock solvent, and ^{1}H NMR spectra were recorded periodically during the reaction on a Bruker DRX-500 spectrophotometer. The ^{1}H-Ala formed was quantitated by the ratio of the integrals of the peaks corresponding to the α- and β-protons (δ 3.8 and 1.5 ppm, respectively, relative to trimethylsilylpropionic acid standard). The rate of transamination under these conditions was measured as described above.

Detection and Characterization of the Quinonoid Intermediate. ACC synthase (20 μM) was incubated with 100 μM *(R,S)*- or *(S,S)*-SAM in reaction buffer (25 mM potassium phosphate, 2.5 μM PLP, 0.5 mM EDTA, 0.1 mM DTT, 7.5% glycerol, pH 8.3) at 25 °C. The characteristic absorbance at 500 nm was followed during the first 200 ms of the reaction with an Applied Photophysics Ltd. SF.17MV stopped-flow spectrophotometer. To determine the rate constants for formation and decay of the intermediate under pre-steady-state conditions, 25 μM ACC synthase was incubated with 5 μM *(S,S)*-SAM in reaction buffer. The resulting data were fit to eq 3:

$$A_{500} = \frac{A_{max}k_1}{k_2 - k_1}(e^{-k_1 t} - e^{-k_2 t}) \quad (3)$$

where k_1 and k_2 are the rate constants, respectively, for formation and decay of the observed intermediate.

Data Collection and Processing. Spectrophotometric and kinetic data were collected in 0.1 mL microcuvettes with a Hewlett-Packard 8453 single-beam spectrophotometer equipped with a diode array detector or with a UVIKON 860 double-beam spectrophotometer. Experimental data were exported into Kaleidagraph (Synergy Software, Reading, PA) and fitted to the corresponding equations by nonlinear regression.

Bioinformatics. BLASTP 2.0.11 (*15*) searches of the nonredundant and Swiss-Prot databases were performed via the National Center for Biotechnology Information Internet site using default settings. Three-dimensional structure alignments were conducted with VAST from the National Center for Biotechnology Information (*16*, *17*), CE (*18*), and DALI (*19*).

Phylogenetic analyses were performed with the PileUp, Pretty, ProfileMake, ProfileGap, PAUPSearch, and PAUP-Display programs from the GCG package (Version 10.0; Genetics Computer Group, Inc., Madison, WI). Profile analysis was applied in the following manner:

(1) The amino acid sequences from apple, pumpkin, and rice ACC synthases were aligned with *Thermus thermophilus* AATase and *Trypanosoma cruzi* TATase using PileUp with default settings. Manual inspection showed no apparent regions of misalignment. One diagnostic of an acceptable alignment includes the four specific residues G197, D222, K258, and R386 (pig cytosolic AATase numbering system). These residues are conserved in the α division of PLP-dependent proteins and in the aminotransferase superfamily (*20*).

(2) A profile, which is a table that indicates the frequency of finding each of the 20 amino acids at each position in an alignment, was generated from the alignment in step 1 with ProfileMake. The profile approach was employed because of its demonstrated success where there is low sequence similarity in comparison sets (*13*).

(3) Several ACC synthases and aminotransferases were then aligned to the profile using ProfileGap (with default settings). The alignment was visually inspected for errors.

(4) Phylogenetic trees were constructed from the sequence alignment in ref *3* using PAUP and Clustal X (*21*) employing all available tree search methods and optimality criteria. All methods yielded similar trees with respect to topology.

RESULTS

ACC Synthase Exhibits Aminotransferase Activity. While the aldehyde form of the cofactor PLP is retained throughout

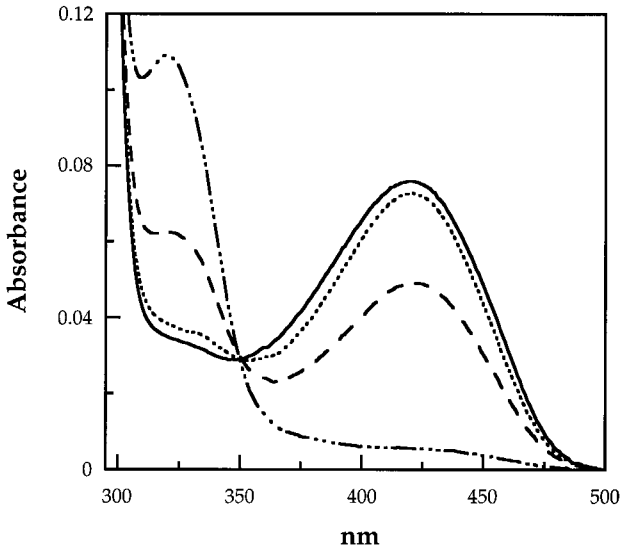


FIGURE 1: Spectra of 10 μM ACC synthase alone (—), and after 1 h incubation with 50 mM L-Asp (· · ·), L-Phe (– –), or L-Ala (– · · · –) in 0.1 M potassium phosphate (pH 8.4) at 25 °C.

