Identification of residues essential for a two-step reaction by malonyl-CoA synthetase from *Rhizobium trifolii*

Jae Hyung AN, Gha Young LEE, Jin-Won JUNG, Weontae LEE and Yu Sam KIM¹

Department of Biochemistry, College of Science, Bioproducts Research Centre, Yonsei University, Seoul 120-749, Korea

Malonyl-CoA synthetase (MCS) catalyses the formation of malonyl-CoA in a two-step reaction consisting of the adenylation of malonate with ATP followed by malonyl transfer from malonyl-AMP to CoA. In order to identify amino acid residues essential for each step of the enzyme, catalysis based on chemical modification and database analysis, Arg-168, Lys-170, and His-206 were selected for site-directed mutagenesis. Glutathione-S-transferase-fused enzyme (GST-MCS) was constructed and mutagenized to make R168G, K170M, R168G/K170M and H206L mutants, respectively. The MCS activity of soluble form GST-MCS was the same as that of wild-type MCS. Circular dichroism spectra for the four mutant enzymes were nearly identical to that for the GST-MCS, indicating that Arg-168, Lys-170 and His-206 are not important for conformation but presumably for substrate binding and/or catalysis. HPLC analy-

sis of products revealed that the intermediate malonyl-AMP is not accumulated during MCS catalysis and that none of the mutant enzymes accumulated it either.

Kinetic analysis of the mutants revealed that Lys-170 and His-206 play a critical role for ATP binding and the formation of malonyl-AMP, whereas Arg-168 is critical for formation of malonyl-CoA and specificity for malonyl-AMP.

Molecular modelling based on the crystal structures of luciferase and gramicidin S synthetase 1 provided MCS structure which could fully explain all these biochemical data even though the MCS model was generated by comparative modelling.

Key words: chemical modification, kinetics, molecular modelling, site-directed mutagenesis.

INTRODUCTION

An important role of malonate metabolism in the symbiotic *Rhizobium trifolii* has been proposed recently [1]. Malonyl-CoA synthetase (MCS), which catalyses the formation of malonyl-CoA from malonate and CoA with the hydrolysis of ATP to AMP and PPi, plays a key role in this pathway. This enzyme was first isolated and characterized from *Bradyrhizobium japonicum* and subsequently from *R. trifolii* [2,3]. Steady-state kinetics and product-inhibition studies of MCS from *B. japonicum* and *R. trifolii*, respectively, suggested that both enzymes follow an ordered Bi Uni Uni Bi Ping Pong Ter Ter mechanism [4,5]. The formation of malonyl-AMP, proposed as an intermediate in the kinetically deduced mechanism, was confirmed by the analysis of ³¹P-NMR spectra of the product AMP isolated from an ¹⁸O transfer experiment using [¹⁸O]malonate [4,5]. According to the mechanism the overall reaction consists of two sequential steps, malonyl-AMP formation and the subsequent CoA transfer to form malonyl-CoA, as shown below.

The *R. trifolii* MCS structural gene (*matB*) has been cloned and sequenced, it encodes a protein of 504 amino acid residues [1]. This enzyme shares, as expected, amino-acid-sequence similarities with the family of acyl-adenylate-forming enzymes [1]. The sequence homology between the enzymes forming acyl-adenylates is known to be typically low (approx. 20–30%) [6,7]. However, three motifs were suggested to be conserved throughout this class of enzymes, as follows:

motif 1: T[SG]-S[G]-G[ST]-T[SE]-G[S]-X-P[M]-K-G[LF]

motif 2: Y[LWF]-G[SMW]-X-T[A]-E

motif 3: Y[FL]-R[KX]-T[SV]-G-D

(the boldface letters represent residues that predominate at that position, with alternatives given in brackets; X represents a hypervariable position). Recently, two conserved motifs present in the *Pseudomonas* sp. strain CBS3 4-chlorobenzoate:coenzyme A (4-CBA:CoA) ligase (motif 1, 161T-S-G-T-T-G-L-P-K-G170, and motif 2, 302Y-G-T-T-E306) were tested for their possible role in substrate binding and/or catalysis. The site-directed mutant G163I, G166I, P168A, K169M and E306Q ligases showed significantly reduced catalytic activity for the adenylation of 4-chlorobenzoate, suggesting that these two motifs are located at the active site [7].

The crystal structure of *Photinus pyralis* luciferase, another member of the enzyme family forming adenylates, was reported, but without bound substrates or other ligands [6]. The protein has a unique fold consisting of a large N-terminal domain (residues 1–436) and a small C-terminal domain (residues 440–550). A majority of the conserved residues are clustered on the surfaces of the two domains opposite each other, suggesting this as a probable location of the active site. It was proposed that a peptide (244HHGF247) obtained through photo-inactivation by 2-(4-benzoylphenyl)-thiazole-4-carboxylic acid (BPTC), a luciferin analogue, may be located at or near the luciferin-binding site [8]. It was subsequently reported that luciferase His-245 mutants showed extremely low specific activity, suggesting that the histidine residue plays a critical role in the active site [9].

