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Identification of Active Site Residues in *Bradyrhizobium japonicum* Acetyl-CoA Synthetase[1]

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Acetyl-CoA synthetase (ACS) catalyses the activation of acetate to acetyl-CoA in the presence of ATP and CoA. The gene encoding *Bradyrhyzobium japonicum* ACS has been cloned, sequenced, and expressed in *Escherichia coli*. The enzyme comprises 648 amino acid residues with a calculated molecular mass of 71,996 Da. The recombinant enzyme was also purified from the transformed *E. coli*. The enzyme was essentially indistinguishable from the ACS of *B. japonicum* bacteroids as to the criteria of polyacrylamide gel electrophoresis and biochemical properties. Based on the results of database analysis, Gly-263, Gly-266, Lys-269, and Glu-414 were selected for site-directed mutagenesis in order to identify amino acid residues essential for substrate binding and/or catalysis. Four different mutant enzymes (G263I, G266I, K269G, and E414Q) were prepared and then subjected to steady-state kinetic studies. The kinetic data obtained for the mutants suggest that Gly-266 and Lys-269 participate in the formation of acetyl-AMP, whereas Glu-414 may play a role in acetate binding.

Key words: acetyl-CoA synthetase, active site residue, *Bradyrhizobium japonicum*, site-directed mutagenesis, steady-state kinetics.

Bacterial acetyl-CoA synthetase (ACS), which belongs to the acyl-adenylate/thioester-forming enzyme family, has been purified and characterized from *Bradyrhizobium japonicum* bacteroids (*1*), *Acetobacter aceti* (*2*), and *Escherichia coli* (*3*). Among the latter, *B. japonicum* is a microorganism that can grow heterotrophically in culture or can exist in a viable but non-growing form symbiotically within soya-bean root nodules (*4*). The symbiotic forms of *B. japonicum* bacteroids metabolize photosynthetically derived carbon compounds from the plant to provide the energy required for the nitrogen-fixation process (*5*). As the symbiotic forms possess a citric acid cycle, a polyhydroxy butyrate cycle and active fatty acid metabolism, acetyl-CoA is thought to be a central molecule in bacteroid metabolism. Also, it has been reported that acetate could support nitrogen fixation in isolated *B. japonicum* bacteroids (*6*). The presence of acetyl-CoA synthetase and acetate kinase was demonstrated in the symbiotic and heterotrophically cultured forms of *B. japonicum*. Previously, acetyl-CoA synthetase was highly purified from a symbiotic nitrogen-fixing bacterium and characterized by steady-state kinetic analysis (*1*).

Acetyl-CoA synthetase catalyses the reversible reaction:

$$\text{Acetate} + \text{MgATP} + \text{CoA-SH} \longleftrightarrow \text{Acetyl-CoA} + \text{MgAMP} + \text{PP}_i$$

The formation of acetyl-AMP, proposed to be an intermediate in the mechanism, was confirmed by the autoradiogram obtained on TLC analysis of a reaction mixture containing the *Pseudomonas fluorecens* ACS, acetate, and [α-^{32}P]ATP (*7*). In the 4-chlorobenzoate:coenzyme A (4-CBA:CoA) ligase, a member of the acyl-adenylate/thioester-forming enzyme family from *Pseudomonas* sp., two conserved motifs (motif I, 161TSGTTGLPKG170; and motif II, 302-YGTTE306) were found and tested as to their possible role in substrate binding and/or catalysis. The kinetic data for the G163I, G166I, P168A, K169M, and E306Q ligase mutants indicated that the two motifs might function primarily in acyl-adenylate formation (*9, 10*). Recently, the *B. japonicum* malonyl-CoA synthetase (MCS) structural gene was cloned and sequenced. It exhibits amino acid sequence similarity with the adenylate/thioester-forming enzyme family. The Lys-175 and Glu-308 residues (residing in motif I or II) in the enzyme were also determined to be essential for catalysis of the MCS reaction by site-directed mutagenesis and kinetic studies (*11*). However, the active site of acetyl-CoA synthetase has not been thoroughly studied yet.

In this report, we describe the molecular cloning of the *B. japonicum* ACS gene and the expression of this gene in *E. coli*. The amino acid residues, which are essential for catalysis and/or substrate binding were identified by site-directed mutagenesis and kinetic analysis.