the course of the preferred ACC synthase reaction (SAM → ACC + MTA), it is reductively aminated to PMP by transamination of an amino acid to its corresponding α-keto acid (Scheme 1). This feature provides a convenient single-turnover spectrophotometric assay that takes advantage of the different absorbance maxima of the PLP (430 nm) and PMP (325 nm) forms of the enzyme. The addition of excess amino acid converts some of the PLP form to the PMP form (Figure 1). The enzyme exhibits substrate specificity in transamination with a preference of L-Ala > L-Phe > L-Asp. Transamination of L-Asp and L-Phe by ACC synthase is slow; only about 5% (L-Asp) or 35% (L-Phe) of the PLP form of the enzyme is converted to the PMP form after a 1 h incubation at 25 °C with 50 mM amino acid. Both reactions, however, reach completion after 18 h of incubation at 25 °C (data not shown). Additional proof that the reaction is transamination is presented in Figure 2. L-Arg, like L-Ala, quantitatively effects the conversion of the A_{430} to A_{325}. Approximately 60% of the modified cofactor dissociates from the protein when the reaction mixture is spun in a Microcon 30, but the retained fraction is rapidly converted back to PLP by the addition of 100 mM pyruvate, thus completing a single cycle of transamination.

Kinetics of L-*Ala and* L-*Arg Aminotransferase Activities of ACC Synthase.* The kinetics describing the reaction of L-Ala with ACC synthase are *pseudo*-first-order. Identical k_{obs} values are obtained by monitoring either the increase in A_{320} or the decrease in A_{435}. The isosbestic point is ~350 nm (Figure 3). The *pseudo*-first-order constants exhibit saturation kinetics with increasing amino acid concentration (Figure 4). The limiting value of k_{cat} of 0.0029 s^{-1} for the reaction with L-Ala is about 2.5-fold greater than that for L-Arg (0.0012 s^{-1}). The apparent K_m values are similar (Table 1).

C_α-Deprotonation of L-*Alanine Is Reversible.* Solvent exchange at the α position of the substrate D,L-alanine was monitored by NMR using C_α-deuterated substrate. The initial rate of hydron exchange (40 μM/s) is 10^4-fold greater than the initial rate of transamination (0.004 μM/s) under identical conditions (Figure 5). The exchange reaction exhibits first-order kinetics, and the first-order rate constant reflects the conversion of the active PLP form of the enzyme to the inactive PMP form. It is consequently equal to the directly measured rate constant for transamination (0.0004 s^{-1}). The large difference in rate between proton exchange and transamination shows that C_α-deprotonation is completely reversible and that the rate-determining step for transamination follows quinonoid formation.

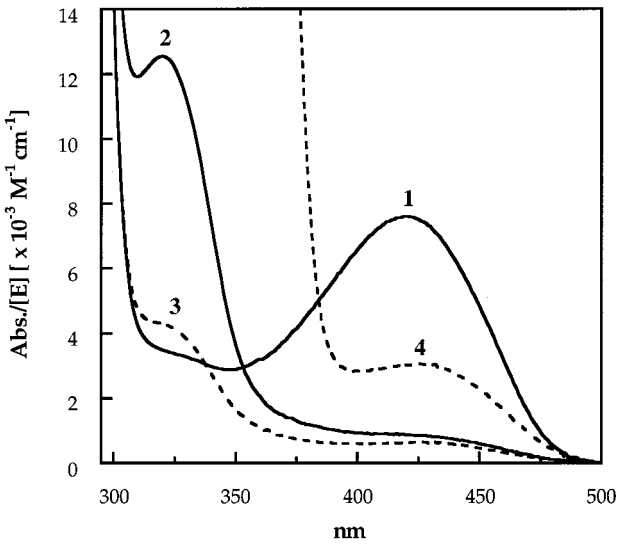


FIGURE 2: Reversible transamination of ACC synthase. WT enzyme (10 μM), with a maximum absorbance at 422 nm (curve 1), was incubated with 50 mM L-Arg in 0.1 M potassium phosphate (pH 8.4) at 25 °C for 1 h (curve 2). The resulting reaction mixture absorbs maximally at 325 nm. About 60% of the chromophore was lost after subjecting the mixture to a Microcon 30 (curve 3). Addition of 100 mM pyruvate converts nearly all of this remaining absorbance at 325 nm back to 425 nm within a minute (curve 4). The high absorbance at λ < 400 nm is from the large excess of pyruvate.

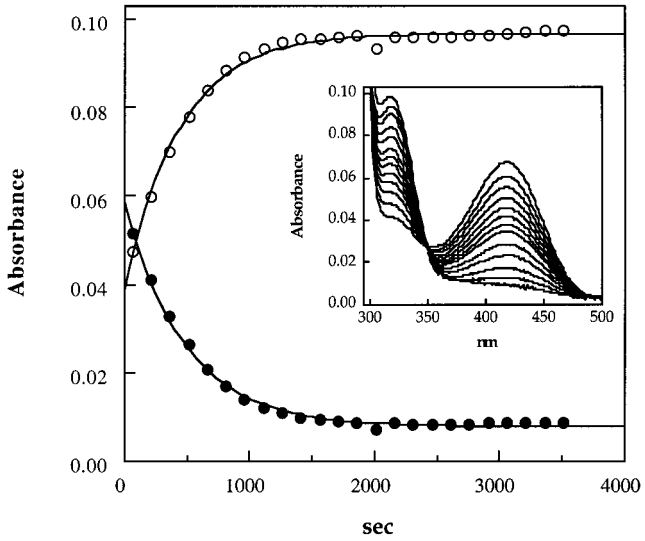


FIGURE 3: Kinetics describing the reaction of ACC synthase with L-Ala. Absorbance at 435 nm (filled circles) or 320 nm (open circles) following the addition of 100 mM L-Ala to 5 μM ACC synthase at 25 °C in 200 mM TAPS (pH 8.4) and 5 μM PLP. The lines represent fits to first-order kinetics. Inset: time-resolved spectra of the above reaction at 1.4, 60, 120, 180, 240, 300, 360, 420, 540, 660, 870, 1170, and 3570 s.

