

# Complementation Analysis of Mutants of 1-Aminocyclopropane-1-carboxylate Synthase Reveals the Enzyme Is a Dimer with Shared Active Sites\*

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The pyridoxal phosphate-dependent enzyme 1-aminocyclopropane-1-carboxylate synthase (ACS, EC 4.4.1.14) catalyzes the rate-limiting step in the ethylene biosynthetic pathway. ACS shares the conservation of 11 invariant residues with a family of aminotransferases that includes aspartate aminotransferase. Site-directed mutagenesis on two of these residues, Tyr-92 and Lys-278, in the tomato isoenzyme Le-ACS2 greatly reduces enzymatic activity, indicating their importance in catalysis. These mutants have been used in complementation experiments either *in vivo* in *Escherichia coli* or in an *in vitro* transcription/translation assay to study whether the enzyme functions as a dimer. When the Y92L mutant is coexpressed with the K278A mutant protein, there is partial restoration of enzyme activity, suggesting that the mutant proteins can dimerize and form active heterodimers. Coexpressing a double mutant with the wild-type protein reduces wild-type activity, indicating that inactive heterodimers are formed between the wild-type and the double mutant protein subunits. Furthermore, hybrid complementation shows that another tomato isoenzyme, Le-ACS4, can dimerize and that Le-ACS2 and Le-ACS4 have limited capacity for heterodimerization. The data suggest that ACS functions as a dimer with shared active sites.

Ethylene is an endogenous plant hormone that regulates many aspects of plant growth and development (1). The rate-limiting step in ethylene biosynthesis is the conversion of *S*-adenosylmethionine to the cyclic amino acid 1-aminocyclopropane-1-carboxylic acid (ACC)<sup>1</sup> and methylthioadenosine catalyzed by the enzyme *S*-adenosyl-L-methionine methylthioadenosine-lyase (ACS, EC 4.4.1.14). ACS is a pyridoxal phosphate (PLP)-dependent enzyme that is proposed to undergo an  $\alpha,\gamma$ -1,3 elimination reaction that is unique among all PLP-dependent enzymes (2, 3). ACS also shares sequence similarity with another group of PLP-dependent enzymes, the aminotransferases (4, 5). A recent alignment indicates that ACSs

have the highest similarity to a sub-group of aminotransferases that includes alanine-, tyrosine-, histidinol phosphate-, phenylalanine-, and aspartate (AspAT) aminotransferases and share all the 11 invariant residues in this subgroup including four conserved residues (Gly-197, Asp-222, Lys-258, Arg-386) present in all aminotransferases (Ref. 6, see Fig. 1I). The homology between ACS and aminotransferases such as AspAT suggests that these two groups of enzymes may be evolutionarily related and raises the possibility that the quaternary structure and co-factor binding sites of these two groups of enzymes may be similar (4, 5).

The subunit structure of ACS is unresolved. Although most published reports indicate that the enzyme is a homodimer (7–10), experimental evidence suggests that ACS purified from tomato (7, 11) and apple fruits (12) are monomers. AspAT is a homodimer with functionally independent active sites formed by the interaction of residues from both subunits and thus cannot function as a monomer (13). It is possible that ACS may also function as a dimer in which the active sites are shared and located at the interface between subunits. The presence of shared active sites in oligomeric proteins can be demonstrated using an elegant approach first described by Wentz and Schachman (14) for aspartyl transcarbamoylase and since then has been applied to other proteins (15–18). The experimental approach is based on the measurement of enzyme activity obtained from hybrid proteins between wild-type and/or inactive mutants of a protein (Fig. 1II).

In this paper we report a series of experiments designed to probe the nature and organization of the active site(s) of the tomato ACS isoenzymes Le-ACS2 and Le-ACS4. In particular, we want to determine whether some of the conserved residues between ACS and aminotransferases are required for active catalysis and thus may be part of the active site of the enzyme. These mutant proteins have also been used to demonstrate that the active site of ACS is located at the interface of the protein subunits, suggesting that the isoenzymes function as dimers. Furthermore the ability of the isoenzymes to heterodimerize has been tested with hybrid complementation experiments.

## EXPERIMENTAL PROCEDURES

### *Enzymes and Chemicals*

Restriction and DNA modifying enzymes were obtained from New England Biolabs and Boehringer Mannheim. Radionucleotides were obtained from Amersham Pharmacia Biotech. Acrylamide and SDS-PAGE gel reagents were from ICN and Bio-Rad. All other chemicals were at least reagent grade and obtained from Sigma.

### *Bacterial Strains and Plasmids*

The *Escherichia coli* strain used for most transformations is Sure: *mcrA*, *D(mcrCB-hsdSMR-mrr)*171, *endA1*, *supE-44*, *thi-1*, *gyrA-96*, *relA1*, *lac*, *recB*, *recJ*, *sbcC*, *umuC*(Kan<sup>r</sup>), *wvrC*(Tet<sup>r</sup>) obtained from Stratagene. *E. coli* CJ236: *dut1*, *ung1*, *thi1*, *relA1/pCJ105*(Cm<sup>r</sup>) (New

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<sup>1</sup> The abbreviations used are: ACC, 1-aminocyclopropane-1-carboxylate; ACS, 1-aminocyclopropane-1-carboxylate synthase; AspAT, aspartate aminotransferase; PLP, pyridoxal 5' phosphate; BS<sup>3</sup>, Bis(sulfosuccinimidyl)suberate; PAGE, polyacrylamide gel electrophoresis; kbp, kilobase pair(s).