MATERIALS AND METHODS

Strains and Plasmids—*B. japonicum* USDA 110 was obtained from the Genetic Engineering Research Institute,

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Abbreviations: ACS, acetyl-CoA synthetase; ATP, adenosine triphosphate; CBA, chlorobenzoate; CD, circular dichroism; GST, glutathione-S-transferase; MCS, malonyl-CoA synthetase; PBS, phosphate-buffered saline.

Daeduck, Korea. *E. coli* XL-1 Blue MRA (P2) and MRA (Stratagene) were used to construct the genomic library of *B. japonicum*. For subcloning, *E. coli* XL-1 Blue MRF' was used as a host strain. A pCR2.1 vector for cloning of PCR products was purchased from Invitrogen. A λ vector (λ DASH II/*Bam*HI vector kit) and a pBluescript KS II plasmid vector for subcloning of genes were purchased from Stratagene. A pGEX-4T-1 for expression of genes was from Amersham Pharmacia Biotech.

Enzymes and Chemicals—Restriction enzymes and *Taq* DNA polymerase were purchased from Roche Molecular Biochemicals. T4 DNA ligase and calf intestinal phosphatase were from New England Biolabs. The sequencing primers and labeling system were from Promega. ^{32}P-labeled nucleotides and nitrocellulose filters were from Amersham Pharmacia Biotech. The synthetic oligonucleotides were from Bioneer, Korea.

Preparation of a λ DNA Library—A *B. japonicum* λ DNA library was prepared according to methods previously reported (*12*). Cells from a 200-ml culture grown in yeast/mannitol at 30°C were harvested. The harvested cells were suspended in 9 ml Tris/EDTA (10 mM Tris/HCl, 1 mM EDTA, pH 8.0), and incubated with proteinase K (1 mg/ml), 1% SDS overnight at 50°C. The lysate was extracted twice with phenol/$CHCl_3$/isoamyl alcohol (25:24:1) and twice with $CHCl_3$/isoamyl alcohol (24:1), and then digested for 30 min at 37°C with ribonuclease A (0.1 mg/ml). The ribonuclease-digested lysate was again extracted with phenol/$CHCl_3$/isoamyl alcohol (25:24:1) and $CHCl_3$/isoamyl alcohol (24:1). The DNA was precipitated by the addition of a 0.1 volume of 3 M sodium acetate, pH 5.2, and 2 volumes of absolute ethanol. The precipitated DNA was spooled onto a glass rod, washed with 70% ethanol, and resuspended in Tris/EDTA buffer, pH 8.0. The DNA isolated from *B. japonicum* was partially digested with *Sau*3AI and analyzed on a 0.6% agarose gel to verify the digestion. The fractions containing fragments of 9–23 kb were pooled, ligated to the λ DASH II/*Bam*HI that had been previously digested with *Bam*HI, and packaged using Gigapack III Gold packaging extract. The packaged λ phage was then used to infect *E. coli* XL-1 Blue MRA (P2).

Preparation of a Probe—The amino acid sequences of two conserved regions for ACS are TYWQTETG and PKDRSGKIM. Based on these amino acid sequences, a sense primer [5'-AC(A/C)TA(C/T)GG(A/T/C)(A/T)C(A/T/C)AC(C/T)GG(C/T)AA(G/C)CC-3'] of amino acid sequence TYWQTETG and an antisense primer [5'-AT(A/G)AT(C/T)TT(A/G)CC(A/G)G(A/T)(A/G)CG(G/C)GT(C/T)TT(G/A/C)GG-3'] of amino acid sequence PKDRSGKIM were prepared to make a hybridization probe. Using the following PCR procedure with the degenerate primers, a PCR product was prepared. The reaction mixtures (50 μl) for PCR contained 100 ng of *B. japonicum* total DNA, 2 mM of each primer, 1.25 units of *Taq* DNA polymerase, 1 mM of each dNTP, and 5 μl of 10 × *Taq* DNA polymerase buffer (Roche). The PCR reaction was carried out in a Techne programmable cycling heat block for 35 cycles of 30 s of denaturation at 95°C, 40 s of annealing at 62°C and 60 s of extension at 72°C followed by one cycle of 50 s of denaturation at 95°C, 50 s of annealing at 58°C and 10 min of extension at 72°C. The 600-bp PCR product was cloned into a pCR 2.1 vector (Invitrogen) according to the manufacturer's protocol and then sequenced.