Characterization of the Quinonoid Intermediate Formed from (R,S)- and (S,S)-SAM. An intermediate absorbing at 500

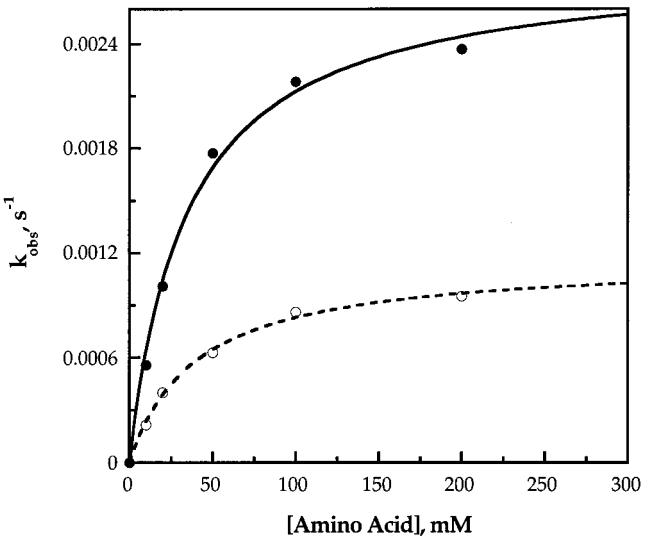


FIGURE 4: Dependence of the first-order rate constant for transamination of ACC synthase on [L-Ala], or [L-Arg]. ACC synthase was reacted with 10−200 mM L-Ala (filled circles) or L-Arg (open circles) in 200 mM TAPS (pH 8.4), 5 μM PLP at 25 °C. The calculated curves (solid line, L-Ala; dashed line, L-Arg) are for rectangular hyperbolas.

Table 1: Kinetic Parameters for Reactions Catalyzed by ACC Synthase

substrate	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (M^{-1} s^{-1})
SAM[a]	9.2	0.012	770000
	(0.3)	(0.002)	(100000)
L-VG[b]	1.8	1.4	1300
	(0.1)	(0.2)	(200)
L-Ala[c]	0.0029	35	0.08
	(0.0001)	(4)	(0.01)
L-Arg[c]	0.0012	40	0.03
	(0.0001)	(4)	(0.01)
L-Phe[c]	ND	ND	<0.01
L-Asp[c]	ND	ND	<0.003

[a] Conversion of SAM to 1-aminocyclopropane-1-carboxylate (*27*). [b] Deamination to α-ketobutyrate and ammonia (*10*). [c] Transamination reaction; assay conditions for L-Ala and L-Arg are given in Figure 4. The upper limits of the k_{cat}/K_m values for L-Phe and L-Asp were estimated from the data of Figure 1.

nm, characteristic of a quinonoid species (Scheme 2), is observed during the reaction of ACC synthase with either *(R,S)*- or *(S,S)*-SAM under steady-state conditions (data not shown). This intermediate is not observed in the reactions of the other natural amino acids. The rate constants for formation and decay of the quinonoid formed from *(S,S)*-SAM under single-turnover conditions (Figure 6) are 100 and 10 s^{-1}, respectively. The corresponding quinonoid arising from *(R,S)*-SAM decays approximately 10^6-fold more slowly (data not shown).

Bioinformatic Analyses. The newly described activity of ACC synthase raises the possibility that ACC synthases and aminotransferases diverged from a common PLP-dependent enzyme with broad substrate specificity. This potential evolutionary relatedness was probed with BLAST searches, three-dimensional structural alignments, multiple sequence alignments, and tree-building methods. Additionally, an exploration was conducted for any signs of evidence of homology between ACC synthases and the β family by including threonine deaminases in the analyses.