England Biolabs) was used for site-directed mutagenesis.

The plasmid vector used for mutagenesis was Bluescript (Stratagene), whereas pGEM3-Z (Promega) was used for *in vitro* transcription and translation. The construction of the two-gene constructs used for the *E. coli in vivo* coexpression experiments is as follows. The vector was derived from pKK233-2 (Amersham) with the removal of the 1.6-kbp *EcoRI*-*AccI* small fragment containing the *Tet* resistance gene and the insertion of an oligonucleotide linker containing restriction sites for *NotI*, *SmaI*, and *XhoI*. An *NcoI* site was engineered into the 5' start codon of the *Le-ACS2* and *Le-ACS4* cDNAs using *ptACS2* and *ptACS4* (4) as templates for polymerase chain reaction. The entire *Le-ACS2* or *Le-ACS4* cDNAs were ligated into the modified pKK233-2 vector as an *NcoI*-blunt fragment to make the single plasmid construct. To make the two-gene constructs, the 2.7-kbp *XhoI*-*ScaI* fragment from the single-gene plasmid construct was gel-purified and ligated to another single-gene plasmid construct that was first digested with *NotI*, made blunt with Klenow, and subsequently digested with *XhoI*.

#### Oligonucleotides

The following oligonucleotides were synthesized using a Perkin-Elmer model 392 DNA synthesizer for constructing the following mutants.

*Le-ACS2*— $\Delta$ C46, GGATCCTAACTTTTCTCAACACCTACG; Y92F, AGCCATGAAAATCTTGA; Y92W, AGCCATGCCAATCTTGA; Y92L, AGCCATGTTAAATCTTGA; K278A, CCATGTCTGCTGAAAGAC.

*Le-ACS4*—Y92Q, GAATTCAGGCAATCCATGCTGATCTTG; Y92E, GAATTCAGGCAATCCATGCTCATCTTG; K278A, GTCCTTCCGCG GACTTAG.

#### Molecular Biology

Routine DNA manipulations were performed as described (19). Single mutants were generated using oligonucleotide-directed mutagenesis (20) or by polymerase chain reaction using the oligonucleotides listed above. Mutants were isolated and identified by restriction analysis and sequencing using the Sanger chain termination method (21) and the Sequenase version 1.0 kit (U. S. Biochemical Corp.). ACS cDNA containing two or three mutations were constructed by swapping appropriate restriction fragments. The final mutant cDNAs were sequenced in their entirety to verify that the desired codon changes were the only mutations in the cDNA.

#### Expression of Wild-type and Mutant ACS in *E. coli*

Plasmids containing one or two copies of the wild-type or mutated ACS cDNA were transformed in *E. coli* Sure and grown in 100 ml of LB medium containing ampicillin (50  $\mu$ g/ml) at 37 °C for 6 h. Aliquots were removed after 6 h for determining the  $A_{600}$  and ACC accumulation as described by Sato *et al.* (8). Crude protein was obtained from cells harvested from the 100-ml culture and used to determine ACS activity as described (8). One unit of ACS activity obtained from crude *E. coli* protein is defined as the amount of enzyme that catalyzes the formation of 1 nmol of ACC/h at 30 °C and in the presence of 200  $\mu$ M *S*-adenosylmethionine. The specific activity is expressed in units/mg of protein. Protein concentration was determined by the Bradford method using the dye reagent from Bio-Rad and bovine serum albumin as a protein standard. The results in Figs. 2 and 4 represent the average of duplicate samples from three independent experiments. The level of protein expression of the various mutants in *E. coli* was assessed by immunoblotting. A value of *E. coli* culture corresponding to  $A_{600} = 1.5$  was resuspended in 60  $\mu$ l of loading buffer (4), and 1  $\mu$ l of the protein extract was analyzed by SDS-PAGE.