Cloning Strategy and DNA Sequence Analysis—The 600-bp DNA fragment obtained on the PCR reaction involving degenerate primers was used as a probe for screening the λ library of *B. japonicum* (*12*). The DNA fragment was purified and radiolabeled with [α-^{32}P]dCTP using a Prime-a-Labeling kit (Promega). Positive plaques were isolated by successive plating and a pure phage was purified using a Plate lysate method (*13*). A 3.5-kb *Bam*HI fragment inserted into the positive λ phage DNA was subcloned into pBluescript KS II (designated as pBACS). These were sequenced using an ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer) and an ABI 310 automated sequencer (Applied Biosystems Division of Perkin Elmer).

Construction of a Recombinant Plasmid for Expression of ACS—The ACS gene was amplified with two oligonucleotides, 5'-AG**GAATTC**ATGTCCGAGAAGATTTACG-3' and 5'-C*GAGCTC*GAGGCTTACGCCGACTTCTTG-3' (bold, *Eco*RI site; italics, *Xho*I site). For PCR amplification, 100 ng of pBACS was used as a template in a 50 μl reaction system consisting of 0.2 mM of each dNTP, 50 pmol of each primer, 1× *Pfu* reaction buffer (Stratagene), and 2.5 units of *Pfu* DNA polymerase (Stratagene). The PCR reaction was carried out in a Techne Progene programmable cycling heat block for 30 cycles of 30 s of denaturation at 95°C, 40 s of annealing at 58°C and 4 min of extension at 72°C, followed by one cycle of 50 s of denaturation at 95°C, 50 s of annealing at 58°C and 10 min of extension at 72°C. The amplified product, which was digested with *Eco*RI and *Xho*I, was subcloned into the corresponding site of GST gene fusion vector pGEX-4T-1 (Amersham Pharmacia Biotech). The resulting plasmid, named pGBACS, permits the production of ACS fused to *Schistosoma japonicum* GST.

Thin Layer Chromatography—To identify acetyl-AMP as a reaction intermediate, TLC analysis was performed. The reaction mixture comprised 100 mM potassium phosphate buffer (pH 7.0), 0.4 mM sodium acetate, 2 mM $MgCl_2$, 0.3 μM [α-^{32}P]ATP and 0.54 μM ACS in a total volume of 25 μl. One μl aliquots of the reaction mixture were spotted onto a PEI-cellulose plate at the indicated time. After that, CoA was added to the final concentration of 0.2 mM. A 1.2 M NaCl solution was used as a developing solvent. The reaction product was analyzed by autoradiography and MALDI-TOF mass spectrometry.

Site-Directed Mutagenesis—Appropriate pairs of mutagenic primers (see Table I) were synthesized and used to generate the mutated ACS by PCR, according to the method of QuickChange site-directed mutagenesis of Stratagene. Here, pGBACS was used as the template for the *Pfu* DNA polymerase (Stratagene). After PCR, the wild-type

TABLE I. **Site-directed acetyl-CoA synthetase gene mutations and corresponding mutagenic oligonucleotides.**

ACS gene allele	Mutagenic primer set[a]
G263I	CCTCTATACCTCAATCTCGACCGGCCAGCCCAA
	CTGGCCGGTCGAGATTGAGGTATAGAGGATGAA
G266I	CTCAGGCTCGACCATCCAGCCCAAGGGCGTGCT
	GCCCTTGGGCTGGATGGTCGAGCCTGAGGTATA
K269G	GACCGGCCAGCCCGGGGGCGTGCTGCACACCTC
	GTGCAGCACGCCCCCGGGCTGGCCGGTCGAGCC
E414Q	CCTGGTGGCAGACCCAGACCGGCGGCATCCTGA
	GATGCCGCCGGTCTGGGTCTGCCACCAGGTATC

[a]Nucleotide sequences corresponding to the mutated amino acid residues are underlined.

parental plasmid remaining in the PCR product was selectively digested with the *Dpn*I restriction enzyme (Roche), and the resultant mixture was used to transform the chemically competent *E. coli* BL21 (DE3) pLysS. The desired mutants were sequenced to identify each mutation and to confirm its fidelity.