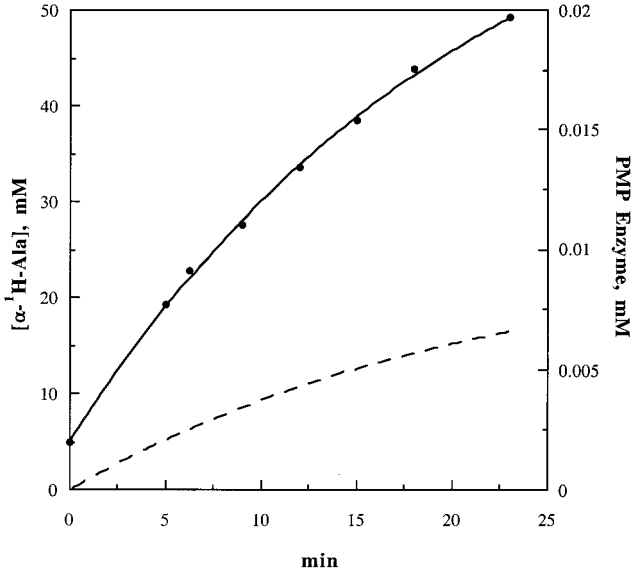
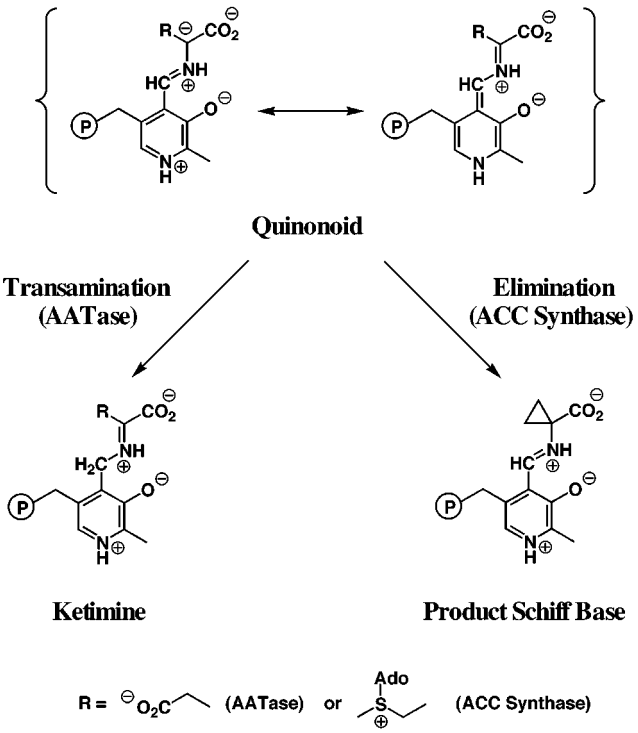


FIGURE 5: Exchange of the C_α-proton of [α-^{2}H]-D,L-Ala with H_2O. ACC synthase (10 μM) was reacted with 600 mM [α-^{2}H]-D,L-Ala, and ^{1}H-Ala formation (filled circles) was followed by ^{1}H NMR. The conditions are given under Materials and Methods. The solid line represents a first-order fit. The dashed line represents the concentration of the PMP form of the enzyme as calculated from the measured rate of transamination under these conditions.

Scheme 2: The ACC Synthase and Aminotransferase Reactions Diverge from the Quinonoid Intermediate[a]



[a] Protonation at C_4' yields the ketimine, which is subsequently hydrolyzed to α-keto acids and the PMP form of the enzyme (not shown), while α,γ-elimination from SAM gives rise to the aldimine of ACC. Transaldimination by the ϵ-NH_2 group of Lys273 yields ACC and the PLP holoenzyme (not shown). α,β-Elimination (not shown) yields α-keto acids and ammonia.

Aminotransferases of varying substrate specificities and from various sources appeared as statistically significant hits in the BLAST searches with apple ACC synthase as the query, but members of the β family did not. Cysteine

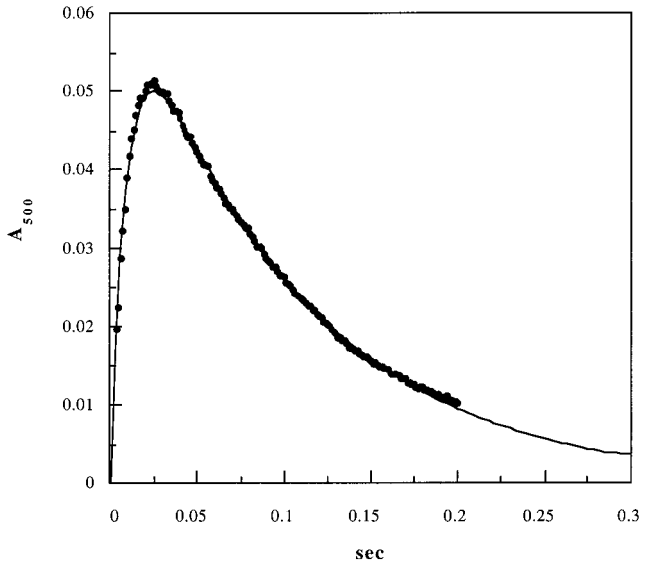


FIGURE 6: Formation and decay of the quinonoid intermediate. Excess ACC synthase (25 μM) was reacted with 5 μM *(S,S)*-SAM in reaction buffer (25 mM potassium phosphate, 2.5 μM PLP, 0.5 mM EDTA, 0.1 mM DTT, 7.5% glycerol, pH 8.3), and the absorbance at 500 nm was followed for 200 ms. The line represents a fit to eq 3.

synthases, threonine synthases, and cystathionine β-synthases were hits when the *E. coli* biosynthetic threonine deaminase sequence was used as the query. All three of these hits have been classified by Mehta and Christen (*11*) as belonging to the β family. Searches in which the query was the biosynthetic threonine deaminase sequence from tomato yielded results similar to those uncovered in the *E. coli* case, except that tryptophan synthase β-chain was an additional hit.