In the present study, we have used site-directed mutagenesis to examine the active site of *R. trifolii* MCS. Chemical modification of MCS from *R. trifolii* with 2,3-butanedione, pyridoxal-5'-phosphate, and diethylpyrocarbonate provided evidence that arginine, lysine and histidine residues of the enzyme located at or near the active site are essentially involved in catalysis and/or substrate binding [10]. However, little is known about the exact location of the amino acid residues in the active site. Here, Arg-

Abbreviations used: MCS, malonyl-CoA synthetase; GST, glutathione-S-transferase; CD, circular dichroism; HPLC, high-performance liquid chromatography.

¹ To whom correspondence should be addressed (e-mail yskim@bubble.yonsei.ac.kr).

168 and Lys-170 of MCS in the conserved motif 1 of acyl-adenylate-forming enzymes and His-206 in another motif, respectively, were selected for site-directed mutagenesis. Each of the mutant enzymes has been purified and characterized with respect to its kinetic parameters for substrates and analysed reaction products. The results reported here indicate that Lys-170 and His-206 are involved in the formation of malonyl-AMP, whereas Arg-168 is critical for the formation of malonyl-CoA from malonyl-AMP.

EXPERIMENTAL

Materials

The protein fusion and purification system was obtained from Pharmacia Biotech. Restriction endonucleases were obtained from Boehringer-Mannheim. T4 DNA ligase and calf intestinal phosphatase were obtained from New England Biolabs. The plasmid purification kit was purchased from Qiagen Inc. The synthetic oligomers were purchased from Bioneer Co., Korea. ATP, GTP, CTP, AMP, CoA, malonyl-CoA, methylmalonyl-CoA, sodium methylmalonate and sodium malonate were obtained from Sigma.

Construction of expression vector inserting glutathione-S-transferase fused malonyl-CoA synthetase (GST-MCS)

The vector pGEX-2T (Pharmacia, UK) was digested with *Bam*HI and *Sma*I. The MCS gene was prepared by PCR from *R. trifolii* genomic DNA using 5' and 3' primers containing *Bam*HI and blunt sites at each end, respectively (Table 1).

Thirty cycles of amplification were performed using the following temperatures: 95 °C for 30 s; 58 °C for 40 s; 72 °C for 30 min 30 s. The amplification produce was digested with *Bam*HI. The insert and vector were ligated overnight and the plasmid was introduced into *E. coli* by electroporation. The resulting construct encoded a chimeric protein with an N-terminal GST domain followed by a C-terminal MCS.

Site-directed mutagenesis

Mutations were created by an overlap extension polymerase chain reaction (PCR) method [11]. Two partial MCS genes encoding each MCS mutant were separately synthesized by PCR from the *R. trifolii* genomic DNA using overlapping antisense or sense oligonucleotides and the 5' or 3' primer (Table 1). The two

PCR products were mixed, then the second PCR was performed using the 5' and 3' primers. The products were digested with *Bam*HI and subcloned into the *Bam*HI and *Sma*I sites of pGEX-2T. The sequence of the mutant was verified by DNA sequencing.

Expression and affinity purification of GST-MCS and GST-MCS mutants

The expression and purification of GST-fused enzymes were carried out according to the manufacturer's instructions (Pharmacia, UK). GST-MCS and its mutants were expressed in *E. coli* strain BL21 (DE3) pLys. The strains were induced by the addition of 1 mM isopropyl-1-thio- β -D-galactopyranoside (IPTG), and the cells were harvested and sonicated. Bacterial cell lysates were loaded onto a Glutathione Sepharose 4B RediPack. Fusion proteins bound to the matrix were eluted with 10 mM glutathione. The purified proteins were dialysed against 0.14 M NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄ (pH 7.3) buffer and stored at 4 °C.

Protein concentration and SDS/PAGE

Concentrations of protein were determined by the method of Bradford [12], with the Bio-Rad protein assay dye. SDS/PAGE was performed according to the method of Laemmli [13].

K_m and k_{cat} measurement

There are four different assay methods for the determination of MCS activity [14]. While the direct spectrophotometric assay, based on the measurement of the increase in absorbance at 232 nm, resulting from the formation of the thioester bond of malonyl-CoA ($\epsilon_{232} = 4.5 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$) was used for kinetic experiments, the malonohydroxamate assay was used for the preparation of the enzymes. The standard reaction mixture for direct spectrophotometric assay contained the following components in a final volume of 1 ml: 100 mM potassium phosphate buffer (pH 7.0, 30 °C), 8 mM sodium malonate, 2 mM MgCl₂, 0.4 mM ATP, 0.2 mM CoA and enzyme. The enzyme concentrations used were 0.037 μM wild type, 0.084 μM R168G, 0.093 μM K170M, 0.28 μM R168G/K170M, and 0.74 μM H206L. The first set of initial velocity measurements were made using varying malonate concentrations and fixed ATP (0.4 mM) and CoA (0.2 mM) concentrations. For the second set of measurements the ATP concentration was varied at fixed malonate (16 mM) and CoA (0.2 mM) concentrations. Finally, the CoA concentration was varied at fixed malonate (16 mM) and ATP (0.2 mM) concentrations. For GST-MCS and mutant enzymes, assays for substrate specificity were run at fixed concentrations of the appropriate substrates. The enzyme concentrations used for methyl MCS activity were 0.074 μM wild type, 0.067 μM R168G, 0.3 μM K170M. The sets of initial velocity measurements for methyl MCS activity were made using methylmalonate in place of malonate. The kinetic data were analysed by constructing Lineweaver-Burk plots of initial velocities.

