The active site and substrates binding mode of malonyl-CoA synthetase determined by transferred nuclear Overhauser effect spectroscopy, site-directed mutagenesis, and comparative modeling studies

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Abstract

The active sites and substrate bindings of *Rhizobium trifolii* malonyl-CoA synthetase (MCS) catalyzing the malonyl-CoA formation from malonate and CoA have been determined based on NMR spectroscopy, site-directed mutagenesis, and comparative modeling methods. The MCS-bound conformation of malonyl-CoA was determined from two-dimensional-transferred nuclear Overhauser effect spectroscopy data. MCS protein folds into two structural domains and consists of 16 α -helices, 24 β -strands, and several long loops. The core active site was determined as a wide cleft close to the end of the small C-terminal domain. The catalytic substrate malonate is placed between ATP and His206 in the MCS enzyme, supporting His206 in its catalytic role as it generates reaction intermediate, malonyl-AMP. These findings are strongly supported by previous biochemical data, as well as by the site-directed mutagenesis data reported here. This structure reveals the biochemical role as well as the substrate specificity that conservative residues of adenylate-forming enzymes have.

Keywords: adenylate-forming enzyme; malonyl-CoA; malonyl-CoA synthetase; molecular modeling; NMR

Rhizobium trifolii malonyl-CoA synthetase (MCS), which is encoded with a polypeptide of 504 residues, catalyzes malonyl-CoA formation directly from malonate and CoA with the hydrolysis of ATP (Kim et al., 1993). The MCS enzyme in *Bradyrhizobium japonicum* has demonstrated a high substrate specificity for malonate, ATP, and CoA. It has been suggested that MCS plays a critical role in nitrogen flow from bacteroids to plant cells with malonamidases (Kim & Chae, 1990). In addition, the steady-state kinetic mechanism of MCS from *R. trifolii* and *B. japonicum* was determined as ordered in a Bi Uni Uni Bi Ter Ter system, based on studies of initial velocity, product inhibition, and intermediate identification (Kim & Kang, 1994). The kinetic parameters of MCS for the three substrates, malonate, CoA, and ATP were measured at 200, 87, and 33.3 μ M, respectively. Data from chemical modification experiments have also supported the hypothesis that histidine, lysine, arginine, and tryptophan residues could be responsible for catalytic activity in MCS (Lee & Kim, 1993a). Especially, it has been proposed that histidine residue could play a critical role in not only ATP binding but also malonate binding (Lee & Kim,

1993b). Lysine residues have also been identified as critical residues in malonate binding during the initial stage of enzymatic reaction, and tryptophan as participating only in CoA binding (Lee & Kim, 1993a, 1993b). The sequence database suggests that the enzymes forming acyl-adenylates have three conserved motifs closely related to their substrate binding and enzymatic activities.

Recently, the crystal structure of firefly luciferase that catalyzes the formation of the adenylate of dehydroluciferin in vitro has been determined by X-ray crystallography as being folded into two compact domains: a large N-terminal domain and a small C-terminal domain (Conti et al., 1996). The conserved residues among the superfamily of adenylate-forming enzymes were also found on the surface of the wide cleft formed by two structural domains. In addition, the crystal structure of the phenylalanine-activating subunit of gramicidin S synthetase 1 (PheA) has also been determined as a ternary complex form of substrates, phenylalanine, and AMP (Conti et al., 1997). PheA also folds into two domains showing a topology similar to that of firefly luciferase. The C-terminal domain of PheA loops back toward the N-terminal region separated by a disordered loop near the active sites. These two structures provided information about the structural role of the conservative residues of adenylate-forming enzymes such as MCS. Very recently, the solution structure (Jung et al., 1999) as well as the

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modeling conformation (Kang & Han, 1997) for free malonyl-CoA based on ab initio calculations has been reported. However, despite the biochemical evidence for MCS as having a different catalytic activity and substrate binding properties (An et al., 1999) compared with luciferase and PheA, no structural information related to either MCS itself or substrate binding has yet been reported. Here, we present the detailed structural model for active sites and substrate binding of MCS based on NMR spectroscopy, site-directed mutagenesis, and comparative modeling calculations.

