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CoA Transferase and Malonyl-CoA Decarboxylase Activity of Malonate Decarboxylase from *Acinetobacter calcoaceticus*

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Abstract : Malonate decarboxylase from *Acinetobacter calcoaceticus* is shown to have malonyl-CoA: acetate CoA transferase, acetyl-CoA: malonate CoA transferase, and malonyl-CoA decarboxylase activity. These enzyme activities were elucidated by isotope exchange reactions. The enzyme modified by *N*-ethylmaleimide completely lost its malonate decarboxylase activity, whereas it still kept CoA transferases and malonyl-CoA decarboxylase activities. The existence of CoA transferases and malonyl-CoA decarboxylase activity is clear, but their physiological significance is obscure. The catalytic reactions for two CoA transfers and malonyl-CoA decarboxylation proceed *via* a cyclic mechanism, which is through two covalent intermediates, enzyme-S-malonyl and enzyme-S-acetyl, proposed for malonate decarboxylation of the enzyme.

Key words : *Acinetobacter calcoaceticus*, CoA transferase,, malonate decarboxylase, malonyl-CoA decarboxylase, the cyclic mechanism

Malonate decarboxylase, which catalyzes the decarboxylation of malonate to acetate and CO_2, was first isolated fiom *Pseudomonas putida* (originally *P. ovalis*) grown aerobically on malonate (Takamura and Kitayama, 1981). Other malonate decarboxylases from aerobic bacteria, such as *Acinetobacter calcoaceticus* (Kim and Byun, 1994), *Pseudomonas fluorescens* (Byun and Kim, 1995), and *Klebsiella pneumoniae* (Schmid *et al*., 1996), were also studied. Anaerobic decarboxylation of malonate to acetate was detected in cell free extracts of *Citrobacter diversus* (Janssen and Harfoot, 1990), *Malonomonas rubra* (Hilbi *et al*., 1992), *Sporomusa malonica, Klebsiella oxytoca*, and *Rhodobacter capsulatus* (Dehning and Schink, 1994). The enzyme from *M. rubra* has been studied extensively to elucidate the relationship between decarboxylation of malonate and energy conservation. *Malonomonas* enzyme does not exist as a tight complex, but rather is composed of at least three proteins in the cytoplasm for decarboxylation of malonate (Hilbi *et al*., 1993) as well as Na^+ pump in membrane for energy conservation. However, it has been suggested that malonate decarboxylases from aerobic bacteria have a common structural design, α, β, γ, and δ, for their subunit organization (Byun and Kim, 1997).

In the bacteria described above, malonate decarboxylation was stimulated by a catalytic amount of malonyl-CoA or acetyl-CoA, and malonyl-CoA decarboxylase activity was found in cell free extracts of the bacteria except for *M. rubra* and *K. pneumoniae*. Malonyl-CoA has been suggested to be an activated form in the aerobic or anaerobic degradation of malonate by *P. fluorescens* (Hayaish, 1955), *P. putida* (Takamura and Kitayama, 1981), *S. malonica, K. oxytoca, R. captsulatus*, and *C. diversus* (Janssen and Harfoot, 1992; Dehning and Schink, 1994). However, a different malonate decarboxylation system for *M. rubra, A. calocoaceticus*, and *K. pneumoniae*, was described recently where the free malonic acid is decarboxylated after activation of enzyme through acetylation (Hilbi *et al*., 1992; Kim and Byun, 1994; Schmid *et al*., 1996). Since the active form of the purified enzymes from *A. calocoaceticus, P. fluorescens*, and *P. putida* were identified to be an acetyl enzyme showing CoA transferase and malonyl-CoA decarboxylase activity, these enzymes were thought to be a good model for the mechanistic study of malonate and malonyl-CoA decarboxylation by the enzyme.

In the present report, evidence is provided that the acetyl enzyme mechanism for decarboxylation of malonate explains the activity of CoA transferase and malonyl-CoA decarboxylase that the enzyme exhibits.