HPLC analysis of products from GST-MCS and mutant enzymes

In order to confirm that malonyl-CoA is the only product formed when using malonate as a substrate and to know whether the intermediate, malonyl-AMP, is accumulated, the reactions were carefully monitored by HPLC at 254 nm. Reaction mixtures (0.25 ml, 37 °C) were prepared for product analysis. Each reaction contained 100 mM potassium phosphate buffer (pH 7.0), 40 mM sodium malonate (or sodium methylmalonate),

Table 1 Oligonucleotides used for site-directed mutagenesis

The mutated nucleotides are shown in bold.

Mutants	Oligonucleotide sequences
GST-MCS	5'-GAGGGGATCCGTGAGCAACCATCTTTTCG-3' 5'-AGAGCGCGGTGCGCTTACGTCCTGG-3'
R168G	5'-GAACGACGGGAG G GCTCCAAGGGGGCGATGCTC-3' 5'-GCATCGCCCCCTTGGAGC C TCCCGTCGTTCCG-3'
K170M	5'-GAACGACGGGACGCTCCATGGGGGCGATGCTCAC-3' 5'-GTGAGCATCGCCCC A TGGAGCGTCCCGTCGTTCC-3'
R168G/H206L	5'-GAACGACGGGAG G GCTCCATGGGGGCGATGCTC-3' 5'-GCATCGCCCC A TGGAGC C TCCCGTCGTTCCG-3'
H206L	5'-GCCTTGCCGATCTTCTC A CGCATGGAGTGTTCG-3' 5'-CGAAGAGTCCATGCGT G AGGAAGATCGGCAAGGC-3'

10 mM MgCl₂, 5 mM ATP, 5 mM CoA and 0.075 μ M GST-MCS (or mutant enzymes). The incubation time of assays for MCS activity was 10 min, whereas the incubation time of assays for methyl MCS activity was 50 min. Acyl-CoAs were separated on an analytical reverse-phase column using a linear gradient of acetonitrile in 0.1 M potassium phosphate, pH 5.2 [15]. HPLC separations were carried out using a column (4.6 mm internal diameter $\times$ 250 mm) of 5 μ m Eclipse XDB-C₁₈ (Hewlett–Packard). Reaction products (2 μ l) were injected on to the column, which had been equilibrated with 1.2% (v/v) acetonitrile in 0.1 M potassium phosphate buffer (pH 5.2) and developed at 0.5 ml/min. This buffer composition was maintained for a further 5 min, and then the acetonitrile concentration was increased linearly over the next 45 min to 12% (v/v). Reference compounds, ATP, AMP, malonyl-CoA, CoA and methylmalonyl-CoA, were calibrated to elute at retention times of around 5, 6, 15, 19 and 21 min, respectively, monitoring absorbance at 254 nm.

Circular dichroism spectroscopy

CD spectroscopy was used to compare the overall secondary structure of wild-type and mutant proteins. All sample proteins eluted from the glutathione Sepharose 4B column were dialysed against 10 mM potassium phosphate buffer, pH 7.3, in order to avoid interference. CD spectra were recorded from 260 to 190 nm at 25 °C on a Jasco J-715 spectropolarimeter (Jasco Corp., Tokyo, Japan) set at 50 mdeg sensitivity, 0.5 nm resolution, 5 unit accumulation, 1 s response and a scanning speed of 200 nm/min. Spectra of protein samples (approx. 0.3 mg/ml) were measured in a 400 μ l quartz cell of 1 mm path length.

Molecular modelling

For homology modelling of MCS, both firefly luciferase (PDB code: 1LCI) [6] and the phenylalanine-activating subunit of gramicidin S synthetase 1 (PDB code: 1AMU) [16] were used for alignment with MCS by the FASTA (<http://www2.ebi.ac.uk/fasta3/>) program. The sequence alignment for homology modelling was based on the structural superposition of the firefly luciferase and gramicidin S synthetase 1. The PredictProtein server at EMBL predicted secondary structures of MCS. Starting from alignment results, the MCS structure was generated by the MODELLER program [17]. The whole structures were fully optimized by conjugate gradient energy minimization and molecular dynamics with simulated annealing procedure in the MODELLER program. This procedure was repeated to generate five refined structures. All calculated structures were evaluated in pairs and the lowest energy structure was selected for further analysis. Since the loop between Thr-162 and Lys-170 was found to distort in both luciferase and gramicidin S synthetase 1, an extensive refinement procedure to define this loop was carried out using loop generation procedure. After finishing all local refinements, a final energy minimization procedure for the overall structure was followed to control bond and angle distortions. The protein evaluation programs, PROCHECK [18], WHATIF [19] and PROSA [20] further confirmed the final MCS structure. The bound conformation of the ATP molecule was generated from X-ray coordinates of ATP complexed with gramicidin S synthetase 1. To refine binding sites of substrates, the atoms in the reaction zone were selected for molecular dynamics simulation. The binding modes of malonyl-CoA and malonate were determined by applying a similar procedure.