#### *In Vitro* Synthesis, Cross-linking of ACS, SDS-PAGE, and Immunoblot Analysis

pGEM-3Z plasmids harboring wild-type or mutant ACS cDNAs were added into a reaction mixture containing either the wheat germ (Fig. 4) or rabbit reticulocyte lysate (Fig. 5) *in vitro* transcription and translation mix from Promega in the presence of [<sup>35</sup>S]methionine and incubated according to the manufacturer's instructions. Samples in Fig. 5 were synthesized using the rabbit reticulocyte lysate system because *Le-ACS4* is more efficiently translated in this system. Cross-linking of the <sup>35</sup>S-labeled proteins was carried out by incubating an aliquot (10  $\mu$ l) of the transcription and translation reaction with an equal volume of a solution containing 400 mM Hepes, pH 8.5, 20 mM PLP and 2 mM bis(sulfosuccinimidyl) suberate (BS<sup>3</sup>; Pierce) at room temperature for 30 min. The cross-linking reaction is then quenched with the addition of 0.1 volume of 1 M Tris, pH 7.5. SDS-PAGE, Coomassie staining, and immunoblot analysis were performed as described previously (4). Im-

munoblots were probed with either a 1:3000 dilution of partially purified *Le-ACS2* rabbit polyclonal antibody obtained previously (4) or with a 1:10,000 dilution of partially purified and concentrated mouse polyclonal antibody for *Le-ACS4* raised from BalbC mouse using standard techniques. The antibody-antigen complex was visualized with alkaline phosphatase conjugated to goat anti-rabbit or goat anti-mouse IgG (Promega). Aliquots of the transcription and translation reaction were assayed for ACS activity using the procedure of Sato *et al.* (8). The specific activity of ACS from the transcription and translation reaction was expressed as nmol of ACC formed/h with 200  $\mu$ M *S*-adenosylmethionine at 30 °C/10<sup>7</sup> cpm of radiolabeled synthesized protein.

#### RESULTS

*Activity of ACS Mutants*—The three-dimensional structure of AspAT has been solved and shows that it functions as a homodimer whose active site is formed from the interaction of residues from the monomeric subunits (13). In particular, the Tyr-70 residue, which helps in anchoring the PLP co-factor to the AspAT apoenzyme, interacts with active-site residues like Lys-258, which forms a covalent Schiff base with the PLP co-factor from the adjacent subunit. The homology of ACS to AspAT suggests that the corresponding residues in ACS may play equivalent roles in PLP binding and catalysis (Ref. 6, Fig. 1J). Site-directed mutagenesis has been carried out by making substitutions at the Lys-92 and Lys-278 residues. Mutant and wild-type *Le-ACS2* cDNAs have been expressed in *E. coli*, and their activity and levels of protein expression have been determined. The data in Fig. 2 show that mutations at these conserved residues severely affect the activity of the enzyme. The decrease in enzyme activity is not due to a decreased expression or stability of the mutant proteins compared with wild-type because immunoblots of wild-type and mutant proteins expressed in *E. coli* show similar levels of expression (see protein expression in Fig. 2).

*Coexpression of ACS Mutants in E. coli*—Fig. 1II illustrates how the presence of shared active sites in a dimeric protein can be tested. A prerequisite of this approach is that the mutations should be targeted on residues that are essential for enzyme activity and occupy distinct domains of the active site. Using AspAT as a model, we postulate that the Lys-278 residue in *Le-ACS2* is located in an active-site domain distinct from that of the Lys-92 residue of the same subunit.

According to the shared active-site model, association between two inactive proteins containing two different active-site mutations results in the formation of active heterodimers (positive complementation; see Fig. 1IIA), while inactive heterodimers result from the association between wild-type and a mutant protein containing both inactivating mutations (negative complementation; see Fig. 1IIB). To allow hybridization to occur between two mutant ACS proteins, we have used an *in vivo* coexpression system in *E. coli*. This coexpression system has the advantage of utilizing the natural efficiency of subunit assembly that occurs in the cytoplasmic environment (15). This approach has been used successfully to demonstrate shared active sites in other proteins; however, in most of these experiments, a two plasmid system has been used (15, 17). Because these two plasmids utilize two different replication origins and antibiotic markers for maintenance, they are often present in different copy numbers in the same cell. Thus, expression of the genes found in these two plasmids can be different and difficult to quantify. To minimize this problem we have designed an expression vector to carry two copies of the ACS gene that may have the same or different mutations under the control of the same promoter. To prevent recombination and DNA rearrangements, the plasmid has been transformed into a recombination-deficient *E. coli* Sure strain. Isolation and sequencing of the two-gene plasmids from *E. coli* Sure have confirmed the presence of the expected mutations, providing evidence that recom-