Materials and Methods

Materials

A. calcoaceticus var. Kim is a bacterium isolated from

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soil and identified on the basis of malonate consumption (Kim and Kim, 1985). *P. fluorescens* ATCC 11250 and *P. putida* IAM 1177 (originally *P. ovalis* IAM 1177 used for malonate decarboxylase study by Takamura and Kitayama) were obtained from the Korea Research Institute of Bioscience and Biotechnology (Taeduck, KOREA) and IAM Culture Collection Center (Tokyo, JAPAN), respectively. Sodium malonate, MOPS, malonyl-CoA, acetyl-CoA, bromoacetate, *N*-ethylmaleimide, and acetic anhydride were purchased from Sigma Chem. Co. [2-^{14}C] Malonate (56.8 mCi/mmol), [2-^{14}C]acetate (58.0 mCi/mmol), and [1-^{14}C]acetyl-CoA (57 mCi/mmol) were obtained from DuPont NEN. All other reagents were of analytical grade.

Growth of bacteria

A. calcoaceticus, *P. fluorescens*, and *P. putida* were grown aerobically at 30℃ for 8 h, 18 h, and 18 h, respectively on malonate as the sole carbon and energy source. The growth medium contained 0.6% malonic acid, 0.3% KH_2PO_4, 0.3% NH_4Cl, 0.04% $MgSO_4$ $6H_2O$, and 0.01% $FeSO_2$ $7H_2O$. The pH was adjusted to 6.8 using KOH for the *Acinetobacter* medium, and also to the same pH using NaOH for the *Pseudomonas* medium. The yields were approximately 6 g, 3 g, and 4 g cells (wet weight) per liter medium for *A. calcoaceticus*, *P. fluorescens* and *P. putida*, respectively.

Enzyme Assays

Measurement of malonate deacrboxylase activity was carried out at 30℃, as described earlier (Byun and Kim, 1994). During the purification of the enzyme, acetate formation was measured by acetate kinase coupling assay, and for the determination of kinetic constants, the initial rate of acetate formation was spectrophotometrically measured by the method using acetyl-CoA synthetase. Malonyl-CoA decarboxylase (EC 4.1.1.9) was spectrophotometrically assayed by the coupling assay method using citrate synthase and malate dehydrogenase, reported previously (Kolattukudy *et al*., 1981). Malonyl-CoA: acetate CoA transferase (EC 2.8.3.3) was also assayed by following acetyl-CoA formation using malonyl-CoA decarboxylase assay system with 20 mM sodium acetate.

Purification

Malonate decarboxylases were purified from three different bacteria, *A. calcoaceticus*, *P. fluorescens* and *P. putida*, by the same procedure. Cells (8 g wet wt), grown on malonate, harvested and stored at -70℃, were suspended in 30 ml buffer A (20 mM MOPS, pH 6.8, 5% glycerol, and 1 mM EDTA) containing 0.5 mM dithiothreitol and disrupted at 4℃ by sonication. After centrifugation at 10,000×g for 20 min, 30% streptomycin sulfate solution was added to the crude extract until 1.5 % saturation was achieved. The precipitations in the preparation were removed by centrifugation at 15,000×g for 30 min. Each supernatant was pumped onto PBE 94 column (3.9×2.0 cm) connected to a low pressure LC apparatus (Pharmacia) at the flow rate of 1 ml/min. The enzyme bound on the column was eluted by application of a linear gradient of buffer A to 60% of buffer B (buffer A containing 1 M KCl) at 2 ml/min. The fractions containing the enzyme were pooled and dialyzed against buffer A for 6 h at 4℃. The enzyme eluted from PBE 94 was applied again on an ω-aminohexyl agarose column (2.0×4.0 cm), followed by washing with 20% buffer B. This enzyme was then eluted with a linear gradient of 20 to 50% buffer B at 1 ml/min. The three malonate decarboxylases were purified from the different bacteria, concentrated by using PM-30 Amicon ultrafiltration membrane (about 2-3 mg/ml) and then stored at 4℃.

Chromatographic identification of products

Acetyl-CoA formed from malonyl-CoA by the enzyme reaction was chromatographically isolated by using a Dionex IC anion exchange column (0.4×25 cm). After removal of enzyme by filtration using centricon, the reaction mixture was injected into the column. Then acetyl-CoA and malonyl-CoA were eluted with 0.01 N of H_2SO_4 solution and monitored at 215 nm.