RESULTS

Identification of putative active site residues

It was previously reported that chemical modification of the *R. trifolii* malonyl-CoA synthetase with pyridoxal-5'-phosphate (PLP), 2,3-butanedione, and diethylpyrocarbonate (DEPC) inhibited MCS, indicating that Lys, Arg, and His residues are involved in substrate binding and/or catalysis [10].

Amino acid sequence comparison between *R. trifolii* MCS and adenylate-forming enzymes provided the consensus sequence, 161YTSGTTGRSK170, which is a putative ATP/AMP binding motif including arginine and lysine (Figure 1). In this sequence Lys-170 is conserved in all cases, whereas Arg-168 is not. However, Arg-168 in addition to Lys-170 was also selected for the site-directed mutagenesis, since an arginine residue was chemically modified and Arg-168 was the sole residue in a putative ATP/AMP binding motif. His-206, which is equivalent to the His-245 proposed as an essential residue for the efficient formation of a luciferyl-AMP intermediate in luciferase [9], was also selected for amino acid replacement (Figure 1).

Expression and purification of GST-MCS and GST-fused mutant enzymes

The pGEX-2T expression vector was used to express MCS as a fusion protein with GST in *E. coli* BL21 (DE3) pLys. The recombinant GST-MCS was purified from soluble *E. coli* extracts by the affinity-chromatography procedure. From 40 ml of culture induced with 1 mM IPTG, the total yield of GST-MCS was 8 mg, representing a 3.7-fold purification. The homogeneity of the purified GST-MCS was judged by SDS/PAGE showing a single 80 kDa protein band which corresponds to the predicted molecular mass of 80.5 kDa of the GST-MCS fusion protein (results not shown). MCS activity of the GST-MCS was the same as that of the enzyme purified from *R. trifolii*, suggesting that GST attached to the N-terminus of MCS does not interfere with the activity. The R168G, K170M, R168G/K170M and H206L mutants were created by an overlap extension PCR method [11].

MCS, Rt	¹⁶¹ YTSGTTGRSK ¹⁷⁰	²⁰³ PIFH ²⁰⁶
CBA, lig. P	YTSGTTGLPK	PLYH
CBA, lig. Ar	YTSGTTGLPK	PIHH
Luci. Lc	NSSGSTGLPK	PFHH
Luci. Pp	NSSGSTGLPK	PFHH
LCFA, lig. H	YTGGTTGVAK	PLYH
LCFA, lig. Aa	YTSGTTGNPK	PFHH
Coum, lig. Pt	YSSGTTGLPK	PLFH
Coum, lig. Gm	FSSGTTGLPK	PLFH

Figure 1 Amino acid sequences of two motifs conserved through CoA ligases and luciferases

MCS.Rt is MCS from *Rhizobium trifolii* [1]. CBA.lig.P is 4-CBA:CoA ligase from *Pseudomonas* sp. [22]. CBA.lig.Ar is 4-CBA:CoA ligase from *Arthrobacter* sp. [23]. Luci.Lc is luciferase from *Luciola cruciata* [24]. Luci.Pp is luciferase from *Photinus pyralis* [25]. LCFA.lig.H is long chain fatty acid CoA ligase from *Haemophilus influenzae* Rd [26]. LCFA.lig.Aa is long chain fatty acid CoA ligase from *Aquifex aeolicus* [27]. Coum.lig.Pt is coumarate:CoA ligase from *Pinus taeda* [28]. Coum.lig.Gm is coumarate:CoA ligase from *Glycine max* [29]. The bold **R**, **K**, and **H** represent residues within the *R. trifolii* MCS subjected to site-directed mutagenesis.

Table 2 Steady-state kinetic constants for MCS activities of the wild type and mutant GST-MCS enzymes

Assay conditions were as described in the Experimental section.

	Malonate	ATP	CoA
Wild type			
K_m (μM)	208 ± 6	52 ± 4.5	46 ± 3.2
k_{cat} (s^{-1})	19 ± 0.3	20 ± 0.4	21 ± 1.1
k_{cat}/K_m ($\text{mM}^{-1} \cdot \text{s}^{-1}$)	91	384	456
R168G			
K_m (μM)	452 ± 42	63 ± 0.9	133 ± 22
k_{cat} (s^{-1})	11 ± 0.5	12 ± 0.1	13 ± 1.2
k_{cat}/K_m ($\text{mM}^{-1} \cdot \text{s}^{-1}$)	24	190	98
K170M			
K_m (μM)	2600 ± 110	76 ± 2.4	54 ± 5.7
k_{cat} (s^{-1})	9.0 ± 1.1	9.0 ± 0.2	9.2 ± 1.6
k_{cat}/K_m ($\text{mM}^{-1} \cdot \text{s}^{-1}$)	3.5	118	170
R168G/K170M			
K_m (μM)	5600 ± 200	106 ± 3	74 ± 0.5
k_{cat} (s^{-1})	3.8 ± 0.1	2.9 ± 0.1	3.2 ± 0.2
k_{cat}/K_m ($\text{mM}^{-1} \cdot \text{s}^{-1}$)	0.68	27	43
H206L			
K_m (μM)	8700 ± 980	59 ± 4.6	7.8 ± 3.4
k_{cat} (s^{-1})	0.53 ± 0.28	0.38 ± 0.10	0.55 ± 0.17
k_{cat}/K_m ($\text{mM}^{-1} \cdot \text{s}^{-1}$)	0.11	6.4	71