Results

NMR assignment and the solution structures of malonyl-CoA

Both proton and carbon chemical shifts of free- and enzyme-bound malonyl-CoA (Fig. 1A) were easily assigned by heteronuclear multiple bonds correlated spectroscopy (HMBC) (Bax & Summers, 1986) and double quantum-filtered COSY (DQF-COSY) (Rance et al., 1983) spectra. The structures of free and bound malonyl-

Fig. 1. **A:** Molecular structure of malonyl-CoA. Atoms are labeled as three groups that are adenine, ribose, and pantetheine, respectively. **B:** One-dimensional ^{31}P -NMR spectra during titration of MCS to malonyl-CoA. The initial NMR sample for the titration was made of 2 mg of malonyl-CoA in 400 μL of PBS buffer. The final sample ratio was 50:1.2:1 (malonyl-CoA:AMP:MCS). **C:** 600 MHz NOESY spectra of both free and MCS-bound malonyl-CoA with mixing time 600 ms. Some intramolecular NOEs between neighboring group are displayed. NOEs that are not detected in MCS-bound form are marked as boxes. (Figure continues on next page.)

Fig. 1. Continued.

CoA were determined from nuclear Overhauser effect (NOE) data based on two-dimensional (2D) NOE spectroscopy (NOESY) (Jeener et al., 1979) and rotating frame NOE spectroscopy (ROESY) (Bax & Davis, 1985). A series of one-dimensional ^{31}P -NMR spectra for malonyl-CoA was also collected during MCS titration (Fig. 1B). The ^{31}P NMR resonances of the adenosine group were easily assigned because of their coupling patterns and characteristic chemical shifts. The downfield shift of a single $3'$ phosphorous resonance as well as upfield shifts of two α, β phosphorous peaks are strong indications of malonyl-CoA binding to the MCS enzyme (Fig. 1A,B). Distance restraints for malonyl-CoA were also deduced from 2D transferred NOESY (Clare & Gronenborn, 1982) data in the presence of MCS protein (Fig. 1C). Figure 2A shows intramolecular NOE contacts of malonyl-CoA based on transferred NOE experiments. Fifth structures were generated using the experimental constraints and ten lowest energy $\langle \text{SA} \rangle_k$ (simulated annealing) structure were displayed for structural analysis (Fig. 2B). The calculated structures show that the adenosine moiety as well as pantetheine groups are well defined; however, high flexibilities are observed in the malonate part due to the lack of experimental constraints for this region (Fig. 2B). It is also observed that the conformation of a pantetheine arm is very similar to that of the coenzyme A bound to the chloramphenicol acetyltransferase enzyme (Barsukov et al., 1996).

Description of a modeling structure for malonyl-CoA synthetase

Figure 3A summarizes the amino acid sequence alignments, folding-threading, and secondary structural elements of MCS together with firefly luciferase and PheA. Table 1 lists the structural statistics calculated by different protein analysis programs that were used to

evaluate final structure of MCS. The Ramachandran plot demonstrates that 97% of residues are found in the structurally allowed regions (Fig. 3B). Especially, it is observed that the general topology of MCS resembles that of both firefly luciferase and PheA in its having 16 α -helices (helix A-P) and 24 β -strands (strands 1–24), as shown in the topology diagram (Fig. 3C). The global fold of MCS shows that the distance between the N- and C-terminal domains is shorter than that in luciferase. The core active site is found in a wide cleft between two structural domains linked by a short flexible loop comprising residues of 396–400. The pocket for substrate binding consists of twisted $\alpha\beta$ structural motif, which is located near three α -helices wrapped by a single two-stranded β -sheet (Fig. 4A,B). A small cavity surrounded by two antiparallel β -strands and a single α -helix is also forming this active site containing a flexible loop comprising residues of Tyr160–Ala171, which are involved in AMP binding. Interestingly, all conserved residues for the AMP binding motif among different ligases were found in this loop region (Fig. 4C).