Acetyl-CoA formed from acetate and malonyl-CoA by the enzyme reaction was also detected by thin layer chromatography. The purified enzyme (10.8 pmole) was incubated in a total volume of 5 μl, with [2-^{14}C]acetate, malonyl-CoA, and CoA or dephosphoCoA. After incubation for 15-60 min at 30℃, [2-^{14}C]acetyl-CoA or [2-^{14}C]acetyl-dephosphoCoA formed was identifed by thin layer chromatograpy with 0.4 M LiCl and 0.25 M ammonium formate, pH 4.2, as a developing solvent (Anke and Spector, 1975). The thin layer chromatography was performed by ascending mode using PEI-cellulose F (Merck). The plate was dried and exposed to an X-ray film at -70℃ for 1 day or to a PhosphorImager screen for 2-3 h.

Results

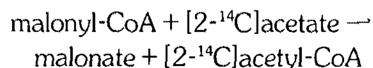
CoA transferase and malonyl-CoA decarboxylase activitiy of malonate decarboxylase

Purified malonate decarboxylase decarboxylated malonyl-CoA as well as malonate and also had CoA transferases activities. To avoid the possibility of contamination, malonate decarboxylase was run on a native PAGE, and the gel was sliced for the determination of the en-

zyme activities. The three different enzyme activities were also located on one protein band (data not shown). Acetyl-CoA production from malonyl-CoA by the enzyme was directly measured by the detection of acetyl-CoA using HPLC. These results suggested that the enzyme has three different activities at least *in vitro*.

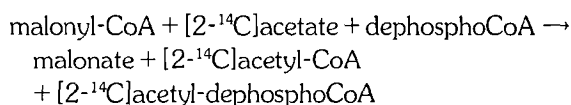
CoA transfer by malonate decarboxylase

When the enzyme was incubated with malonyl-CoA and [2-^{14}C]acetate, [2-^{14}C]acetyl-CoA was formed (Fig. 1, lane 3).



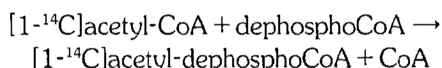
malonyl-CoA + [2-^{14}C]acetate $\rightarrow$
malonate + [2-^{14}C]acetyl-CoA

The formation of [2-^{14}C]acetyl-CoA depended on the concentration of malonyl-CoA (Fig. 1, lane 3, 5, 6). Furthermore, the rate of [2-^{14}C]acetyl-CoA formation was increased by the addition of CoA (Fig. 1, lane 4). When dephosphoCoA instead of CoA was used, acetyl-dephosphoCoA was also formed (Fig. 1, lane 7).



malonyl-CoA + [2-^{14}C]acetate + dephosphoCoA $\rightarrow$
malonate + [2-^{14}C]acetyl-CoA
+ [2-^{14}C]acetyl-dephosphoCoA

[2-^{14}C]Acetyl-CoA was not formed in the reaction mixture containing [2-^{14}C]acetate, acetyl-CoA, and enzyme (Fig. 1, lane 8). Fig. 1, lane 9 clearly showed that there was no exchange reaction between acetyl-enzyme and acetate. However, [1-^{14}C]acetyl-dephosphoCoA was produced by the enzyme mixture containing [1-^{14}C]acetyl-CoA and dephosphoCoA (Fig. 1, lane 10).



[1-^{14}C]acetyl-CoA + dephosphoCoA $\rightarrow$
[1-^{14}C]acetyl-dephosphoCoA + CoA

Decarboxylation of malonyl-CoA by malonate decarboxylase

With increasing concentration of malonyl-CoA, the formation of acetyl-CoA was measured, and K_m and V_{max} were determined. Also, the formation of acetate from malonate was measured with increasing concentration of malonyl-CoA or acetyl-CoA or acetic anhydride. The results presented in Table 1 show that K_m for the decarboxylation of malonyl-CoA by enzyme was similar value to that of malonate. But the K_m was 10^4 times higher than Km for activation of the inactive malonate decarboxylase (deacetylated form) by malonyl-CoA.