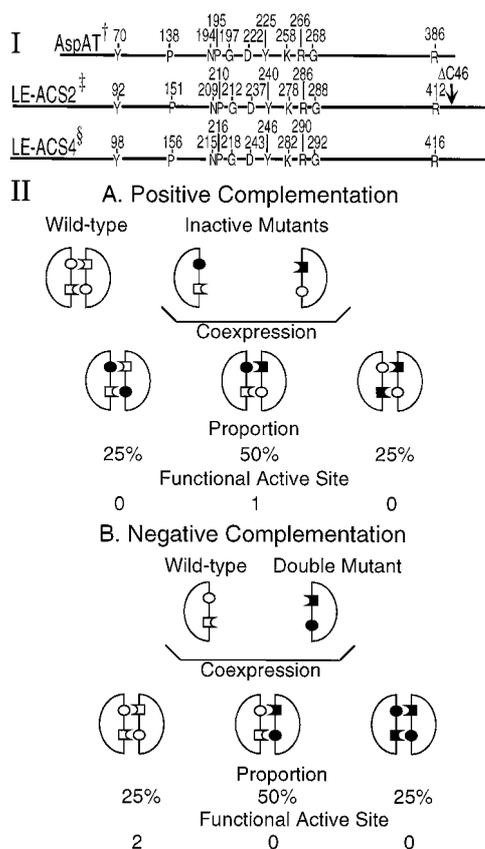


FIG. 1. *I*, amino acid residues conserved in Le-ACS2, Le-ACS4, and AspAT. <sup>†</sup>, numbering is based on chicken cytosolic AspAT. <sup>‡</sup>, numbering is from tomato Le-ACS2; the position of the  $\Delta$ C46 deletion at the carboxyl terminus is indicated by an arrow. <sup>§</sup>, numbering is based on tomato Le-ACS4. *II*, schematic presentation of positive and negative complementation to demonstrate shared active sites. The open symbols denote the unmodified active-site residues in the wild-type enzyme, and the mutated residues are represented by the corresponding closed symbols. *A*, positive complementation; formation of active heterodimers from inactive monomers. *B*, negative complementation; inactivation of the wild-type enzyme in heterodimers with mutant proteins containing two active-site mutations. The expected proportions and the number of functional active sites are indicated assuming random association of equimolar amounts of the parental subunits.

bination has not occurred. The level and activity of ACS protein accumulated in *E. coli* expressing a plasmid with one or two copies of the ACS cDNA are similar (data not shown).

To test for positive complementation, the Y92L mutant has been used for coexpression with the K278A single mutant proteins. Coexpression of two inactive mutant ACSs in the same *E. coli* results in partial restoration of enzyme activity as shown in Fig. 2 (K278A/Y92L, 16%). To test for negative complementation, wild-type Le-ACS2 has been coexpressed with cDNAs containing one mutation as well as with cDNAs containing two inactivating mutations in the same *E. coli* cell. In agreement with the shared active-site model, activity is reduced to 25% when wild-type and a mutant containing two mutations are coexpressed (Fig. 2, Y92L+K278A/WT), whereas coexpression of the wild type with a mutant with only one inactivating mutation reduces the activity to about 50% that of wild-type activity (Fig. 2, K278A/WT, 55%; Y92L/WT 41%).

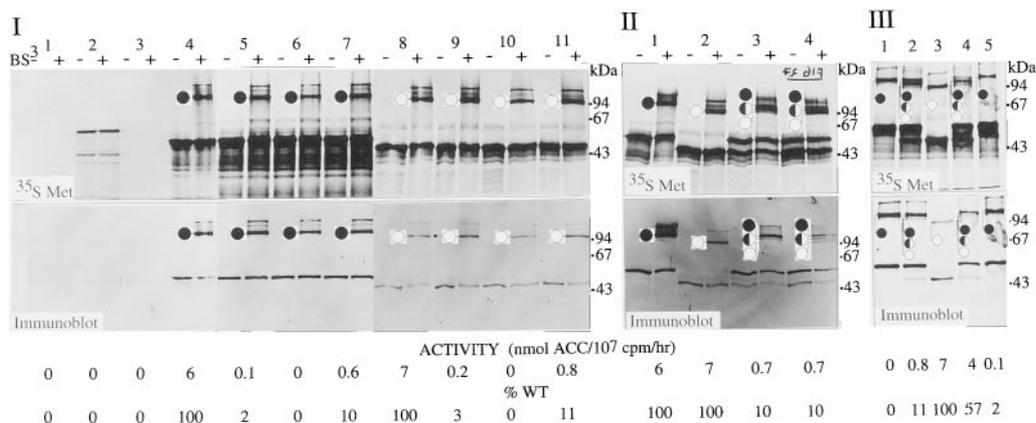
**Demonstration of Shared Active Sites in a Cell-free System**—To verify the results from the *in vivo* coexpression system, further experiments have been carried out in a cell-free system by expressing the mutant proteins in an *in vitro* transcription and translation system. ACS has a detectable enzymatic activity when synthesized *in vitro* either in a wheat germ or a rabbit reticulocyte lysate system. Since the wild-type and

Mutation	Activity		Protein Expression <sup>¶</sup>
	nmol ACC/mg Protein/hr	%	
<b>Single-Gene Constructs</b>			
Wild-type (WT) LE-ACS2	529±65	100	
Y92F	177±13	33	
Y92W	25±5	5	
Y92L	4±1	1	
K278A	0	0	
Y92L + K278A	0	0	
<b>Double-Gene Constructs</b>			
<b>Positive Complementation</b>			
K278A/Y92L	62±3	16	
<b>Negative Complementation</b>			
K278A/WT	215±60	55	
Y92L/WT	160±30	41	
Y92L+K278A/WT	100±30	25	

FIG. 2. **Positive and negative complementation of Le-ACS2 in *E. coli*.** <sup>¶</sup>, Protein bands were obtained from immunoblots of crude *E. coli* lysates expressing wild-type or mutant Le-ACS2 proteins probed with Le-ACS2 antibody as described under "Experimental Procedures." WT, wild type.