The ATP binding mode of MCS

The conserved ATP binding site comprising residues of Ser274–Leu280 and Ile378–Gly383 was easily identified (Fig. 5A). During modeling calculations, loops generated by Ser274–Leu280 and Ile378–Gly383 are considered to be involved in ATP binding; this demonstrates that dramatic conformational transitions occur upon ATP docking. Several possible intermolecular hydrogen bonds between ATP and MCS enzymes were clearly identified. Mutagenesis data based on our modeling structure have clearly revealed that Arg168, Lys170, and His206 residues are definitively involved in ATP binding via the electrostatic interactions between side chains and ligand (Fig. 6). Our structure demonstrated that the residues responsible for both catalysis and reaction intermediate generation were located about 4–5 Å apart from the ATP molecule. The side chains of the Lys170 and Lys487 residues were placed to effect direct hydrogen bonding with the phosphate oxygens in ATP (Fig. 5A). In addition, the backbone carbonyl oxygens of Ser379, Gly380, and Arg395 residues are closely located to the adenosine ring of ATP. The carboxyl group of Glu303 is involved in an

Table 1. Structure validation of malonyl-CoA synthetase

Procheck	
Residue in allowed region of Ramachandran plot	97%
G-factor ^a	
Overall	−0.23
Dihedral angle	−0.14
Covalent	−0.40
Whatif ^b	
2nd generation packing Z-score	−3.244
Prosa ^c	
Combined energy	−171.06
Z-score rank	1

^aG-factor that is equal or greater than −0.5 is good.

^bWhatif's Z-score should be between −4.0 and 4.0. For the packing score, the score of a good structure is above −1.3

^cProsa calculates the energy of model using the backbone potential from the library.

Fig. 2. **A:** NOE diagram for malonyl-CoA in the presence of MCS. The observed NOEs are summarized, and its relative bonding distances are arranged with the sequence of atoms. Off-diagonal squares denote medium- and long-range NOEs. **B:** Solution structures of malonyl-CoA in the presence of MCS. A stereoview of the 10 (SA)_k structures is displayed.

extensive network of charge-charge interaction with both Mg^{2+} and phosphate group of ATP.

The implication of malonate binding

A malonate binding site has been suggested from both a primary sequence alignment and preliminary biochemical studies. Because

the amino acid sequences of a malonate binding site are well conserved among those of the MCS family, we expected that the helix F, comprising residues of Leu135–Arg140, would be specifically responsible for malonate binding. Our MCS structure in the absence of substrate shows that malonate should be located near helix F. In our results, a significant structural change upon malonate binding was also observed for the Arg480–Gln489 loop next

Fig. 3. A: The sequence alignment and folding-threading of malonyl-CoA synthetase with firefly luciferase and gramicidin S synthetase 1. The alignment was based on structural homology of three enzymes. The secondary structural elements of MCS determined by the algorithm of Kabsch and Sanders using DSSP program (Kabsch & Sander, 1983) are indicated as H (helix, in black) and S (strand, in gray). Residues conserved within the superfamily of adenylate-forming enzymes as well as AMP binding motif are marked as boxes. **B:** Distribution of all Φ, Ψ dihedral angle values for the final modeling structure of MCS. The glycine residues are represented by triangles. This figure was produced with PROCHECK (Laskowski et al., 1993). **C:** Topological diagram demonstrating the secondary structural arrangement of MCS. The secondary structures are represented as gray circles (helices) and arrows (strands), respectively.

to helix O. A small loop comprising the residues Thr162–Arg170 also demonstrated an outward structural reorganization in malonate binding. During the calculation for the MCS/ATP/malonate complex, the malonate was trapped in-between His206 and a pocket formed by both ATP and MCS; this implies that His206 is important in the catalytic reaction needed to make reaction intermediate malonyl-AMP.

The CoA binding mode

The adenylate group of CoA interacts with both a loop of Thr333–Ala340 and helix M. The flexible pantetheine group shows hydrophobic interactions with not only helix M but also Lys398. Recently, we reported that the chemical modification of tryptophan residues caused a profound decrease of CoA binding (Lee & Kim, 1993b).