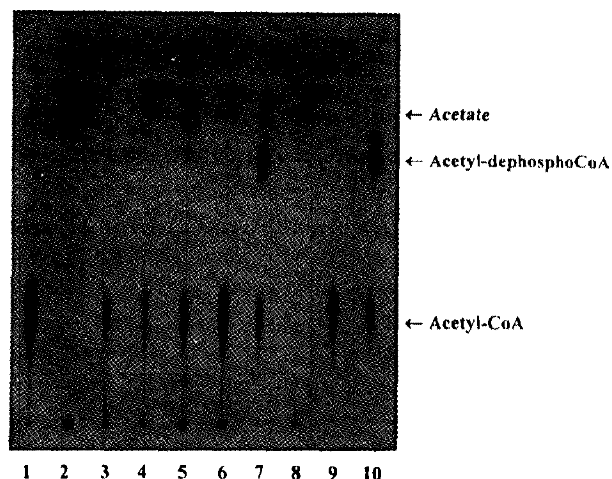


Fig. 1. TLC analysis of products by malonyl-CoA: acetate CoA transferase catalysis. Purified deacetyl malonate decarboxylase (10.8 pmol) from *A. calcoaceticus* was used in a total of 5 μl reaction mixture. After incubation for 30 min at 30°C, aliquots (0.5 μl) were spotted on the plate. (Lane 1): 0.9 nmol [1-^{14}C]acetyl-CoA, (2): 10 nmol [2-^{14}C]acetate, (3): 0.1 nmol malonyl-CoA and 10 nmol [2-^{14}C]acetate, (4): 0.1 nmol malonyl-CoA, 10 nmol [2-^{14}C]acetate, and 5 nmol CoA, (5): 0.5 nmol malonyl-CoA and 10 nmol [2-^{14}C]acetate, (6): 1.0 nmol malonyl-CoA and 10 nmol [2-^{14}C]acetate, (7): 0.1 nmol malonyl-CoA, 10 nmol [2-^{14}C]acetate, and 5 nmol dephosphoCoA, (8): 1.0 nmol acetyl-CoA and 10 nmol [2-^{14}C]acetate, (9): 0.9 nmol [1-^{14}C]acetyl-CoA and 10 nmol acetate, (10): 0.9 nmol [1-^{14}C]acetyl-CoA and 5 nmol dephosphoCoA.

Modification of thiol group in δ-subunit

When the deacetyl enzymes from *Acinetobacter or Pseudomonas* were pretreated with thiol group-specific

Table 1. The kinetic constants of malonate decarboxylase

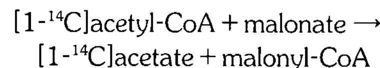
Catalysis	K_m(M)/V_{max} (μmol/min/mg)		
	A. calcoaceticus	*P. fluorescens*	*P. putida*
Malonate decarboxylase			
malonate[a]	5.3×10^{-5}/9.23	3.6×10^{-4}/3.43	1.7×10^{-4}/21.1
malonyl-CoA[b]	5.2×10^{-8}	N.D.[c]	N.D.
acetyl-CoA[b]	3.5×10^{-7}	N.D.	N.D.
acetic anhydride[b]	7.1×10^{-5}	N.D.	N.D.
Malonyl-CoA decarboxylase			
malonyl-CoA[a]	7.5×10^{-4}/1.11	1.5×10^{-4}/0.43	2.5×10^{-4}/2.77

[a]as a substrate.
[b]as an activator for acylation of the enzyme.
[c]Not determined.

reagents such as bromoacetate and *N*-ethylmaleimide, these enzymes were not reactivated by the treatment of malonyl-CoA. From the determination of initial velocity for inactivation by bromoacetate and *N*-ethylmaleimide, second order rate constant, k, for inactivation were calculated to be 230 $M^{-1}min^{-1}$ and 2462 $M^{-1}min^{-1}$, respectively. However, when these enzymes were acylated with malonyl-CoA or acetic anhydride prior to the treatment with the reagents, these acylated enzymes were fully protected from the inactivation by the modification. To detect the acyl-thioester carrier subunit of malonate decarboxylase, the purified deacetyl enzymes were treated with bromo[1-^{14}C]acetate or *N*-[ethyl-1-^{14}C]maleimide. It was found by a native PAGE that the three malonate decarboxylase from different bacteria were labelled. The analysis of the labeled enzyme by SDS/PAGE revealed that δ-subunits were specifically labeled, indicating that the smallest subunit, δ, was catalytically important acyl-carrier subunit (Byun and Kim, 1997). As expected, however, acetyl enzymes, prepared by incubation with malonyl-CoA, failed to label the δ-subunit.