mutant proteins are identical with regard to their electrophoretic migration, heterodimer formation can only be inferred from the activity observed from coexpression. In order to distinguish the formation of homo- and heterodimeric proteins, we have used a truncated Le-ACS2 that lacks 46 amino acids at the carboxyl terminus ( $\Delta$ C46) which retains full enzymatic activity (9). The results of both positive and negative complementation experiments are shown in Fig. 3. The enzymatic activity is very low when full-length (Fig. 3*I*, lanes 5 and 6) or truncated proteins (Fig. 3*I*, lanes 9 and 10) containing either the Y92L or K278A mutations are expressed individually. Co-translation of either full-length or truncated proteins containing either the Y92L or the K278A mutations yields 10–11% wild-type activity (Fig. 3, *I* (lanes 7 and 11) and *II*, (lanes 3 and 4)). The activity of the truncated wild-type (WT $\Delta$ C46) ACS is reduced to 57% when co-translated with the full-length ACS containing the Y92L mutation, whereas it is reduced to 10% when cotranslated with ACS containing both the Y92L and K278A mutations (negative complementation; Fig. 3*III*, lane 2).

To demonstrate that dimeric proteins are formed, the *in vitro* translation products have been cross-linked with a homo-bifunctional cross-linker, BS<sup>3</sup>, followed by electrophoresis on SDS-PAGE. As shown in Fig. 3, full-length and truncated synthesized proteins can be cross-linked to produce protein complexes with sizes corresponding to the expected dimeric proteins. The estimated sizes are full-length monomer, 56 kDa; truncated monomer, 49 kDa; full-length dimer, 110 kDa; truncated dimer, 98 kDa; heterodimer, ~105 kDa (Figs. 3, *II*, lanes 3 and 4) and *III* (lanes 2 and 4)). Minor bands corresponding to even higher molecular mass complexes are also observed that may correspond to higher oligomeric forms of the enzyme (Fig. 3*I*, *II*, and *III*). When a full-length protein is synthesized simultaneously with a truncated protein, an intermediate-sized cross-linked species is formed that has the molecular mass between a full size and truncated dimer, as seen in Figs. 3, *II* (lanes 3 and 4) and *III* (lanes 2 and 4). The cross-linked species represents the dimeric ACS because they are also detected by immunoblotting using an Le-ACS2 polyclonal antibody (see Fig. 3*I*, *II*, and *III*). It should be pointed out that the Le-ACS2 antibody has more affinity for full-length protein than for truncated. The antibody is less efficient in detecting both truncated



**FIG. 3. Positive and negative complementation of Le-ACS2 *in vitro*.** Panel I, positive complementation among inactive mutants Le-ACS2. *In vitro* synthesized mutants of Le-ACS2 were incubated with or without the cross-linking agent BS<sup>3</sup> and analyzed by SDS-PAGE. Lane 1, wheat germ lysate; lane 2, luciferase; lane 3, pGEM3-Z vector; lane 4, wild-type Le-ACS2; lane 5, Y92L mutant; lane 6, K278A mutant; lane 7, Y92L/K278A coexpressed mutant proteins; lane 8, wild-type Le-ACS2 ΔC46; lane 9, Y92L ΔC46; lane 10, K278A ΔC46; lane 11, Y92L ΔC46/K278A ΔC46. Panel II, positive complementation between full-length and truncated Le-ACS2. Lane 1, wild-type Le-ACS2; lane 2, wild-type Le-ACS2 ΔC46; lane 3, Y92L/K278A ΔC46; lane 4, Y92L ΔC46/K278A. Equal cpm were loaded in each lane in panels I and II. Panel III, negative complementation between wild-type Le-ACS2 ΔC46 and inactive Y92L single mutant protein or inactive Y92L/K278A double mutant protein. Lane 1, Y92L/K278A double mutant protein; lane 2, Y92L/K278A/wild-type ΔC46 coexpressed proteins; lane 3, wild-type Le-ACS2 ΔC46; lane 4, Y92L/wild-type ΔC46 coexpressed proteins; lane 5, Y92L mutant protein. All samples loaded have been cross-linked with BS<sup>3</sup> and are of equal cpm. The circles indicate the position of the cross-linked homo- and heterodimers formed. Filled circles indicate the cross-linked full-length homodimers, whereas open circles indicate the truncated homodimers formed after cross-linking. Half-filled circles show the position of the heterodimers formed between full-length and truncated ACS protein. In all panels, the upper figures represent autoradiograms of [<sup>35</sup>S]Met *in vitro* synthesized proteins, whereas the lower figures show immunoblots of the same gels with Le-ACS2 antibodies. Activity and expression of *in vitro* synthesized wild-type and mutant Le-ACS2, cross-linking with BS<sup>3</sup>, and immunoblotting were carried out as described under "Experimental Procedures." WT, wild type.

homodimers and the heterodimers that are formed. We have also observed that there is less cross-linked protein for the K278A mutant than for the Y92L mutant protein (see Fig. 3I, compare lanes 5 and 9 with lanes 6 and 10). This may reflect differences in the ability of the mutant proteins to dimerize.