Fig. 4. **A:** Stereoview of the lowest energy structure of MCS. The residues are marked with numbers. **B:** Model of the malonyl-CoA synthetase drawn in ribbon plot. This figure was generated using the program MOLSCRIPT (Kraulis, 1991) and RASTER3D (Merritt & Bacon, 1997). **C:** Electropotential surface of MCS displaying some of the conserved residues in the active site. The negative electrostatic potential is represented in red, the positive in blue, and the neutral in white. The surface was generated with GRASP program (Nicholls et al., 1991). Clustering of positive charged residues including Lys170 and Arg168 is clearly observed in the substrate binding pocket.

Fig. 5. A: Stereoview of ATP binding site with the residues involved in ATP binding. **B:** Molecular model of malonyl-CoA/AMP/MCS complex. The structure was generated from NMR structure of malonyl-CoA combined with MCS model.

The preliminary analysis performed by us suggested that Trp361 residue could be involved in CoA binding via hydrophobic interaction. Our modeling structure shows that both Trp361 and Phe376 residues are not likely to interact directly with CoA. Instead, the side chains of Phe376, Lys366, and Ile378 combined with Trp361 residue allow an extensive network of hydrophobic interactions with CoA molecule. Therefore, we assume that the chemical modification of tryptophan residue may disrupt the hydrophobic network and thus change the local conformation of CoA binding site.

Mutagenesis data and modeling structure

Figure 6 shows the relative activities of four mutant types of MCS compared to a wild-type. It is clear that the mutagenesis data strongly supports the structural model presented here. The replace-

ment of Arg168 by Gly168 (R168G) reduces 63% of enzyme activity, and the Lys170 mutant (K170M) retains only 35% of its activity. The double mutant protein (R168G + K170M) shows a dramatic decrease in enzyme activity, retaining only 12% of its activity (Fig. 6). This result indicates that Arg168 and Lys170 could play a synergetic role in MCS activity. Especially, the H206L mutant lost most of its malonate binding activity, showing only a 2.4% binding. This result could be clearly explained using our structural model demonstrating that His206 has been directly involved in not only malonate binding but also ATP binding (Fig. 5A).

Discussion

Even though the sequence homologies of three proteins as shown in Figure 3A are relatively low [29.5% (luciferase) and 16%

Fig. 6. Specific activity plot of wild-type and mutant proteins. Enzyme activities for three substrates were measured by spectrophotometric assay method. The reaction mixture for activity measurement contained 8 mM sodium malonate, 2 mM MgCl_2 , 0.4 mM ATP, 0.2 mM CoA, and 0.2 mM enzyme in 100 mM potassium phosphate buffer at pH 7.0. The temperature was maintained at 30 °C during measurements.

(gramicidin S synthetase 1)], they have conserved their common AMP binding motifs by distributing sharing common conserved amino acids among these proteins. In addition, all these enzymes generate an AMP molecule as a final reaction product; this implies that they have similar reaction mechanisms. As a result, these proteins can be classified as a family of adenylate-forming enzymes, like long-chain fatty acid ligase. However, PheA does not have a histidine residue at its active site like other adenylate forming enzymes do. Therefore, we propose that the unique binding mode as well as substrate specificity of malonate to MCS enzyme would mainly come from His206 residue, as shown in mutagenesis data. A recent study for malonate binding mode as well as the stereo specificity of malonate has been examined by us, and it showed that MCS retained catalytic activity for methylmalonate. Even though methylmalonate is a prochiral molecule, when CoA is ligated on one carboxyl group, it turns to be chiral. In this manner, only (S)-methylmalonyl-CoA is produced by MCS (Y.S. Kim & J.H. An, unpubl. obs.). These results suggest that the methyl group of methylmalonate should be located apart from ATP to reduce steric hindrance, whereby only one binding mode would be available, in which an (S)-conformation of methylmalonyl-CoA is formed. The low energy conformation of an MCS/malonyl-CoA/AMP complex (Fig. 5B) showed a significant rearrangement around ligand binding site to accommodate optimum binding of ligands. The adenylate group of malonyl-CoA has close proximity to a short loop of the residues Thr333–Ala340 and helix-L; moreover, the pantetheine part of CoA directly interacts with the side chain of Lys398. The helix P is also considered to be involved in the coordination of an AMP molecule (Fig. 5B). In addition, Arg168 residue is found near the final enzymatic product, malonyl-CoA; this thereby supports supporting mutagenesis data (Fig. 6). Therefore, we conclude that our findings would provide a structural basis for the interpretation of the biochemical data of the MCS enzyme as well as further structural understanding of other adenylate/thioester-forming enzymes.