Expression and Purification of Wild-Type and Mutant ACS—The expression and purification of the wild-type enzyme were carried out according to the manufacturer's instructions (Amersham Pharmacia Biotech). *E. coli* cells transformed with pGBACS were cultured for 12 h in 3 ml of LB medium containing ampicillin (50 μg/ml), and then transferred to 200 ml of the same medium. The culture was incubated at 37°C for 2 h and isopropyl-1-thio-β-D-galactoside (final conc., 0.5 mM) was added, followed by incubation of the culture at 22°C for 20 h. The cells were harvested, resuspended in 15 ml phosphate-buffered saline (PBS), and then sonicated. The cell lysates were mixed with a Glutathione-Sepharose 4B resin (1.33 ml), and then after incubation for 30 min at 25°C with gentle shaking, the slurry was packed and washed with 10 bed-volumes of PBS. Then, thirty units of thrombin was added to the slurry to separate the GST part of the fusion enzyme. After the slurry had been incubated for 12 h at 22°C with gentle shaking, the enzyme was eluted with 3 bed-volumes of PBS. Each mutant was also expressed and purified under the same conditions as above.

Circular Dichroism Spectroscopy—CD spectroscopy was performed to compare the overall secondary structures of the wild-type and selected mutant proteins. Measurements were made at room temperature from 190 to 250 nm using a Jasco J-720 spectropolarimeter with a 1 mm cell and 0.2 nm wavelength increments. Each spectrum was normalized for protein concentration, and the observed ellipticity (θ) was background-corrected against the spectrum obtained for the dialysis buffer, 10 mM potassium phosphate, pH 7.0.

Enzyme Assays and Kinetic Experiments—There are two different assay methods for the determination of ACS activity (*8*). The acetohydroxamate assay was used for preparation of the enzyme, while the direct spectrophotometric assay, based on measurement of the increase in absorbance at 232 nm resulting from the formation of the thioester bond of acetyl-CoA (ε_{232} = 4.5 × 10^3 M^{-1}·cm^{-1}), was used for kinetic experiments. The standard reaction mixture for the direct spectrophotometric assay comprised the following components in a final volume of 1 ml: 100 mM potassium phosphate buffer (pH 7.0), 0.4 mM sodium acetate, 2 mM $MgCl_2$, 0.5 mM ATP, 0.2 mM CoA, and enzyme. The enzyme concentrations used were 0.054 μM wild-type, 1.0 μM G263I, 0.675 μM G266I, 1.11 μM K269G, and 1.39 μM E414Q, respectively. The first set of initial velocity measurements was made using varying acetate concentrations, and fixed ATP (0.5 mM) and CoA (0.2 mM) concentrations. For the second set of measurements the ATP concentration was varied with fixed acetate (0.4 mM) and CoA (0.2 mM) concentrations. Finally, the CoA concentration was varied with fixed acetate (0.4 mM) and ATP (0.5 mM) concentrations. For wild-type ACS, assays for substrate specificity were performed with fixed concentrations (0.1 mM) of appropriate substrates. The kinetic data were analyzed by constructing Lineweaver-Burk plots of initial velocities.

RESULTS AND DISCUSSION

Cloning and Nucleotide Sequence of the ACS Gene from B. japonicum USDA 110—Degenerate oligonucleotides corresponding to the conserved amino acid sequence for ACS were synthesized and used to clone a 600-bp DNA fragment from *B. japonicum* total DNA. This partial clone was used as a probe to screen a *B. japonicum* total DNA library for full-length clones. The DNA prepared from the positive clones was subjected to sequencing analysis. A 3.5-kb *Bam*HI DNA fragment, which contained the entire gene, was isolated and subcloned into pBluescript KS II (designated as pBACS). The nucleotide sequence of this ACS gene has been deposited in the GenBank database under accession number AF290478. The ACS gene encodes a polypeptide of 648 amino acids with a calculated molecular mass of 71,996 Da, which is almost identical to that of ACS purified from *B. japonicum* bacteroids. A data base search showed that the ACS protein sequence exhibits close homology to most ACS enzymes from organisms as diverse as *A. rhizogenes* (72% identical residues) (*14*), *P. aeruginosa* (67%) (*15*), and *Homo sapiens* (53%) (*16*). Also, the amino acid sequence of this enzyme showed good similarity with those of the acyl-adenylate forming enzymes (*10–12*).