**Evidence for Shared Active Sites in Le-ACS4 and Heterodimerization between Le-ACS2 and Le-ACS4**—Because ACS is encoded by a multigene family in tomato (4), it is of interest to determine whether other isoenzymes of ACS can function as dimers with shared active sites. In addition, because Le-ACS2 and Le-ACS4 have similar amino acid sequences (69% amino acid identity) and similar expression characteristics, particularly in ripening tomato fruits (4), we wanted to determine whether the two isoenzymes are able to heterodimerize using the positive complementation approach. Mutations in the Tyr-98 and Lys-282 residues of Le-ACS4 have been constructed and used for coexpression experiments both *in vivo* and *in vitro* (Figs. 4 and 5). The Y98Q and Y98E mutants as well as the K282A mutant protein do not have ACS activity when expressed in *E. coli* (Fig. 4) or in the *in vitro* transcription and translation system (see Fig. 5II, lanes 1–3). When either the Y98Q or Y98E mutated Le-ACS4 protein is coexpressed with the K282A-mutated Le-ACS4 protein, ACS activity is partially restored to about 14% that of the Le-ACS4 wild-type activity in the *in vivo E. coli* coexpression system (Fig. 4) and 32 and 37% in the *in vitro* transcription and translation system (Fig. 5II, lanes 4 and 5).

To determine whether Le-ACS2 and Le-ACS4 are able to heterodimerize, we have performed experiments whereby we coexpressed the Le-ACS2 Y92L mutant with the Le-ACS4 K282A mutant protein and also did the converse experiments where the Le-ACS4 Y98Q or Le-ACS4 Y98E mutant proteins were coexpressed with the Le-ACS2 K278A mutant. The results shown in Fig. 4 and Fig. 5II (lanes 6–8) indicate that there is some restoration of ACS activity and that the activity obtained varies depending on the functional active-site residue on the isoenzyme. For example, coexpression of the Le-ACS2 Y92L with the Le-ACS4 K282A mutant proteins showed 11% wild-type activity *in vivo* (Fig. 4) and 35% wild-type activity *in*

Mutation	Activity		Protein Expression	
	nmol ACC/mg Protein/hr	%	Detected with Antibodies of LE-ACS2	LE-ACS4**
<b>Single-Gene Constructs</b>				
WT,LE-ACS2	541±16	100		
WT,LE-ACS4	85±7	100		
Y98Q,LE-ACS4	0	0		
Y98E,LE-ACS4	0	0		
K282A,LE-ACS4	0	0		
<b>Double-Gene Constructs</b>				
<b>Positive Complementation</b>				
Y98Q/K282A,LE-ACS4	12±1	14		
Y98E/K282A,LE-ACS4	11±1	14		
<b>Coexpression of LE-ACS2 and LE-ACS4</b>				
Y92L,LE-ACS2 / K282A,LE-ACS4	9±0.6	11		
Y98Q,LE-ACS4 / K278A,LE-ACS2	4±1.7	5		
Y98E,LE-ACS4 / K278A,LE-ACS2	2±0.4	2		

**FIG. 4. Positive complementation of Le-ACS4 and heterodimerization of Le-ACS2 and Le-ACS4 in *E. coli*.** ACS activity and expression of wild-type and mutant Le-ACS2 and Le-ACS4 in *E. coli*. \*\*, Protein bands were obtained as described in the legend of Fig. 2.

*in vitro* (Fig. 5II, lane 6), whereas coexpression of the Le-ACS4 Tyr-92 with the Le-ACS2 K278A mutant proteins showed only 2–5% wild-type activity both *in vivo* (Fig. 4) and *in vitro* (Fig. 5II, lanes 7 and 8). The presence of these two isoenzymes in the coexpression experiments is confirmed using polyclonal antibodies for Le-ACS2, which can also detect Le-ACS4, and a more specific antibody for Le-ACS4 as shown in Figs. 4 and 5. Cross-linking with BS<sup>3</sup> produces cross-linked proteins corresponding to the homodimers of Le-ACS2 (110 kDa) and high molecular