Materials and methods

The purification and characterization of malonyl-CoA synthetase

Vector transformed *Escherichia coli* was cultured in LB broth and the cell was harvested by centrifugation and resuspension in Tris-HCl buffer (pH 7.4). The enzyme was purified by the three chromatography steps DEAE-Shepecel, Affi-Gel Blue, and hydroxyapatite column. A linear gradient of 0–0.5 M NaCl (in Tris-HCl buffer) was applied to elute proteins. The enzyme activity at each peak of the profile was assayed as in the below method, and the fractions showing activity were pooled and dialysed with Tris-HCl buffer. Affi-gel blue column was followed using the same procedure with above except the salt gradient from 0 to 1 M. Hydroxyapatite column was equilibrated with phosphate buffer, and the protein solution was loaded. After a sample was loaded, the eluted solution was immediately collected and assayed. To monitor the activity of the enzyme, the reaction product of chemical coupling was measured. The reaction buffer contains 100 mM KH_2PO_4 , 200 mM NH_2OH , 40 mM sodium-malonate, 500 μM CoA, 10 mM ATP, and 10 mM MgCl_2 at pH 7.0. The MCS enzyme was added to the reaction buffer and the mixed solution was incubated for 30 min at 37 °C. The reaction was quenched by a 5% TCA, 0.7% HCl, and 10% FeCl_3 solution. The FeCl_3 detected the reaction product and the malonylhydroxamate concentration was measured by optical absorbance at 540 nm. The pooled solution after the activity test was concentrated for NMR experiments.

Site-directed mutagenesis

Point mutations were introduced by an overlap extension of the polymerase chain reaction (PCR) approach (Pan & McEver, 1993). The PCR products of the mutant proteins were cloned in pGEX-2T vector (Pharmacia, Buckinghamshire, UK). The expression and purification of GST-fused mutant MCS were carried out according to the manufacture's instructions. The amino acid sequences of the mutant proteins were confirmed by DNA sequencing. The activities of the mutant MCS were assayed by the methods described previously.

NMR sample preparation

A total of 3 mg of malonyl-CoA synthetase enzyme was dissolved in 195 μL in PBS buffer solution at pH 7.4. For substrate titration, 50 μL of enzyme stock solution was added to malonyl-CoA up to a total volume of 150 μL of protein solution. The final molar ratios between enzyme and substrate for NMR measurements were 1:200, 1:100, and 1:66.7, respectively. One-dimensional ^1H and ^{31}P spectra were collected during titration.

NMR spectroscopy

All NMR experiments were performed on either a Bruker DMX600 or an AMX500 spectrometer in quadrature detection mode equipped with an SGI INDY computer. Most of the data were acquired at a temperature of 25 °C, and pulsed-field gradient techniques combined with a water-gated pulse sequence were used for solvent suppression. One-dimensional spectra were collected for malonyl-CoA with the titration of MCS enzyme. All 2D NMR measurements were performed on both free and enzyme-bound malonyl-

CoA. For free malonyl-CoA, ROE mixing times of 50–400 ms were used for ROESY experiments. Two-dimensional NOESY experiments with mixing times of 100–600 ms were also performed for both free and enzyme-bound forms of malonyl-CoA. DQF-COSY spectra were collected in a 90% H₂O/10% ²H₂O solution. All NMR data were recorded in the phase-sensitive mode using the time-proportional phase increment method, with 2,048 data points in the acquisition domain and 256 in the time domain. HMBC spectroscopy data were also collected.