Malonyl-CoA decarboxylase and CoA transferase activity by malonate decarboxylase modified with N-ethylmaleimide

Although the modification of catalytically important thiol-group in δ-subunit resulted in a complete loss of malonate decarboxylase activity, the enzyme modified with N-ethylmaleimide interestingly showed catalytic activity for the formation of acetyl-CoA from malonyl-CoA (data not shown). Furthermore, the formation of acetyl-CoA from malonyl-CoA and acetate, showing CoA transfease activity, by the thiol-modified enzyme was almost the same in its catalytic rate as that of the native enzyme. For further analysis of CoA transferase reactions, two forms of malonate decarboxylases from *A. calocoaceticus*, *P. fluorescens*, and *P. putida* were prepared. That is, one was malonate decarboxylase modified with *N*-ethylmaleimide (E-S-NEM) and the other was the active enzyme incubated with *N*-ethylmaleimide (E-S-ac:NEM). Those inactivated enzymes (E-S-NEM) catalyzed the formation of [2-^{14}C]acetyl-CoA from malonyl-CoA and [2-^{14}C]acetate (Fig. 2, lane 1, 5, 9), and [2-^{14}C]acetyl-dephosphoCoA from malonyl-CoA, [2-^{14}C]acetate, and dephosphoCoA (Fig. 2, lane 2, 6, 10). However, when E-S-NEM and E-S-ac:NEM from three bacteria incubated with [1-^{14}C]acetyl-CoA and malonate, only E-S-NEM catalyzed CoA transfer to malonate (Fig. 2, lane 3, 7, 11), whereas E-S-ac:NEM did not (Fig.2, lane 4, 8, 12). E-S-NEM also catalyzed the formation of [1-^{14}C]acetate from [1-^{14}C]acetyl-CoA and malonate (Fig.3, lane 1-3), and produced [2-^{14}C]acetyl-CoA from acetyl-CoA and [2-^{14}C]malonate (Fig.3, lane 4-6).



$$[1\text{-}^{14}C]\text{acetyl-CoA} + \text{malonate} \longrightarrow [1\text{-}^{14}C]\text{acetate} + \text{malonyl-CoA}$$

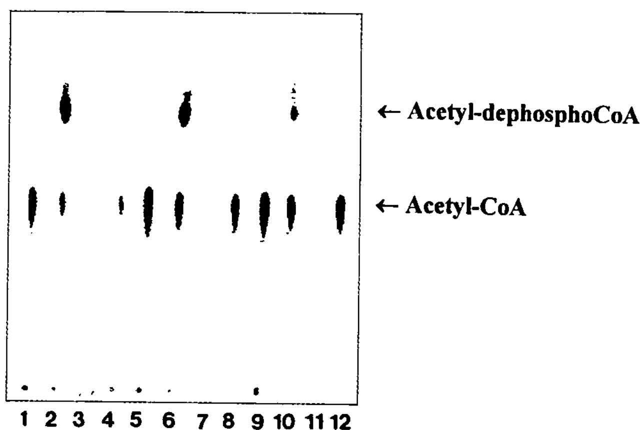


Fig. 2. TLC analysis of products by CoA transferase reactions of inactivated enzymes (E-S-NEM) or acetyl enzymes (E-S-ac: NEM). Inactivated enzymes modified by *N*-ethylmaleimide (10.8 pmol, lanes 1-3, 5-7, 9-11) and acetyl-enzymes (10.8 pmol, lanes 4, 8, 12) from *A. calcoaceticus* (lanes 1-4), *P. fluorescens* (lanes 5-8), and *P. putida* (lanes 9-12) were used in a total of 5 μl reaction mixture. After incubation for 30 min at 30°C, aliquots (0.5 μl) were spotted on the plate. (Lanes 1, 5, 9): 1.0 nmol malonyl-CoA and 10 nmol [2-^{14}C]acetate. (lanes 2, 6, 10): 1.0 nmol malonyl-CoA, 10 nmol [2-^{14}C]acetate, and 5 nmol dephosphoCoA, (lanes 3, 4, 7, 8, 11, 12): 0.9 nmol [1-^{14}C]acetyl-CoA and 10 nmol malonate.