Purification and Characterization of ACS Expressed in E. coli—ACS was purified from a fusion protein, GST-ACS. The GST-ACS bound to Gluthathione-Sepharose 4B resin was treated with thrombin to obtain ACS without GST (Fig. 1). Because three substrates are involved in the ACS reaction, determination of kinetic constants was carried out for the overall reaction. The k_{cat} and K_m values were determined at pH 7.0 and 30°C by carrying out initial velocity experiments in which two of the substrates were each held at a fixed, saturating concentration while the third was varied in the range of (0.5–10) K_m. The results obtained are shown in Table III. The specific activity of the purified recombinant enzyme was 11.69 μmol/min per mg of protein, which was similar to the reported value of 16.1 μmol/min per mg of protein for the ACS purified from *B. japonicum* bacteroids (*1*). However, for the ACS purified from *B. japonicum* bacteroids, the K_m values for acetate, ATP, and CoA were 146, 275, and 202 μM respectively, which differ from the values of 66, 138, and 36 μM for the recombinant

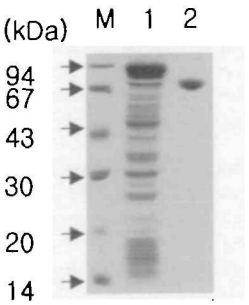

Fig. 1. **SDS/PAGE analysis of ACS purified from the transformed *E. coli*.** Lane M was loaded with the following molecular mass standards: phosphorylase (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa), and α-lactalbumin (14 kDa). Lane 1, crude extracts; lane 2, enzyme from a Glutathione-Sepharose 4B column after treatment with thrombin.

ACS from *E. coli*. The substrate specificity of the recombinant ACS was determined with substrate and substrate analogues (Table II). The activity of the enzyme toward propionate was 61% of that for acetate. Butyrate, malonate, and methylmalonate were also reactive with this enzyme (13, 5, and 11%, respectively).

Identification of Acetyl-AMP as a Reaction Intermediate—As shown in Fig. 2, an autoradiogram after TLC analysis showed three different spots. On MALDI-TOF mass analysis, these spots were identified as ATP, acetyl-AMP, and AMP, respectively. This suggested that the overall reaction of ACS consists of two sequential steps; acetyl-AMP formation (adenylation) and subsequent CoA transfer to form acetyl-CoA (thioesterification step).

Identification of Putative Active Site Residues—In a previous study, ACS purified from *B. japonicum* bacteroids was found not to be inhibit on 0.5 h preincubation with reagents (1 mM each) that modify thiol groups (iodoacetamide), serine (phenylmethanesulphonyl fluoride), or lysine residues (pyridoxal phosphate). However, it was rapidly inhibited by diethyl pyrocarbonate, indicating that histidine and cysteine residues are involved in substrate binding and/or catalysis (*1*).

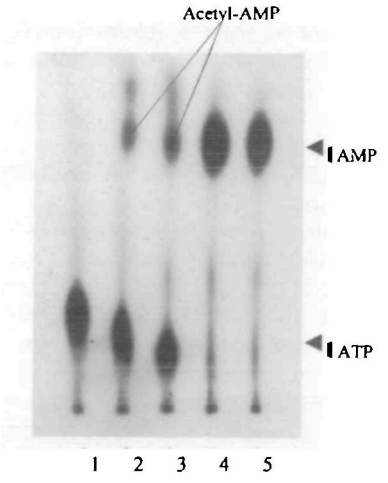

Fig. 2. **Autoradiogram after TLC analysis of the partial reaction product with acetyl-CoA synthetase using [α-^{32}P]ATP.** The reaction mixture contained [α-^{32}P]ATP and acetate as substrates. After 10 min, 20 μl aliquots were taken and mixed with CoA. The reaction mixture was separately incubated and 1 μl aliquots were spotted onto a PEI-cellulose plate at the indicated time. Lane 1, control (no enzyme); lanes 2 and 3, after 4 min and 8 min incubation in the absence of CoA, respectively; lanes 4 and 5, after 4 min and 8 min incubation in the presence of CoA, respectively.