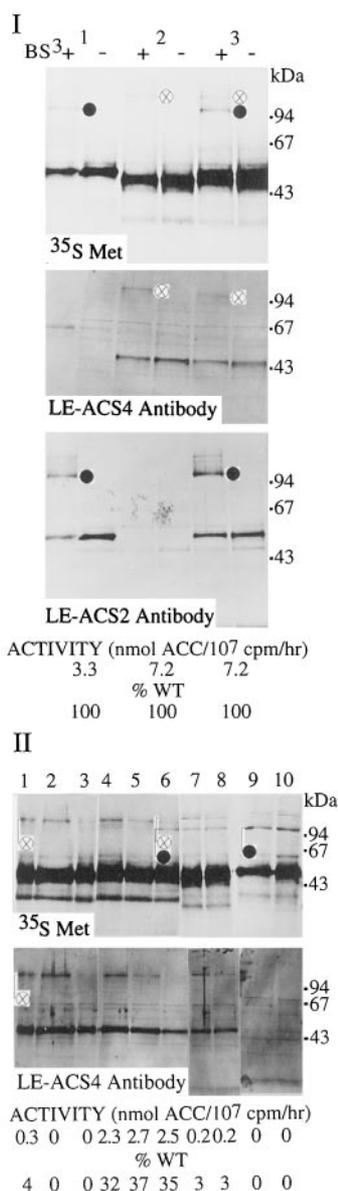


FIG. 5. *Panel I*, coexpression of Le-ACS2 and Le-ACS4 *in vitro*. *In vitro* synthesized Le-ACS2 and Le-ACS4 were incubated in the presence or absence of the cross-linking agent BS<sup>3</sup> and analyzed by SDS-PAGE. *Lane 1*, wild-type Le-ACS2; *lane 2*, wild-type Le-ACS4; *lane 3*, coexpression of wild-type Le-ACS2 and Le-ACS4. The *first panel* is an autoradiogram of [<sup>35</sup>S]Met-labeled proteins; the *second panel* is an immunoblot of a gel loaded with the same samples and probed with Le-ACS4 antibody; the *third panel* is an immunoblot of the gel in *panel I* probed with Le-ACS2 antibody. The gel used for the *first* and *third panels* was run longer than that for the *second panel*. *Panel II*, positive complementation of Le-ACS4 and heterodimerization of Le-ACS2 with Le-ACS4 *in vitro*. *In vitro* synthesized mutants of Le-ACS2 and Le-ACS4 were incubated with BS<sup>3</sup> and analyzed by SDS-PAGE. *Lane 1*, Le-ACS4 Y98Q; *lane 2*, Le-ACS4 Y98E; *lane 3*, Le-ACS4 K282A; *lane 4*, Le-ACS4 Y98Q/K282A coexpressed proteins; *lane 5*, Le-ACS4 Y98E/K282A; *lane 6*, Le-ACS2 Y92L/Le-ACS4 K282A; *lane 7*, Le-ACS4 Y98Q/Le-ACS2 K278A; *lane 8*, Le-ACS4 Y98E/Le-ACS2 K278A; *lane 9*, Le-ACS2 Y92L; *lane 10*, Le-ACS2 K278A. The *first panel* is an autoradiogram of [<sup>35</sup>S]Met-labeled proteins, and the *second panel* is immunoblot of the same gel probed with an Le-ACS4 polyclonal antibody enzyme. The *circles* indicate the putative position of oligomers formed after cross-linking. *Filled circles* refer to Le-ACS2 homodimeric protein, whereas *crossed circles* indicate Le-ACS4 oligomeric protein. See "Experimental Procedures" for experimental details. WT, wild type.

mass complexes of Le-ACS4 (Fig. 5I, lanes 1 and 2). The cross-linked proteins of Le-ACS4 are detected by a specific Le-ACS4 antibody but have a higher molecular mass (~120 kDa) than

the expected dimeric protein (109 kDa). The slower migration of the cross-linked protein may indicate that Le-ACS4 cross-links with a contaminating protein, but it can also be due to a partial unfolding of the protein during the cross-linking reaction. We have been unable to detect any heterodimers that may be formed between Le-ACS2 and Le-ACS4 (Fig. 5, I (lane 3) and II (lanes 6–8)). This negative result, however, does not rule out the formation of heterodimers. The possibility exists that the heterodimers may be inefficiently cross-linked or may migrate close to the Le-ACS2 and Le-ACS4 homodimeric cross-linked proteins.

#### DISCUSSION

Although the three-dimensional structure of ACS remains unresolved, critical active-site residues have been inferred based on sequence similarity with aminotransferases and functional analysis of mutant proteins. In this work, we have made mutations in the tomato Le-ACS2 and Le-ACS4 isoenzymes in the amino acid residues that correspond to Tyr-70 and Lys-258 of AspAT. Mutations in these residues decrease or completely abolish ACS activity, indicating that these residues play an important role in catalysis. Mutagenesis in some of these residues in the apple ACS shows that the resulting mutant proteins also have reduced enzymatic activity and altered kinetic properties (10). This is consistent with the proposition that the conserved residues may have a similar role in catalysis as their counterparts in the aminotransferases (10). Thus, the sequence similarity between ACS and aminotransferases such as AspAT may extend to a similarity in structure.