Three-dimensional structure comparison is a powerful tool for recognizing relatives, especially those that are ancient (*23*). Therefore, published three-dimensional structures of apple ACC synthase (PDB code 1b8g; *24*), aminotransferases, and *E. coli* threonine deaminase (1tdj; *25*)—the only threonine deaminase whose structure has been solved—were examined with the VAST, CE, and DALI programs. The parameters were set to identify all structures in the Protein Data Bank that are similar to apple ACC synthase. A subset of the results is shown in Table 2. All programs generated similar results: the closest structural neighbors are AATase from *Thermus thermophilus* (VAST results: 17.2% identical residues in the aligned region; NRES = 366) and TATase from *Trypanosoma cruzi* (19.4% identical residues in the aligned region; NRES = 351). Other structures that align well with ACC synthase include pig cytosolic aspartate aminotransferase, an *E. coli* bifunctional protein that is a β-cystathionase and a repressor of the maltose regulon called MalY, and 8-amino-7-oxononanoate synthase. All of these "hits" are members of the vitamin B_6-dependent enzyme superfamily Fold Type I based on the classification of Schneider et al. (*12*). Additional proteins, all having Fold Type I, superimposed well with ACC synthase (VAST similarity scores = 24.8−33.2; NRES = 247−303). Threonine deaminase, having Fold Type II, did not appear as a statistically significant structural neighbor. Additionally, only tryptophan synthase was uncovered as a structural neighbor of threonine deaminase by VAST. Attempts to align the three-dimensional structures of ACC synthase and threonine deaminase resulted in low *Z* scores (1.6−3.7, from CE) and high RMSD values (3.4−8.6 Å), indicating that the alignments are quite poor and that the overall folds of the two proteins are dissimilar.

Multiple sequence alignments and tree-building were performed with ACC synthases and various aminotransferases. The low sequence similarities between the two enzyme groups frustrated initial efforts to construct alignments with a diverse set of these proteins via programs such as PileUp. By restricting the choice of comparison sequences only to those identified as the closest relatives of ACC synthase by the three-dimensional structure analyses, *Thermus thermophilus* AATase and *Trypanosoma cruzi* TATase, it was possible to generate the alignment shown in Figure 7 (see step 1, *Bioinformatics* section of Materials and Methods). A new alignment was subsequently generated with additional sequences by constructing and employing a profile from this figure (see steps 2 and 3, *Bioinformatics* section of Materials and Methods).

Armed with an adequate alignment containing several ACC synthases and aminotransferases, phylogenetic trees such as the one shown in Figure 8 could be built. ACC synthases form two main branches: one contains the apple and rice proteins while the second embraces those from pumpkin, tobacco, and *Arabidopsis*. Two primary clusters of aminotransferases are present: *Thermus thermophilus* AATase, *Bacillus* AATase, *Trypanosoma cruzi* TATase, and rat TATase form one cluster while the remaining aminotransferases, consisting mainly of AATases, form a second group. Interestingly, AATases from some species appear to be more closely related to TATases than to AATases from other species.

DISCUSSION

Aminotransferase Activity of ACC Synthase and Evolutionary Implications. The physiological role of ACC synthase is to catalyze the α,γ-elimination of MTA from SAM, and that of AATase is to effect the amino group transfer from dicarboxylic amino acids to the corresponding α-keto acids. Transamination or γ-elimination occurs depending on whether the electron flow is to the C_4' of the cofactor or to the γ-carbon of PLP-bound SAM from the common quinonoid intermediate (Scheme 2). The aminotransferase activity of ACC synthase described herein suggests that the active site, which is optimized for catalyzing the α,γ-elimination, also encodes the basic elements required for transamination.

Homology between aminotransferases and ACC synthases is evident from a combination of BLAST searches, multiple sequence alignments, profile analyses, and three-dimensional structure alignments. The similarity of the two enzymes at the primary structure level has been noted (*13*, *26*−*28*). *Bacillus* sp. AATase was reported as the closest relative of ACC synthases based on profile analysis (*13*). Zarembinski and Theologis (*28*) noted that all aminotransferases and ACC synthases contain four conserved residues: Gly197, Asp222, Lys258, and Arg386. These remain conserved in the comparison set that includes sequences determined since that time. The present phylogenetic trees indicate that ACC synthases are more similar to each other than to aminotransferases. Mehta and Christen (*11*) recently constructed a tree from family profile analysis data that also shows that ACC synthase is part of a cluster that is distinct from aminotrans-

Table 2: Structural Neighbors of Apple ACC Synthase[a]

protein	source	PDB code (ref)	VAST similarity score[b] (RMSD)[c]	DALI *Z*-score[d] (RMSD)[c]	CE *Z*-score[e] (RMSD)[c]
AATase	*T. thermophilus*	1bjw (*41*)	42.3 (2.6)	39.7 (2.5)	7.4 (2.4)
TATase	*T. cruzi*	1bw0 (*42*)	39.5 (2.7)	38.6 (2.6)	7.2 (2.7)
AATase	pig (cytosolic)	1ajs (*43*)	39.5 (3.4)	15.2 (3.6)	6.8 (3.1)
MalY	*E. coli*	1d2f (*44*)	nd[f]	33.3 (2.6)	7.2 (2.5)
8-amino-7-oxononanoate synthase	*E. coli*	1bs0 (*45*)	34.2 (4.5)	22.1 (4.4)	6.6 (4.2)

[a] Apple ACC synthase was queried to discover the nearest structures in the PDB with the aid of the programs indicated. [b] The VAST structure-similarity scores correspond to the number of secondary structure elements that are superimposed and the overall quality of the superposition. A higher VAST score correlates with a greater degree of similarity. [c] The RMSD values are the square root of the mean square distances between equivalent C_α atoms. [d] *Z*-Scores are a measure of the statistical significance of the result relative to an alignment of random structures. [e] A *Z*-score $\geq$ 3.5 from CE usually indicates that the aligned proteins have a similar fold (*18*). [f] This structure was not identified as a neighbor with this program.