Acetyl-CoA synthetase belongs to the acyl-adenylate/thioester-forming enzyme family. The sequence homology among acyl-adenylate–forming enzymes is known to be typically low (approx. 20–30%) (*17*). However, stretches of sequence that are highly conserved in all of these enzymes were identified. The most easily recognized sequence is the **T**[SG]-**S**[G]-**G**-[ST]-**T**[SE]-**G**[S]-X-**P**[M]-**K**-**G**[LF] signature motif (motif I) (the boldface letters represent residues that predominate at that position, with alternatives given in brackets; X represents a hypervariable position). A second highly conserved sequence stretch is **Y**[LWF]-**G**[SMW]-X-**T**[A]-**E** (motif II), and the third motif is **Y**[FL]-**R**[KX]-**T**[SV]-**G**-**D** (motif III) (*9*). In a recent study involving site-directed mutagenesis, it was found that motif I and motif II of the *B. japonicum* malonyl-CoA synthetase may function primarily in malonyl-adenylate formation (*10*). Steady-state kinetic data for the MCS E308Q mutant also suggested that motif II plays a role in binding the carboxylate substrate. As shown in Fig. 3, amino acid sequence comparison among *B. japonicum* ACS and adenylate-forming enzymes revealed the following consensus sequences, 261-TSGTTGQPK269 (corresponding to motif I), 410WWQ TE414 (corresponding to motif II), and 493YFTGD497 (corresponding to motif III). However, no histidine or cysteine residue was found in these conserved motifs. Considering the sequence comparison described above, G263I, G266I, K269G, and E414Q were prepared and then subjected to steady-state kinetic studies.

Construction, Purification, and Characterization of Mutant Enzymes—The mutant enzymes were successfully produced in *E. coli* BL21 (DE3) pLysS and purified from GST-fused proteins with treatment of thrombin under the same conditions as used for purification of the wild-type ACS (Fig. 4). Their CD spectra in the far UV region were similar

TABLE II. **Substrate specificity of acetyl-CoA synthetase purified from transformed *E. coli*.** In order to determine the substrate specificity, the formation of the thioester bond was monitored spectrophotometrically at 232 nm (ε_{232} = 4.5 × 10^3 M^{-1}·cm^{-1}) in the presence of substrates or substrate analogues. The standard reaction mixture comprised the following components in a final volume of 1 ml: 100 mM potassium phosphate buffer (pH 7.0), 2 mM $MgCl_2$, 0.5 mM ATP, 0.2 mM CoA, 0.054 μM enzyme, and 0.1 mM of an appropriate substrate.

Substrates and their analogues	Activity (μmol/min)	Relative rate (% of control)
Acetate	11.69	100
Propionate	7.09	61
Butyrate	1.51	13
Malonate	0.58	5
Methylmalonate	1.29	11

Fig. 3. **Amino acid sequences of three conserved regions in CoA ligases.** ACS Bj is ACS from *Bradyrhizobium japonicum*. ACS Pa is ACS from *Pseudomonas aeruginosa*. (*9*). MCS Bj is MCS from *B. japonicum*. MCS (*10*). Rt is MCS from *Rhizobium trifolii* (*11*). CBA. lig. P is 4-CBA:CoA ligase from *Pseudomonas* sp. (*9*). CBA. lig. Ar is 4-CBA:CoA ligase from *Arthrobacter* sp. (*21*). LCFA. lig. H is long chain fatty acid CoA ligase from *Haemophilus influenzae* Rd (*22*). LCFA. lig. E is long chain fatty acid CoA ligase from *Escherichia coli* (*23*). White letters on a black background indicate the amino acid residues that correspond to Gly-263, Gly-266, Lys269, and Glu414 of acetyl-CoA synthetase from *B. japonicum* examined in this experiment.

	^{260}YTS●ST●QP^{269}	^{410}WWQT^{414}	^{493}YFTGD^{497}
ACS.Bj	YTS●ST●KP■	WWQT■	VRKGD
ACS.Pa	YTS●ST●KP■	WWQT■	VRKGD
MCS.B	YTS●TT●RS■	YGMT■	FITGD
MCS.Rt	YTS●TT●RS■	YGMT■	FISGD
CBA.lig.P	YTS●TT●LP■	YGTT■	FRKAG
CBA.lig.Ar	YTS●TT●LP■	YGTT■	YRTGD
LCFA.lig.H	YTG●TT●VA■	YGMT■	MATGD
LCFA.lig.E	YTG●TT●AP■	YGLS■	LHTGD

to that of the wild-type enzyme (Fig. 5), indicating that essentially no major change in the overall conformation had occurred in the mutant enzymes. The activities of the mutant enzymes as to acetyl-CoA formation were drastically reduced compared with that of the wild-type ACS. Among the mutants, the specific activity of the G263I mutant was below the detection level (≤0.3% of the original activity).