All mutant enzymes were cloned in the pGEX-2T vectors and expressed in *E. coli* BL21 (DE3) pLys used for expression of the wild-type enzyme described above. The mutants were also purified from soluble *E. coli* extracts by the affinity-chromatography procedure (results not shown). All mutant enzymes were soluble and their yield was high (7.6 mg of R168G, 8.4 mg of K170M, 8.1 mg of R168G/K170M and 8.4 mg of H206L GST-fused mutant enzyme per 40 ml of liquid culture, respectively).

Circular dichroism spectroscopy

As described above CD spectroscopy was used to compare the overall secondary structures of wild-type and selected mutant proteins. Mutations at positions 168, 170, 168/170, 206 do not appear to be associated with structural changes, as the CD spectra indicate no substantial differences from that of the wild type (results not shown). Evidently, this result suggests that Arg-168, Lys-170 and His-206 are not critical for structure but important for substrate binding and/or catalysis.

Steady-state kinetic analysis

Michaelis constants of *R. trifolii* GST-MCS were $208 \pm 6 \mu\text{M}$, $52 \pm 4.5 \mu\text{M}$ and $46 \pm 3.2 \mu\text{M}$ for malonate, ATP, and CoA, respectively (Table 2), which is similar to those for the wild-type MCS of *R. trifolii* and *B. japonicum* [2,3].

The k_{cat} value of the H206L mutant was approx. 40-fold lower than that of the wild type, whereas the k_{cat} values of the R168G, K170M and R168G/K170M mutants were reduced 1.7-, 2.2- and 6-fold, respectively (Table 2). The k_{cat}/K_m value for CoA of the R168G mutant was 4.7-fold lower than that of the wild type, whereas the k_{cat}/K_m values for malonate of the K170M, R168G/K170M and H206L mutants were reduced to 3.8-, 144- and 827-fold, respectively. The k_{cat}/K_m values for ATP of K170M, R168G/K170M, and H206L mutants were reduced 3.3-, 14- and 60-fold, respectively.

Figure 2 Methyl MCS activity of the wild-type and mutant GST-MCS enzymes

Methyl MCS activity was measured by direct spectrophotometric assay ($\epsilon_{232} = 4.5 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$). The reaction mixture contained the following components in a final volume of 1 ml: 100 mM potassium phosphate buffer (pH 7.0, 30 °C), 8 mM sodium methylmalonate, 2 mM MgCl_2 , 0.4 mM ATP, 0.2 mM CoA and enzyme.

Table 3 Steady-state kinetic constants for methyl MCS activities of the wild type and mutant GST-MCS enzymes

Assay conditions were as described in the Experimental section.

	Methylmalonate	ATP	CoA
Wild type			
K_m (μM)	373 ± 19	20 ± 0.4	8.1 ± 1.2
k_{cat} (s^{-1})	2.7 ± 0.1	2.7 ± 0.1	3.3 ± 0.2
k_{cat}/K_m ($\text{mM}^{-1} \cdot \text{s}^{-1}$)	7.2	135	407
R168G			
K_m (μM)	2240 ± 80	58 ± 4.3	17 ± 3.5
k_{cat} (s^{-1})	3.0 ± 0.1	3.0 ± 0.1	3.1 ± 0.1
k_{cat}/K_m ($\text{mM}^{-1} \cdot \text{s}^{-1}$)	1.3	52	182
K170M			
K_m (μM)	4100 ± 100	72 ± 7.8	7.8 ± 1.1
k_{cat} (s^{-1})	0.59 ± 0.01	0.60 ± 0.05	0.56 ± 0.1
k_{cat}/K_m ($\text{mM}^{-1} \cdot \text{s}^{-1}$)	0.14	8.3	72

Substrate specificity

The activities of the wild-type and mutant proteins for various substrates were measured. None of the mutant enzymes showed any thioester-forming activity with acetate, propionate, or succinate in place of malonate, or with GTP or CTP in place of ATP. Though the activity of the R168G mutant for malonyl-CoA synthesis showed only 65% activity compared to that of wild type, the R168G mutant enzyme showed slightly higher methyl MCS activity than the wild type (Figure 2).

Determination of kinetic parameters for methylmalonyl-CoA formation

As the kinetic parameters for malonyl-CoA formation, the k_{cat} and K_m values for methylmalonyl-CoA formation were determined by carrying out initial velocity experiments (Table 3).