ferases. The low sequence identity between the two enzyme classes prompted a need for three-dimensional structure comparisons in order to address the homology issue firmly. Capitani et al. (*24*) compared the newly solved ACC synthase structure with all others in the Protein Data Bank using DALI. Two aminotransferases, the pig cytosolic and chicken mitochondrial AATases, were identified as being most similar to ACC synthase. A new analysis that includes the more recently deposited structures (those available as of March 2000 in the Protein Data Bank) and employs DALI, VAST, and CE finds *Thermus thermophilus* AATase and *Trypanosoma cruzi* TATase as the closest structural neighbors, thereby providing additional evidence for the relatedness of ACC synthase and aminotransferases. Babbitt and Gerlt (*29*) pointed out that a single structural scaffold can accommodate a fairly broad range of catalytic functions for several superfamilies. This generalization clearly applies to ACC synthase and aminotransferases within the PLP-dependent enzyme superfamily.

The level of aminotransferase activity of ACC synthase is extremely low. The k_{cat}/K_m value of 0.08 M^{-1} s^{-1} for L-Ala is about 6 orders of magnitude less than that of *E. coli* WT AATase for L-Asp (9×10^4 M^{-1} s^{-1}) (Table 1 and ref *30*). The major difference of $\sim$50 000-fold is reflected in k_{cat}, which may be due to the fact that the orientation of the cofactor and the position of the active site base Lys273 in ACC synthase are not fine-tuned for transamination. The reversibility of C_α-deprotonation, as shown by NMR, demonstrates that the rate-determining step in the transamination reaction occurs after the quinonoid formation and that the rate constant for proton abstraction ($\geq 4 s^{-1}$) is only 25-fold less than the corresponding reaction with SAM (100 s^{-1}). This result is anticipated since quinonoid formation is also required for α,γ-elimination, but the following two steps, C_4' protonation and hydrolysis, are unique to transamination. Solvent exclusion may therefore be a factor in slowing the undesirable transamination reaction rate. The observation that the rate-determining step for α,γ-elimination from SAM also follows C_α-deprotonation supports this concept. During single-turnover reactions with *(S,S)*-SAM and excess ACC synthase, a 500 nm-absorbing intermediate, presumed to be a quinonoid species, is formed quickly and decays with a rate constant similar to k_{cat} (10 s^{-1}). This result suggests that quinonoid decay is the primary rate-determining step, with little or no contribution from C_α-deprotonation. The rates of transamination of four representative amino acids [one cationic (L-Arg), one anionic (L-Asp), one aromatic (L-Phe), and L-Ala] were investigated. The reactivity order is L-Ala > L-Arg > L-Phe > L-Asp (Figure 1 and Table 1). The charge selectivity, L-Arg $\gg$ L-Asp, is the opposite of that with AATase. The order is consistent with the fact that the natural substrate of ACC synthase, SAM, has a cationic side chain.

A single active site mutation in AATase reduces its specific transamination activity to that exhibited by WT ACC synthase. Arg292 of AATase is essential for recognition of the β- or γ-carboxylate of dicarboxylic amino and keto acids. The R292D mutant of AATase exhibits k_{cat}/K_m values for a number of amino acids that are on the order of 0.01$-$100 M^{-1} s^{-1} (*31*). Substituting Arg292 in AATase by valine or leucine causes an enhanced specificity for nonpolar amino acids (*32*). ACC synthase is not an α-ketoglutarate-coupled aminotransferase and correspondingly lacks Arg292. The aminotransferase activity of ACC synthase therefore might reflect the basal activity of a rudimentary aminotransferase lacking the major specificity determinant. This is not to say that introduction of Arg292 into ACC synthase would convert it into an efficient aspartate aminotransferase, but that this exercise might be accomplished with relatively few mutations.