All data were transferred to a SGI Indigo² workstation and processed using nmrPipe (Biosym/Molecular Simulations Inc., San Diego, California) or Bruker XWIN-NMR (Bruker Instruments) software. The DQF-COSY spectra were processed to 8,192 × 1,024 data matrices to obtain a maximum digital resolution for the measurement of coupling constants (Marion & Wüthrich, 1983).

The structure calculation of malonyl-CoA

NMR structures of enzyme-bound malonyl-CoA were calculated using hybrid distance geometry and a dynamical simulated annealing procedure (Nilges et al., 1988a, 1988c; Driscoll et al., 1989; Lee et al., 1994) on X-PLOR 3.1 (Brünger, 1993). The initial structure of malonyl-CoA was generated by adding malonate moiety to the sulfonyl end of CoA deduced from the X-ray crystal coordinates of succinyl-CoA synthetase (Protein Data Bank (PDB) code: 1CTS). Here, topology and parameter files were modified for malonyl-CoA. Interproton distance constraints were derived from both NOESY experimental data based on NOE build-up curves. NOE intensities were converted to three distance ranges, strong (1.8–2.7 Å), medium (1.8–3.3 Å), and weak (1.8–5.0 Å). The upperbound distances for methyl and methylene protons were calibrated for pseudoatom correction. The coupling constant information for ribose and pantetheine protons was also used for the final stage of modeling calculations.

Homology modeling of malonyl-CoA synthetase

Homology modeling of Malonyl-CoA synthetase was based on the sequence alignment of MCS in both firefly luciferase and gramicidin S synthetase 1 using the FASTA (<http://www2.ebi.ac.uk/fasta3/>) program. The sequence alignment for a structurally conserved region was performed using the structural data extracted from the X-ray coordinates of firefly luciferase (PDB code: 1LCI) and the phenylalanine activating subunit of gramicidin S synthetase 1 (PDB code: 1AMU). Secondary structures of malonyl-CoA synthetase were predicted by PredictProtein server at EMBL (<http://www.embl-heidelberg.de/predictprotein/predictprotein.html>). The initial template structures of malonyl-CoA synthetase were generated for only heavy atoms. The modeling structures were further optimized by combined use of conjugate gradient energy minimization and molecular dynamics calculations in the MODELLER program (Säli et al., 1995). Structurally variable regions were engineered by the combined use of PDB coordinate searching and fragment fitting routine, and then further relaxed by 1,000 steps of conjugate-gradient energy minimization until its energy derivation reached to 0.001 kcal/mol. The molecular dynamics simulation of 10 ps at 298 K was also performed in the MODELLER program. After all the coordinates were assigned, a conformational search for side chains was made using a side-chain rotamer library. In addition, a local structure refinement procedure was also employed for each structural segment by the successive use of energy min-

imization and molecular dynamics simulations. Especially, an extensive refinement procedure was manipulated for the active site loop comprising residues from Thr162 to Lys170. All local refinements including energy minimization were effected by regulating the distortions of bond and angle. Rather than using something constant for electrostatic energy calculations, a distance-dependent dielectric term for mimicking solvent effects was tried. The final MCS structure was evaluated by PROCHECK (Laskowski et al., 1993), PROSA (Sippl, 1993), and WHATIF (Vriend & Sander, 1993) programs. The quality of the modeling structure has been proven sufficiently good for ligands fitting.

The molecular modeling of MCS/ATP/malonate complex

A bound conformation of ATP was generated from the X-ray coordinate of gramicidin S synthetase 1 complexed with ATP. After MCS was superimposed with gramicidin S synthetase 1, an AMP molecule was docked to MCS. To regulate the binding site of MCS, all the atoms of MCS within a 15 Å range from ATP were selected for refinement. The regulation procedure is as follows: first, energy minimization was performed until RMS energy deviation is <0.01 kcal/mol. Then, 5 ps of molecular dynamics was performed at 300 K, followed by final energy minimization. Because the binding site of MCS was generated from that of luciferase, a high steric hinderance arose due to the smaller binding pocket of MCS. To overcome this steric problem, the refinement procedure was repeated twice. A similar procedure was applied to substrate malonate also. The binding mode of malonyl-CoA to MCS was also determined by applying exactly the same procedure used for AMP.

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