Figure 3 Schematic diagram of important residues for ATP binding

His-206 and Lys-170 as well as other charged side chains near the ATP molecule were demonstrated to display molecular interactions with ATP. Hydrogen bonding between MCS and ATP are drawn based on molecular modelling.

The k_{cat} value of the R168G mutant was similar to that of wild type, but the k_{cat}/K_m values of the R168G mutant were significantly lower than that of wild type. The kinetic parameters by the R168G/K170M and H206L mutant enzymes were not able to be determined due to low methyl MCS activity.

Identification of the reaction products

The reaction products of the wild type and mutant proteins for MCS and methyl MCS activities can be easily resolved by HPLC. Malonyl-CoA and methylmalonyl-CoA were eluted at around 15 and 21 min, respectively (results not shown). As expected, the chromatographs of the reaction products were similar to results of the spectrophotometric assay and no accumulation of intermediate malonyl-AMP was observed. In malonyl-CoA formation, the activities of the R168G, K170M, R168G/K170M and H206L mutants were 0.81, 0.62, 0.3 and 0.07 times that of the wild type, respectively, and in methylmalonyl-CoA formation they were 0.92, 0.31, 15 and 0.08 times that of the wild type, respectively (results not shown). The specific activity of the R168G mutant for methyl MCS shown in Figure 2 was higher than that of wild type. However, the methylmalonyl-CoA peak of the R168G mutant reaction products was smaller than that of the wild type. This result can be explained by the fact that the k_{cat}/K_m value of R168G mutant is lower than that of the wild type.

Three-dimensional structure of MCS

The general fold of the modelled structure resembles those of both firefly luciferase and gramicidin S synthetase 1. The three-dimensional structure of MCS showed that the distance between the N-terminal domain of MCS is shorter than that of luciferase. Since malonate, which is the specific substrate for MCS, is smaller than luciferin (the substrate of firefly luciferase), the substrate binding pocket would be also smaller than that of luciferase. The secondary structural elements of MCS were determined to be 15 helices and 18 strands, including many turns. The core activity region was found in the N-terminus near a wide cleft, which is characterized by two structural domains. The C-terminus of the protein forms a small separate $\alpha\beta$ structure, and three α -helices in the small C-terminal domain are surrounded by a two-stranded β -sheet. The major active site regions are surrounded by two anti-parallel β -strands and a single α -helix. All of the residues conserved among the ligase family based on sequence homology are easily identified [6]. The binding site for ATP consists mainly of two loops comprising residues of 274–277 and 378–383. Several hydrogen bonds were also observed between ATP and the protein. Figure 3 shows a schematic diagram of molecular interactions between enzyme and ATP molecule. Mg^{2+} was also incorporated into the model and displayed. The side chain of Glu-303 has been observed to be involved in a charge–charge interaction with Mg^{2+} . The side chains of Lys-487 and Lys-170 show possible hydrogen bonds with ATP and the

Figure 4 Active site and binding pocket of MCS generated by molecular modelling

(A) ATP, malonate and critical residues (Arg-168, Lys-170 and His-206) of MCS enzyme are displayed. (B) AMP and malonyl-CoA molecules are found to have close molecular interactions with Arg-168, Lys-487 and His-206. The substrates were drawn in stick model and side chains in ball and stick model. These figures were prepared with MOLSCRIPT [30] and Raster3D [31].

backbone carbonyl oxygens of Gly-380, Ser-379 and Glu-297 are proven to be good candidates for hydrogen bond acceptors. The critical residues His-206 and Arg-168 for both catalysis and reaction intermediate are positioned at a 4–5 Å distance from ATP (Figure 3). Since the malonate is the unique substrate for the formation of the malonyl-AMP, its location should be near both the ATP molecule and the catalytic residue His-206. Our model clearly supports this hypothesis, demonstrating that the substrate malonate is found between ATP and His-206 (Figure 4A). Previously, chemical modification results suggested that tryptophan residues might be involved in CoA binding [21]. However, our model points to an alternative notion, namely that the majority interaction of the protein with CoA is through hydrophobic forces between CoA and residues Phe-376, Lys-366 and Ile-378 (Figure 4B).

DISCUSSION

The k_{cat} values for malonyl-CoA formation by the R168G, K170M, R168G/K170M and H206M mutant enzymes, which were constructed according to suggested chemical modifications and sequence homology, were reduced 1.7-, 2.2-, 6.1- and 40-fold, respectively (Table 2). The CD spectrum of the wild type was nearly identical to those of the mutant forms. These results indicate that the residues Arg-168, Lys-170 and His-206 influence not the conformation of the protein but substrate binding and/or catalysis.

We reported previously that the MCS reaction consists of an adenylation step followed by a thioesterification step, and malonyl-AMP is formed as an intermediate [4,5]. Further, we proposed that MCS catalysed the reaction by an ordered Bi Uni Uni Bi Ping Pong Ter Ter mechanism as follows:

where Mal is malonate, Mal-CoA is malonyl-CoA, and Mal-AMP is malonyl-AMP.