The reactivities of PLP-dependent enzymes are, in general, strongly influenced by pH, because the pH dependencies of k_{cat}/K_m require that either the internal aldimine or the amino acid (but not both) be protonated. The pK_a of the internal aldimine, therefore, is optimized to complement the amine pK_a of the substrate. For example, the aldimine pK_a for AATase is about 7 (*33*), while that for ACC synthase is 9.2 (*34*). This difference is required because the pK_a values of the α-amino groups of L-Asp and SAM are 9.6 and 7.5, respectively (*34*, *35*). Consequently, the k_{cat}/K_m vs pH profile for ACC synthase-catalyzed transamination exhibits a narrow bell-shaped curve that can be fit to an ascending pK_a of 9.2 and a descending pK_a of 9.6 (data not shown). At pH 8.4, only about 10% of the maximal activity is realized [(k_{cat}/

```
                          10                                                  50
p37821  ~~~~~~~~~~ mrmLSrnAtf NSHGQDSSYF LGWQEYEKNP yHeVhNtnGi IQMG...LAE NQLcFDLlEs WLaKnPEAaa fkk..nGesi
q07215  MvsqvvaEek pqLLSKkAgc NSHGQDSSYF LGWQEYEKNP FdPVsNPSGi IQMG...LAE NQLSFDLlEe WLeKnPhAlg LrreggGasv
p23599  MefhqidErn qaLLSKiAvd dgHGenSpYF dGWkaYdndP FHPedNPlGv IQMG...LAE NQLSFDmivd WirKhPEAsi ctpk..Gler
q56232  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~mrglsr rvqamkPSat vavnAKaLel rrqgvDLvaL taGepdfd.. ...tpehvke
p33447  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~m ssWdvsmsNh aglVfNPirt vsdnAKpsps pk...piikL svGdptldkn Lltsaaqikk
Consen  M------E-- --LLSK-A-- NSHGQDSSYF LGWQEYEKNP FHPV-NPSG- IQMGAK-LAE NQLSFDL-EL WLGK-PEA-- L-----G---

                                        100                                                 150
p37821  FaELALFQD. ..YH...GLP aFkkAmv... .dFMaEiRGN KVTFDPnhlV LTAGATSANE TfIFCLADPG eAvLiPtPYY PGFDRDLKWR
q07215  FrELALFQD. ..YH...GLP aFknALA... .rFMsEqRGy KVvFDPSNIV LnAGATSANE aLmFCLADhG DAffiPtPYY PGFDRDLKWR
p23599  FksiAnFQD. ..YH...GLP EFRngiA... .sFMgkvRGg rVqFDPSrIV mggGATgAsE TvIFCLADPG DAfLVPsPYY aaFDRDLKWR
q56232  aarrALaQgk tkYaPpaGiP ElReALAekf R......ReN glsvtPeeti vTvGgkqAlf nLfqailDPG DeviVlsPYw vsypemvrf.
p33447  lkEaidsQec ngYfPtvGsP EaReAvAtww Rnsfvhkeel KsTivkdNvV LcsGgshgil maItaicDaG DyaLVPqPgf PhyetvcKa.
Consen  F-ELALFQD- --YHP--GLP EFR-ALA--- R-FM-E-RGN KVTFDPSNIV LTAGATSANE TLIFCLADPG DA-LVP-PYY PGFDRDLKWR

                                                200
p37821  TGvEIVPiHC tSsNGFQiTE tALEEAYqeA eKRNLRVKGV lVTNPSNPLG TTmtRneLyl LlsFVEdkgI HLISDEIYsG TaFssP..sF
q07215  TGAEIVPVHC aSaNGFrVTr AALddAYRrA QKRrLRVKGV liTNPSNPLG TasPRadLET iVdFVaakgI HLISDEIYaG TaFaePpagF
p23599  TrAqIirVHC nSsNnFQVTk AALEiAYkkA QeaNikVKGV iiTNPSNPLG TTYdRdtLkT LVtFVnqhdI HLIcDEIYsa TvFKaP..tF
q56232  aGgvvVeVet lpeeGF...v pdpErvrRai tp...RtKal vVnsPnNPtG avYPkevLEa LaRlavEhdf yLvSDEIYeh llyeGe..hF
p33447  yGigmhfynC rpeNdw...E AdLdEirRlk dd...ktKll iVTNPSNPcG snfsRkhvEd iVRlaEElrl pLfSDEIYaG mvFKGkdpna
Consen  TGAEIVPVHC -S-NGFQVTE AALEEAYR-A QKRNLRVKGV -VTNPSNPLG TTYPR--LET LVRFVEE--I HLISDEIY-G T-FKGP---F

          250                                                 300
p37821  ISvmEVlkdR ncdenseVwq RVHvVYSLS**K** DLGLPGFRVG AIYSnd.... ..DmVVaAAT KMSSFGLVSS QT...QhLLs AmLs.DkklT
q07215  vSalEVvagR d.gggagVsd RVHvVYSLS**K** DLGLPGFRVG AIYSan.... ..aaVVsAAT KMSSFGLVSS QT...QyLLa ALLg.DrdFT
p23599  ISiaqiveEm ehckk....e liHilYSLS**K** DmGLPGFRVG iIYSyn.... ..DvVVrrAr qMSSFGLVSS QT...QhLLa AmLs.DedFv
q56232  .SpgrVapEh T....l.... ...tVngaa**K** afamtGwRiG yac..... GPkeVikAma svSSqsttSp dTiaQwatLe ALtn..Qeas
p33447  .tftsVadfe Ttvprv.... ...ilggta**K** nLvvPGwRlG wllyvdphgn GP.sfleglk rv.gmlvcgp cTvvQaaLge ALLntpQehl
Consen  IS--EV--ER T------V-- RVH-VYSLS**K** DLGLPGFRVG AIYS------ GPD-VV-AAT KMSSFGLVSS QT--QQ-LL- ALL--DQ-FT

                          350                                                 400
p37821  knyiAENhkR LKqRqkkLVs GLqKsGIsCL NgNAGlFcWv DMRHLLrSnT FeAEMELWkK ivyEVhLNiS PGSScHcTEP GWF**R**VCFANl
q07215  rsyVAENkrR iKERHDqLVd GLreIGIgCL psNAGlFcWv DMsHLmrSRs FagEMELWkK vvfEVGLNiS PGSScHcrEP GWF**R**VCFANM
p23599  dkflAENskR LaERHarftk eLdKmGItCL NsNAGvFvWm DlRrLLkdqT FKAEMELWrv iinEVkLNVS PGSSFHvTEP GWF**R**VCFANM
q56232  rafVemarea yrrRrDlLlE GLtalGlkav rps.GaFyvl ....mdtSpi apdEvraaer ll.EaGvaVv PGtdFaa..f Ghv**R**lsyAt.