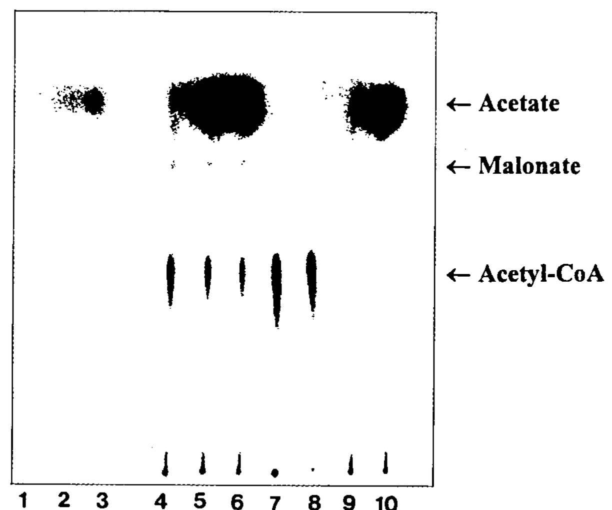
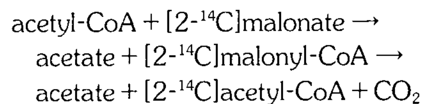
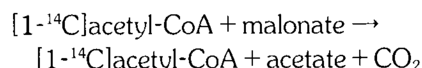


Fig. 3. TLC analysis of the products by acetyl-CoA: malonate CoA transfer reaction. Enzyme modified by *N*-ethylmaleimide (E-S-NEM, lanes 1-6) and deacetyl-enzyme (E-S, lanes 7-10) purified from *A. calcoaceticus* were used in a total of 5 μl reaction mixture. After incubation for 15 min (lanes 1, 4), 30 min (lanes 2, 5, 7, 9), or 60 min (lanes 3, 6, 8, 10) at 30°C, aliquots (0.5 μl) were spotted on the plate. (Lanes 1-3, 7, 8): 0.9 nmol [1-^{14}C]acetyl-CoA and 3 nmol malonate, (lanes 4-6, 9, 10): 0.9 nmol acetyl-CoA and 3 nmol [2-^{14}C]malonate.

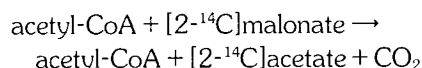


$$\text{acetyl-CoA} + [2\text{-}^{14}\text{C}]\text{malonate} \rightarrow \text{acetate} + [2\text{-}^{14}\text{C}]\text{malonyl-CoA} \rightarrow \text{acetate} + [2\text{-}^{14}\text{C}]\text{acetyl-CoA} + CO_2$$

We failed to detect [2-^{14}C]malonyl-CoA from the reaction mixture containing acetyl-CoA, [2-^{14}C]malonate, and E-S-NEM. It seems that the formed [2-^{14}C]malonyl-CoA was further decarboxylated to [2-^{14}C]acetyl-CoA and CO_2. E-S-ac:NEM did not show CoA transfer activity from acetyl-CoA to malonate (Fig. 3, lane 7, 8), but decarboxylated malonate to acetate (Fig. 3, lane 9, 10).



$$[1\text{-}^{14}\text{C}]\text{acetyl-CoA} + \text{malonate} \rightarrow [1\text{-}^{14}\text{C}]\text{acetyl-CoA} + \text{acetate} + CO_2$$



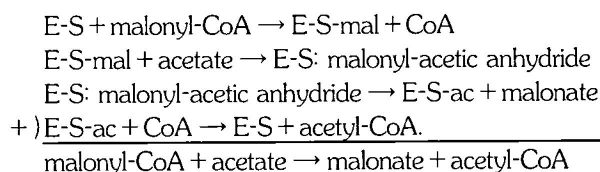
$$\text{acetyl-CoA} + [2\text{-}^{14}\text{C}]\text{malonate} \rightarrow \text{acetyl-CoA} + [2\text{-}^{14}\text{C}]\text{acetate} + CO_2$$

