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

Alice S. Tarun and Athanasios Theologis†
From the Plant Gene Expression Center, Albany, California 94710

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) 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 α,γ-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 sub-group 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 evolutionary related and raise 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 Wente 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. III).

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. Radiolabeled chemicals were obtained from Amersham Pharmacon 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, Δ(mcrCB-hsdSMR-mrr)171, endA1, supE44, thi-1, gyrA96, relA1, lac, recB, recC, sbeC, umuC(Can), umvC(Tet) obtained from Stratagene. E. coli CJ236: dut1, ung1, thi1, relA1/pCJ105(Cm) (New...
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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 pPK233–2 (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 pACS2 and pACS4 (4) as templates for polymerase chain reaction. The entire Le-ACS2–Le-ACS4 cDNA constructs with the modified pE. coli vector as an NcoI-blunt fragment to make the single plasmid construct. To make the two-gene constructs, the 2.7-kbp XhoI-NcoI fragment from the single-gene plasmid construct was gel-purified and ligated to another single-gene plasmid construct that was first digested with NcoI, 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—ΔC46, GGATCTAACATTCTCAACACCTAGC; Y92F, AGCCATGAAAATCTTGA; Y92L, AGCCATGCCAATCTTGA; Y92Q, GAATTCAGGCAATCCATGCTGATCTTG; Y92E, GAATTCAGGCAATCCATGCTCATCTTG; K278A, GTCTTTCCCGCGGAC; K278R, AGATCCGATCGCGGAC; K278N, GGCTGGTCCGCGGAC.

Expression of Wild-type and Mutant ACSs in E. coli

Plasmids containing one or two copies of the wild-type or mutated ACS cDNAs were transferred in E. coli Sure and grown in 100 ml of LB medium containing ampicillin (50 μg/ml) at 37 °C for 6 h. Aliquots were removed after 6 h for determining the A600 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). The unit of ACS activity obtained from strain E. coli protein is defined as the amount of enzyme that catalyzes the formation of 1 nmol of ACC/h at 30 °C in the presence of 200 μM 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. 1–3 are expressed as specific activity of ACS from the transcription and translation reaction with an equal volume of a standard assay mix from Promega in the presence of [35S]methionine and incubation of the transcription and translation reaction with an equal volume of a solution containing 400 m units Hepes, pH 8.5, 20 mM PLP and 2 mM bis(sulfosuccinimidyl) suberate (BS2; 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). Immunoblots 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 in BalbC mouse using standard techniques. The antibody-antigen complex was visualized with alkaline phosphatase-conjugated 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 ACSs from the transcription and translation reaction was expressed as nmol of ACC formed/h with 200 μM S-adenosylmethionine at 30 °C/107 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. 1). Site-directed mutagenesis has been carried out by making substitutions at the Lys-92 residue. Site-directed mutagenesis has been used 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. 1B 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-258 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. 1A), while inactive heterodimers result from the association between wild-type and a mutant protein containing both inactivating mutations (negative complementation; see Fig. 1B). 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 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-
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 when wild-type and a mutant containing two mutations are coexpressed (Fig. 2, K278A/WT, 16%). To test for negative 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 inactive 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 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 (ΔC46) 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. 3I, lanes 5 and 6) or truncated proteins (Fig. 3I, lanes 9 and 10) containing either the Y92L or K278A mutations are expressed individually. Coexpression of either full-length or truncated proteins containing the Y92L mutation, whereas it is reduced to 10% when cotranslated 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. 3III, lane 2).

To demonstrate that dimeric proteins are formed, the in vitro translation products have been cross-linked with a homo-bifunctional cross-linker, BS3, 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. 3I, II, III and IV). Minor bands corresponding to even higher molecular mass complexes are also observed that may correspond to higher oligomeric forms of the enzyme (Fig. 3I, 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. 3I, 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. 3I, 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...
In vitro studies showed that Le-ACS2 and Le-ACS4 are able to dimerize to form functional dimers with shared active sites. However, differences in the ability of the mutant proteins to dimerize suggest that the two isoenzymes have different dimerization properties.

In vivo studies using the positive complementation approach revealed that mutations in the Tyr-98 and Lys-282 residues of Le-ACS4 have different effects on dimerization. The Y98Q and Y98E mutated Le-ACS4 proteins showed 11% wild-type activity in vitro, whereas the Y92L and Y92E mutated Le-ACS4 proteins showed 2–5% wild-type activity both in vivo and in vitro. The presence of these two isoenzymes in the coexpression experiments 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 BS3 produced cross-linked proteins corresponding to the homodimers of Le-ACS2 (110 kDa) and high molecular weight dimers of Le-ACS4.
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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 BS3 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 [35S]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 1 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 BS3 and analyzed by SDS-PAGE. Lane 1, Le-ACS4 Y98Q; lane 2, Le-ACS4 Y98E; lane 3, Le-ACS4 K278A; lane 4, Le-ACS4 Y98Q/K278A coexpressed proteins; lane 5, Le-ACS4 Y98E/K282A; lane 6, Le-ACS2 Y92L/Le-ACS4 K278A; 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 [35S]Met-labeled proteins, and the second panel is an 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.

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 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. III). 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 ΔC46 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 and Le-ACS4 with Y92L and K278A mutations also show complementation (Fig. 3I, lane 11). Thus, contrary to their claim that Le-ACS2 ΔC46 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 BS3 (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 protozoon and mouse heterodimers of ornithine decarboxylase (22). Osterman et al. (22) found that the binding partner 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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