Steady-State Kinetic Analysis of Mutant ACS Enzymes—According to the proposed mechanism, the overall reaction of ACS consists of two sequential steps; acetyl-AMP formation (adenylation) and subsequent CoA transfer to form acetyl-CoA (thioesterification step). The conservation of motifs I and II by all acyl-adenylate forming enzyme family members suggests that the two motifs may contribute to the ATP binding site, since ATP is a substrate common to all members (*8, 9*).

Steady-state kinetic analysis of the mutant enzymes showed that mutations of three residues, Gly266, Lys269, and Glu414, resulted in ACS with significant catalytic defects (Table III). The k_{cat} value, which is 4.85 s^{-1} for the wild-type, is reduced 27-fold for the G266I mutant, 16-fold for the K269G mutant, and 17-fold for the E414Q mutant (Table III). Thus, Gly266 and Lys269 in motif I, and Glu414 in motif II were shown to be important for catalysis of the overall reaction. Motif I mutants G266I and K269G showed relatively normal K_m values for CoA, and slightly increased K_m values for acetate (1.9 fold and 3.2-fold increases, respectively) and ATP (4.0- and 1.5-fold increases, respectively), with much lower k_{cat} values for each substrate (Table III). On the other hand, steady-state kinetic analysis of motif II mutant E414Q revealed normal K_m values for ATP and CoA but a high K_m value for acetate, suggesting a possible role of motif II in binding the carboxylate substrate.

Comparison of the Kinetic Properties of ACS Site-Directed Mutants with Those of Malonyl-CoA Synthetase and 4-CBA:CoA Ligase Mutants—In several ATP-binding proteins of known structure (*13–20*), the motif I consensus

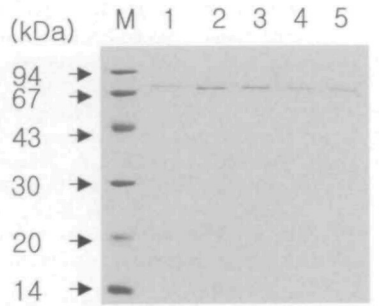

Fig. 4. **SDS/PAGE of the wild-type and mutant ACS enzymes purified from extracts of the *E. coli* transformants.** Lane 1, wild-type; lane 2, G263I; lane 3, G266I; lane 4, K269G; lane 5, E414Q.

TABLE III. **Steady-state kinetic constants for acetyl-CoA synthetase activities of the wild-type and mutant ACS enzymes.** In order to determine the enzyme activity, the formation of the thioester bond of acetyl-CoA was monitored spectrophotometrically at 232 nm. The reaction mixture was prepared as described under "MATERIALS AND METHODS."

	Acetate	ATP	CoA
Wild type			
K_m (μM)	66.4 ± 2.6	138 ± 13	36.1 ± 2.0
k_{cat} (s^{-1})	2.33 ± 0.31	5.46 ± 0.42	6.76 ± 1.6
k_{cat}/K_m (mM^{-1} s^{-1})	35.1	39.6	187
G266I			
K_m (μM)	129 ± 9.4	549 ± 3.6	32.6 ± 2.6
k_{cat} (s^{-1})	0.11 ± 0.07	0.34 ± 0.04	0.09 ± 0.19
k_{cat}/K_m (mM^{-1} s^{-1})	0.837	0.619	2.76
K269G			
K_m (μM)	215 ± 5.5	201 ± 13	22.4 ± 6.3
k_{cat} (s^{-1})	0.35 ± 0.19	0.40 ± 0.07	0.20 ± 0.10
k_{cat}/K_m (mM^{-1} s^{-1})	1.63	2.06	8.93
E414Q			
K_m (μM)	2020 ± 10	146 ± 7.3	62.0 ± 6.8
k_{cat} (s^{-1})	0.27 ± 0.10	0.07 ± 0.01	0.50 ± 0.12
k_{cat}/K_m (mM^{-1} s^{-1})	0.13	0.48	8.06