The subunit structure of ACS is controversial. ACS is reported to be either dimeric or monomeric depending on from which species it has been purified. To obtain further insight on the subunit structure and active-site localization of ACS, we have carried out *in vivo* and *in vitro* complementation experiments that allow the determination of whether tomato Le-ACS2 and Le-ACS4 also have shared active sites and function as dimers. We observe partial restoration of ACS activity when expression plasmids harboring two inactive Le-ACS2 mutants are expressed in a recombination-deficient *E. coli* Sure strain. Cells harboring these plasmids exhibit 16% wild-type ACS activity, which is close to the theoretical value of 25% (Fig. 1II). This result has been verified by *in vitro* transcription and translation reactions of the inactive Y92L and K278A mutant ACS. This positive complementation result demonstrates an interaction between the two inactive mutants to form partially active heterodimers, indicating that ACS must be able to dimerize. Protein level complementation, however, can result from different mechanisms. In the conformational correction model, folding of the mutant enzyme is defective, and the adjacent subunit serves as a template to aid in the folding process (14). Complementation can also occur when active sites are made up of amino acid residues from the two interacting subunits, and a functional active site can thus be formed from the properly folded components of the two defective subunits. To rule out the first model and thus provide evidence for shared active sites in ACS, a negative complementation experiment has been carried out. Negative complementation is demonstrated when association between wild-type and double-mutant subunits results in a significant reduction in wild-type activity, reflecting the formation of completely inactive heterodimers. In both *in vivo* and *in vitro* coexpression experiments, it has been observed that although coexpression of wild-type with a single mutant protein decreased ACS activity about 50%, coexpression with a double mutant protein decreased activity to about 25% *in vivo* and 10% *in vitro* of wild-type activity (Figs. 2 and 3). The formation of heterodimers is clearly demonstrated from cross-linking experi-

ments of *in vitro* synthesized full-length and truncated Le-ACS2.

The Le-ACS2  $\Delta$ C46 deletion mutant has been reported to be monomeric and with 450% of the specific activity as the full-length protein (9). Although our data indicate that this truncated protein is indeed highly active, the specific activity is only 120% that of the full-length wild-type protein. The difference could be due to the fact that the previous paper based their specific activity on the apparent amount of the ACS protein as detected from Western blots. Using the same Le-ACS2 rabbit polyclonal antibody that they had used, we have observed that the antibody has a lower titer for the truncated protein than for the full-length protein (Fig. 3). This can account for an underestimation of the amount of truncated Le-ACS2 that will inflate its specific activity. Our results indicate that the truncated Le-ACS2 produces a cross-linked band of the expected size of a dimeric protein. Coexpression experiments of Le-ACS2  $\Delta$ C46 with Y92L and K278A mutations also show complementation (Fig. 3I, lane 11). Thus, contrary to their claim that Le-ACS2  $\Delta$ C46 is monomeric, our data clearly show that it is able to dimerize.

Complementation studies with another tomato ACS isoenzyme, Le-ACS4, provide strong evidence that it is also able to dimerize and has shared active sites. However, coexpression studies between inactive mutants of Le-ACS2 and Le-ACS4 are unclear as to whether these two isoenzymes can heterodimerize. Although complementation studies between inactive mutants of Le-ACS2 and Le-ACS4 show some restoration of enzyme activity, the restored activity is very low and is not corroborated by the results from the cross-linking with BS<sup>3</sup> (Fig. 5). However, it is interesting that there is a difference between the activity of the coexpressed constructs depending on the mutation (or the functional amino acid) that is present on the isoenzymes. For instance when the Tyr-92 mutation is on Le-ACS2 and the Lys-278 mutation is on Le-ACS4, the coexpressed proteins are more active than when the Tyr-92 mutation is on Le-ACS4 and the Lys-278 mutation is on Le-ACS2. Furthermore some of our preliminary studies indicate that the former resembles Le-ACS2 in its kinetic properties, particularly in the time course of reaction with *S*-adenosylmethionine, whereas the latter is more like Le-ACS4 (data not shown). It is possible that the functional heterodimers that are formed in the two co-expressions have different kinetic properties. A similar result has been previously reported from *in vitro* complementation studies to form functional protozoan and mouse heterodimers of ornithine decarboxylase (22). Osterman *et al.* (22) found that the substrate binding properties of the restored active site of the cross-species heterodimer mimic the characteristics of the wild-type enzyme from the species that contributed the subunit with the functional Lys that serves as the Schiff base of the PLP co-factor.

In summary, by utilizing *in vivo* and *in vitro* complementation experiments, we provide compelling evidence that ACS has an intersubunit or shared arrangement with its active sites and that it functions as a dimer. This active site arrangement has been previously found for a number of oligomeric enzymes;

however, in most of these cases dissociation of the oligomeric protein produces inactive monomers. Bovine serum RNase is the only example in the literature of a dimeric enzyme with shared active sites that is also functional as a monomer (23). In view of the fact that previously reported experimental evidence indicates that tomato ACS is able to function as a monomer, the question that can be asked is whether ACS must dimerize to function or whether ACS may be active both as a monomer and as a dimer and have shared active sites. Our experimental results cannot distinguish between these two possibilities. The experimental strategy we have used, however, can be used to screen which other residues are part of the active site of ACS. Our complementation studies also show that a dominant negative effect is observed when mutant proteins are coexpressed with the wild type, resulting in an inhibition of ACS activity. This raises the prospect that expression of mutant ACS isoforms in transgenic plants using their own promoters may inhibit ACS expression in a cell- and tissue-specific manner. This reverse genetics strategy may allow the elucidation of the role of each ACS isoform during plant growth and development.

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