In the present study, the comparison of the k_{cat}/K_m values between the wild type and the mutant enzymes showed that the R168G mutant had a dramatic decrease in CoA affinity, whereas the K170M, R168G/K170M and H206M mutant enzymes had a decrease in affinity for malonate and ATP. This result suggests that Lys-170 and His-206 participate in the formation of malonyl-AMP, whereas Arg-168 participates in the late stage of MCS activity.

Both wild-type and mutant GST-MCS demonstrate methyl MCS activity. Surprisingly, the R168G mutant had the highest specific activity for the methyl MCS reaction among the wild type and mutant enzymes, whereas the activity of the R168G mutant for malonyl-CoA synthesis showed only 65% activity compared to that of wild type. HPLC analyses showed that the peak of methylmalonyl-CoA formed in the reaction catalysed by the wild type is slightly higher than that with R168G mutant. From the steady-state kinetic data of the methyl MCS reaction, we also found that the k_{cat}/K_m values measured for three substrates with the R168G mutant are significantly lower than those for the wild type (Table 3). However, the k_{cat} value of the R168G mutant is somewhat higher than that of the wild type. These results indicate that the methyl MCS activity of the R168G mutant at saturating concentrations of substrates is relatively higher than that of the wild type, whereas the methyl MCS

activity of the R168G mutant at low concentrations of substrates is relatively lower than that of the wild type.

Mutational studies of 4-CBA:CoA ligase, a member of the acyl-adenylate-forming enzymes, have been reported [7]. The 4-CBA:CoA ligase K169M (Lys-170 in MCS) mutation resulted in a 4-fold drop in catalytic activity and significant accumulation of the 4-CBA-AMP intermediate. Moreover, the K_m measured for CoA with the K169M (Lys-170 in MCS) mutant was unchanged while those for 4-CBA and ATP were significantly increased. These results suggested that the mutation at Lys-169 (Lys-170 in MCS) inhibited the adenylation partial reaction and not the thioesterification partial reaction. With respect to the reduction of catalytic activity and the change of the K_m values, the K170M MCS mutant is similar to the K169M 4-CBA:CoA ligase mutant. However, MCS and 4-CBS:CoA ligase utilize different acyl substrates, malonate and 4-chlorobenzoic acid, respectively. Therefore, it is strongly suggested that the lysine is responsible for the binding and/or hydrolysis of ATP.

Recently, the structure functions of luciferase, which is a family of the acyl-adenylate-forming enzymes, have been reported [9]. The specific activity of the luciferase H245D (His-206 in MCS) mutant was approx. 300-fold lower than that of wild type. The molecular modelling studies demonstrated that the imidazole side chain of His-245 (His-206 in MCS) is located in the vicinity of a luciferin carboxylate oxygen and the γ -phosphate of ATP. They reported that a histidine at position 245 of luciferase (His-206 in MCS) is important for the efficient formation of a luciferyl-AMP intermediate or assists in positioning luciferyl-AMP. The H206L mutant of MCS showed the lowest specific activity among the four mutants. On the other hand, while the MCS H206L mutant showed a 6.4-fold reduction of the k_{cat}/K_m for CoA, it showed 830- and 60-fold reductions of the k_{cat}/K_m for malonate and ATP, respectively. Considering the biochemical data of mutant H206L and an ordered Bi Uni Uni Bi Ping Pong Ter Ter mechanism reported previously, we suggested that His-206 is critical for the formation of malonyl-AMP.

Three-dimensional structure of MCS from molecular modelling resembles the general fold of both luciferase and gramicidin S synthetase 1. It is interesting to note that the topology of the N-terminal domain of MCS is very similar to that of luciferase, whereas the C-terminal region is closest to that of gramicidin S synthetase 1. It is also clear that our model explains all mutagenesis results presented here. Especially, the role of Lys-170 is explained by the MCS/ATP complex structure as shown in Figure 3. Since the charged side chain of Lys-170 helps to establish an optimum orientation of the ATP molecule by a favourable electrostatic interaction with a phosphate group of ATP, the Lys-170 mutant demonstrates only 45% activity compared with that of the wild type. Therefore, we suppose that Lys-170 would lock the ATP molecule and keeps this molecule during the initial catalytic steps of MCS. In addition, the catalytic role of His-206 is clearly explained by our structure (Figures 3 and 4) and steady-state kinetic data of the H206L mutant protein (Table 2). In the modelling structure, His-206 is placed near both the malonate and ATP molecules, demonstrating that it participates in substrate binding as well as catalytic procedure. Especially, Figure 4B shows that Arg-168 is oriented close to final enzymic product, malonyl-CoA, supporting the view that Arg-168 could be involved in the generation of malonyl-CoA.

From previous work and the mutational, biochemical and molecular modelling studies, we propose that Lys-170 is important for the binding of ATP and His-206 catalyses the formation of malonyl-AMP as a reaction intermediate. In addition, Arg-168 would assist in the formation of malonyl-CoA from malonyl-AMP. We believe that our findings of the MCS

substrate-binding kinetics could provide a key to the understanding of other adenylate/thioester-forming enzyme mechanisms.

We thank Professor Heinz G. Floss for critical review of the manuscript and comments. This work was supported by a grant (97-05-01-06-01-5) from the Korea Science and Engineering Foundation.