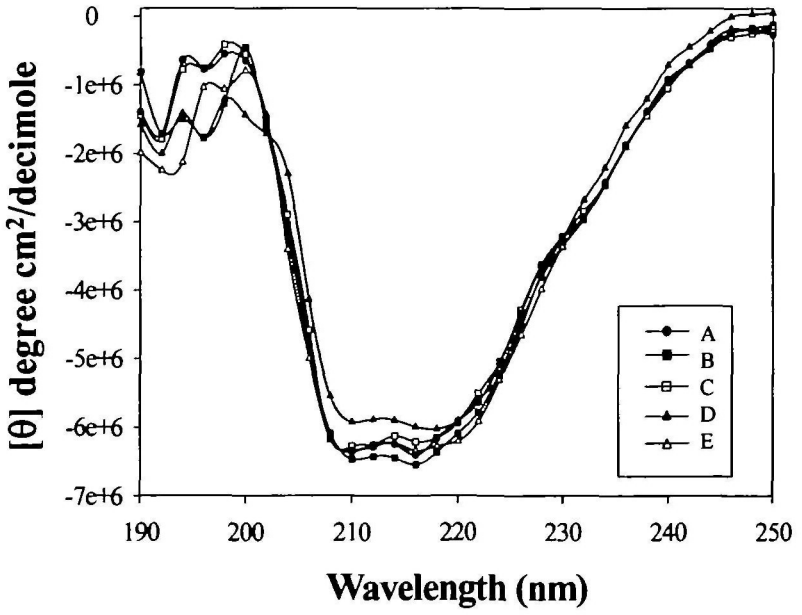

Fig. 5. **Circular dichroism spectra of the wild-type and mutant ACS enzymes.** The spectra were recorded in 10 mM potassium phosphate, pH 7.0, in the far-UV region between 190 and 250 nm at 25°C. Spectra of protein samples (approximately 0.3 mg/ml) were measured in a 300 μl quartz cell of 1 mm path length. A, B, C, D, and E represent the wild-type, G263I, G266I, K269G, and E414Q, respectively.

sequence, T[SG]-S[G]-G-[ST]-T[SE]-G[S]-X-P[M]-K-G[LF], was proposed to be related to the phosphate-binding loop. In the case of 4-CBA: CoA ligase, in which the first and second glycine residues of motif I (G163 and G166 corresponding G266 and G269 in ACS, respectively) were separately replaced with isoleucine residues, the catalytic activity was also significantly reduced (*9*). In *B. japonicum* malonyl-CoA synthetase (MCS), replacement of the MCS Lys175 (Lys269 in ACS) of motif I resulted in only a 3.8-fold decrease in the k_{cat}/K_m value for CoA compared to that of the wild type, whereas the values for malonate and ATP were reduced 51- and 17-fold, respectively (*10*). Also, it was suggested that Lys175 participates in the formation of malonyl-AMP (*10*).

In the case of ACS motif I mutants G266I and K269G, catalytic activity was significantly reduced. Also, these mutants showed relatively normal K_m values for CoA, and increased K_m values for acetate and ATP (Table III). This suggests that glycine residues in the ACS motif I might play a role in the formation of acetyl-AMP.

Motif II, which is highly conserved in all of the sequences representing the acyl-adenylate/thioester-forming enzyme family, contains the stringently conserved glutamate residue. In the case of replacement of *B. japonicum* MCS Glu308 (Glu414 in ACS), the k_{cat}/K_m value for malonate was reduced 2,080-fold, whereas the k_{cat}/K_m values for ATP and CoA were 296- and 46-fold lower than that of the wild type. This suggested that Glu308 might play a role in malonate binding. Also, in *Pseudomonas* 4-CBA:CoA ligase, the K_m value for 4-CBA of the E306Q mutant (Glu414 in ACS) was significantly increased, while those for ATP and CoA were slightly changed, suggesting a possible role of motif II in binding the carboxylate substrate. In the case of ACS, steady-state kinetic analysis of the E414Q mutant revealed a significantly reduced k_{cat}/K_m value for acetate (270-fold decrease), and slightly changed k_{cat}/K_m values for ATP and CoA (83-fold and 23-fold decrease, respectively), suggesting a possible role of motif II in binding acetate.

In summary, based on the results of both mutational and biochemical studies, we propose that Gly266 and Lys269, residing in motif I, are important for the formation of acetyl-AMP as a reaction intermediate and that Glu414 is crucial for the binding of acetate. These findings could help us to understand the mechanism of other enzymes belonging to the acyl-adenylate/thioester-forming enzyme family.

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