p33447  dqiVAkiees amylynhigE ...cIGlapt mpr.Gamylm sridLekyRd iKtdvEffeK lleEenvqVl PGtiFHa..P Gft**R**ltttr.
Consen  ---VAEN--R LKERHD-LVE GL-KIGI-CL N-NAG-F-W- DMRHLL-SRT FKAEMELW-K ---EVGLNVS PGSSFH-TEP GWF**R**VCFANM

                                        450
p37821  pErTLDlAmq RlKaFvGeyy nVpevnGGsq sshLshS... .RrqS...lt KWvsRL..SF dDRgpipGr~ ~~~~~~~~~~ ~~~~~~~~
q07215  sakTLDVAmq RlrsF..... .VdsatGGgD NaaLrraavp vRsvScplai KWaLRLtpSi aDRkaer~~~ ~~~~~~~~~~ ~~~~~~~~
p23599  ddnTvDVAln RihsF..... .VenidkkeD NtvampSktr rRenk..... ...LRLsfSF sgRrydeGnv lnsphtmsph splviakn
q56232  sEEnLrkAle RfarvlGRa~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~
p33447  pvEvyreAve RiKaFcqRha aV~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~
Consen  -EETLDVA-- R-K-F-GR-- -V----GG-D N--L--S--- -R--S----- KW-LRL--SF -DR----G-- ---------- --------
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FIGURE 7: Multiple sequence alignment of five ACC synthases and aminotransferases. The aminotransferases shown were selected for inclusion because their three-dimensional structures superimposed the best with apple ACC synthase relative to all other PDB entries. The alignment was produced by PileUp with the default settings. The consensus sequence (Consen) was generated with the program Pretty. Uppercase letters indicate that their comparison value with the consensus residues meets or exceeds the default threshold. Residues below the threshold are in lowercase. The catalytic lysine and the arginine involved in substrate binding are in boldface type. Sequences are denoted by their Swiss-Prot numbers, i.e., p37821, apple ACC synthase; q07215, rice ACC synthase; p23599, pumpkin ACC synthase; q56232, *Thermus thermophilus* AATase; p33447, *Trypanosoma cruzi* TATase.

$K_m)/(k_{cat}/K_m)_{max} = 1/(1 + 10^{pK_1-pH} + 10^{pH-pK_2})]$. If the p$K_a$ of ACC synthase were reduced to 7 (equal to that of AATase), the k_{cat}/K_m value for transamination at pH 8.4 would be about 10-fold greater.

Both the PLP and PMP forms of aminotransferases have sufficiently small cofactor dissociation constants such that loss of cofactor rarely occurs (*36*, *37*). The PMP form of ACC synthase, a species that would be formed rarely in vivo and to no advantage, is quite unstable as seen by the experiment described in Figure 2. This instability would generate apoenzyme in the rare event that ACC synthase were inactivated by transamination in vivo. The nascent apoenzyme would capture PLP to restore the physiologically active form of ACC synthase.

Final Remarks. ACC synthase converts L-VG to α-KB and ammonia (*10*); therefore, similar chemistry might effect the conversion of L-Thr to the same products plus water as in the threonine deaminase reaction (*22*). Surprisingly, ACC synthase is incapable of deaminating L-Thr (Dr. Keith A. Koch, unpublished results). This result implies that Fold Type II is essential for threonine deaminase activity. It has not yet been shown that a Fold Type I enzyme catalyzes this reaction effectively.

Both the present analysis and the earlier work of Christen's group (*14*, *38*) support the conclusion that the α and β families are not homologous. However, short segments of structural similarity do exist between these two families (*39*). The common mechanistic features of ACC synthase and threonine deaminase may have been forced to converge by the chemical properties of PLP, as suggested by Mehta and Christen (*11*), rather than being inherited from a common ancestor.

The evolutionary analyses in this paper were conducted with ACC synthases from plants. The sequence for a possible homologue in animals has been discovered (*40*), but the activity of the encoded protein has not yet been evaluated.

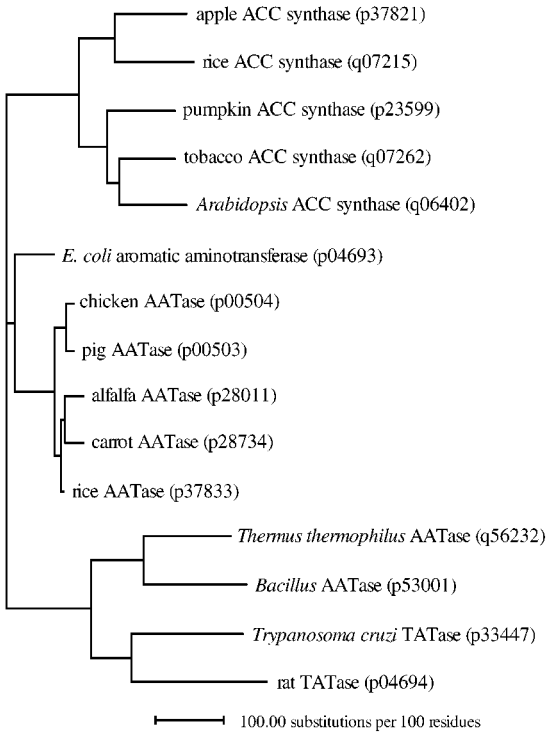

FIGURE 8: Phylogram of ACC synthases and aminotransferases. The tree was built using PAUP with a branch-and-bound tree search method, parsimony optimality criterion, and the midpoint method for rooting. Swiss-Prot numbers are given in parentheses.

Therefore, it was excluded from the present analysis. The finding of this gene is noteworthy, because ACC has no known function outside of plants and microorganisms.

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