REFERENCES

- An, J. H. and Kim, Y. S. (1998) *Eur. J. Biochem.* **257**, 395–402
- Kim, Y. S. and Chae, H. Z. (1991) *Biochem. J.* **273**, 511–516
- Kim, Y. S., Kwon, S. J. and Kang, S. W. (1993) *Korean Biochem. J.* **26**, 176–183
- Kim, Y. S. and Kang, S. W. (1994) *Biochem. J.* **297**, 327–333
- Kang, S. W., Hong, S. Y., Ryoo, H. D., Rhyu, G. I. and Kim, Y. S. (1994) *Bull. Korean Chem. Soc.* **15**, 394–399
- Conti, E., Franks, N. P. and Brick, P. (1996) *Structure* **4**, 287–298
- Chang, K. H., Xiang, H. and Dunaway-Mariano, D. (1997) *Biochemistry* **36**, 15650–15659
- Thompson, J. F., Geoghegan, K. F., Lloyd, D. B., Lanzetti, A. J., Magyar, R. A., Anderson, S. M. and Branchini, B. R. (1997) *J. Biol. Chem.* **272**, 19359–19364
- Branchini, B. R., Magyar, R. A., Murtiashaw, M. H., Anderson, S. M. and Zimmer, M. (1998) *Biochemistry* **37**, 15311–15319
- Lee, S. C. and Kim, Y. S. (1993) *Korean Biochem. J.* **26**, 286–290
- Pan, J. and McEver, R. P. (1993) *J. Biol. Chem.* **268**, 22600–22608
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Kim, Y. S. and Bang, S. K. (1988) *Anal. Biochem.* **170**, 45–49
- Roughan, G. (1994) *Biochem. J.* **300**, 355–358
- Conti, E., Stachelhaus, T., Marahiel, M. A. and Brick, P. (1997) *EMBO J.* **16**, 4174–4183
- Sali, A., Potterton, L., Yuan, F., van Vlijmen, H. and Karplus, M. (1995) *Proteins* **23**, 318–326
- Laskowski, R. A., MacArthur, M. W., Moss, D. S. and Thornton, J. M. (1991) *J. Appl. Crystallogr.* **26**, 283–291
- Vriend, G. and Sander, C. (1993) *J. Appl. Crystallogr.* **26**, 47–60
- Sippl, M. J. (1993) *J. Comput.-Aided. Mol. Des.* **7**, 473–501
- Lee, S. C. and Kim, Y. S. (1993) *Korean Biochem. J.* **26**, 378–382
- Babbitt, P. C., Kenyon, G., Martin, B. M., Charest, H., Sylvestre, M., Scholten, J. D., Chang, K.-H., Liang, P.-H. and Dunaway-Mariano, D. (1992) *Biochemistry* **31**, 5594–5604
- Schmitz, A., Gartemann, K.-H., Fiedler, J., Grund, E. and Eichenlaub, R. (1992) *Appl. Environ. Microbiol.* **58**, 4068–4071
- de Wet, J. R., Wood, K. V., DeLuca, M., Helinski, D. R. and Subramani, S. (1987) *Mol. Cell. Biol.* **7**, 725–737
- Masuda, T., Tatsuta, H. and Nakano, E. (1989) *Gene* **77**, 265–270
- Fleischmann, R. D., Adams, M. D., White, O., Clayton, R. A., Kirkness, E. F., Kerlavage, A. R., Bult, C. J., Tomb, J., Dougherty, B. A., Merrick, J. M., McKenney, K., Sutton, G. G., FitzHugh, W., Fields, C. A., Gocayne, J. D., Scott, J. D., Shirley, R., Liu, L. I., Glodek, A., Kelley, J. M., Weidman, J. F., Phillips, C. A., Spriggs, T., Hedblom, E., Cotton, M. D., Utterback, T., Hanna, M. C., Nguyen, D. T., Saudek, D. M., Brandon, R. C., Fine, L. D., Fritchman, J. L., Fuhrmann, J. L., Geoghegan, N. S., Gnehm, C. L., McDonald, L. A., Small, K. V., Fraser, C. M., Smith, H. O. and Venter, J. C. (1995) *Science* **269**, 496–512
- Deckert, G., Warren, P. V., Gaasterland, T., Young, W. G., Lenox, A. L., Graham, D. E., Overbeek, R., Snead, M. A., Keller, M., Aujay, M., Huber, R., Feldman, R. A., Short, J. M., Olson, G. J. and Swanson, R. V. (1998) *Nature* **392**, 353–358
- Voo, K. S., Whetten, R. W., O'Malley, D. M. and Sederoff, R. R. (1995) *Plant Physiol.* **108**, 85–97
- Uhlmann, A. and Ebel, J. (1993) *Plant Physiol.* **102**, 1147–1156
- Kraulis, P. J. (1991) *J. Appl. Crystallogr.* **24**, 946–950
- Merritt, E. A. and Bacon, D. J. (1997) *Methods in Enzymol.* **277**, 505–524

Received 1 June 1999/28 July 1999; accepted 10 September 1999