Discussion

Inactive malonate decarboxylase from *A. calcoaceticus* can be reactivated by catalytic amount of malony-CoA or acetyl-CoA through self-acylation on specific thiol group in the δ-subuint of the enzyme. At present it is not known whether there is any specific ligase for acetylation of malonate decarboxylase in *A. calcoaceticus* and *P. fluorescens* similar to the ligase detected in *M. rubra* and *K. pneumoniae*. But the incubation of the enzyme with acetate and ATP resulted in reactivating the enzyme by the catalysis of ligase within the cell extract, even though acetyl phosphate cannot reactivate the enzyme. However, incubation with malonate, ATP, CoA, and cell extract (Hilbi *et al.*, 1993; Dehning and Schink, 1994) might result in the formation of acetyl-CoA *via* malonate decarboxylase, acetate kinase, phosphoacetyltransferase or acetyl-CoA synthetase (Byun and Kim, 1995), which would reactivate deacetylated enzyme in *A. calcoaceticus* and *P. fluorescens*.

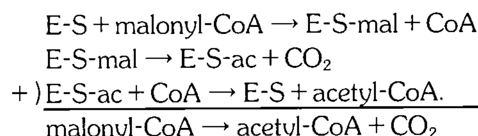
Acylation of the enzyme by malonyl-CoA or acetyl-CoA, which activates the enzyme, depended on the concentration of acyl-CoAs. When the malonate decarboxylase activity at various concentrations of malonyl-CoA or acetyl-CoA was measured, the activity increased hyperbolically showing a high affinity for the enzyme (Table 1). K_m for the self-acylation of the enzyme by malonyl-CoA was much lower than that by acetyl-CoA, indicating that malonyl-CoA is a physiological activator. Similar results for reactivation of malonate decarboxylase in *K. pneumoniae* (Schmid *et al.*, 1996), and in anaerobic bacteria (Dehning *et al.*, 1994) were reported previously. Malonate decarboxylase can catalyze the formation of acetyl-CoA from malonyl-CoA as a substrate in two different ways, decarboxylation of malonyl-CoA by malonyl-CoA decarboxylase and CoA transfer from malonyl-CoA to acetate by malonyl-CoA: acetate CoA transferase. However, a high concentration of malonyl-CoA is required for these catalyses. Therefore, it appears that the activity of malonyl-CoA decarboxylase or CoA transferase of malonate decarboxylase does not imply that malonyl-CoA is an activated form of malonate for its decarboxylation.

In the proposed cyclic mechanism for decarboxylation of malonate by the enzymes from *M. rubra, A. calcoaceticus,* and *K. pneumoniae,* two acyl forms of the enzyme, malonyl-enzyme (E-S-mal) and acetyl-enzyme (E-S-ac), are catalytically important intermediates. It seems that these acyl enzymes are also crucial intermediates for the catalysis of malonyl-CoA decarboxylation and CoA transfer by the enzyme. On malonyl-CoA: acetate CoA transferase, the formation of [2-^{14}C]acetyl-dephosphoCoA from the incubation of malonyl-CoA, [2-^{14}C]acetate, dephosphoCoA, and the enzyme (Fig. 1, lane 7) strongly suggested that malonyl-enzyme, mixed anhydride, and the subsequently formed acetyl-enzyme are involved as intermediates in CoA transferase reaction. It indicates that the reactions might proceed through the following order:



$$\begin{aligned} &\text{E-S} + \text{malonyl-CoA} \rightarrow \text{E-S-mal} + \text{CoA} \\ &\text{E-S-mal} + \text{acetate} \rightarrow \text{E-S: malonyl-acetic anhydride} \\ &\text{E-S: malonyl-acetic anhydride} \rightarrow \text{E-S-ac} + \text{malonate} \\ +)\ &\underline{\text{E-S-ac} + \text{CoA} \rightarrow \text{E-S} + \text{acetyl-CoA.}} \\ &\text{malonyl-CoA} + \text{acetate} \rightarrow \text{malonate} + \text{acetyl-CoA} \end{aligned}$$

Likewise, it seems that the breakage and rejoining of the thioester bond of acyl-CoA is involved in the decarboxylation of malonyl-CoA. Malonyl-CoA is decarboxylated to acetyl-CoA through these reactions:



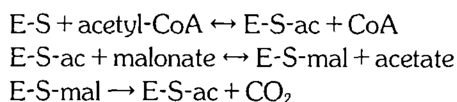
$$\begin{aligned} &\text{E-S} + \text{malonyl-CoA} \rightarrow \text{E-S-mal} + \text{CoA} \\ &\text{E-S-mal} \rightarrow \text{E-S-ac} + CO_2 \\ +)\ &\underline{\text{E-S-ac} + \text{CoA} \rightarrow \text{E-S} + \text{acetyl-CoA.}} \\ &\text{malonyl-CoA} \rightarrow \text{acetyl-CoA} + CO_2 \end{aligned}$$

CoA exchange between acetyl-CoA and CoA by the enzyme was confirmed by the production of [1-^{14}C]acetyl-dephosphoCoA from the mixture containing [1-^{14}C]acetyl-CoA, dephosphoCoA, and the enzyme (Fig. 1, lane 10). This indicates that the formation of acetyl enzyme intermediate with acetyl-CoA occurs first, followed by the attack of dephosphoCoA to this covalent intermediate. By incubation of the enzyme with borohydride and the acyl-CoA substrates, any catalytic inhibitions for the enzyme were not observed, unlike other CoA transferases which have been demonstrated to form enzyme-CoA covalent intermediate (Buckel *et al.*, 1981). Therefore, these results suggest that CoA transferases in *Acinetobacter* and *Pseudomonas* are similar to those CoA

transferases detected in citrate lyase system from *K. aerogenes* or malonate decarboxylase system from *M. rubra*.

Though inactivated enzyme (E-S-NEM), prepared by alkylation of proposed acylating thiol group, did not decarboxylate malonate, it produced acetyl-CoA from malonyl-CoA or malonyl-CoA and acetate. It means that enzyme-S-acetyl (E-S-ac) is an essential intermediate for malonate decarboxylation, but it can be replaced by acyl-CoAs for malonyl-CoA decarboxylation and CoA transfer of the enzyme. Acyl-CoA itself may exert catalytic effect on CoA transfer by replacing non-covalently enzyme-bound CoA-S-acyl instead of natural enzyme-S-acyl (E-S-ac). It is the very same case with citrate lyase in which enzyme-S-acyl is an essential intermediate in citrate cleavage. Though thiol-modified citrate lyase (enzyme-S-CH_2COOH) lost its catalytic activity, it could be revived by incubation with acetyl-CoA, which means acetyl-CoA might replace enzyme-S-acetyl in citrate cleavage (Buckel *et al.*, 1973). It was reported that CoA-like prosthetic group is covalently linked to acyl-carrier protein (ACP) of the enzyme, and α-subunit of the enzyme acts as an ACP transferase in *M. rubra* (Berg *et al.*, 1996; Hilbi and Dimroth, 1994) and *K. pneumoniae* (Schmid *et al.*, 1996).

On the other hand, the other CoA transfer reaction, acetyl-CoA: malonate CoA transfer, was fundamentally different from the reaction of malonyl-CoA: acetate CoA transferase. Detection of malonyl-CoA production from acetyl-CoA and malonate was unsuccessful. Inactivated enzyme (E-S-NEM) with alkylating agent showed isotope exchange between acetyl-CoA and malonate to produce acetate and acetyl-CoA, not malonyl-CoA. This result suggests that acetyl-CoA, unlike malonyl-CoA, was easily released from the enzyme. And this low affinity of acetyl-CoA for the enzyme explains the reason that acyl-CoAs cannot replace enzyme-S-acetyl (E-S-ac) in decarboxylation of malonate by inactivated enzyme (E-S-NEM). This CoA transfer reaction was not observed in the reaction with deacetyl enzyme (E-S), because acetyl-enzyme (E-S-ac), which was formed by incubation of enzyme with acetyl-CoA, decarboxylated malonate continuously to acetate in a cyclic manner:



$$\text{E-S} + \text{acetyl-CoA} \leftrightarrow \text{E-S-ac} + \text{CoA}$$
$$\text{E-S-ac} + \text{malonate} \leftrightarrow \text{E-S-mal} + \text{acetate}$$
$$\text{E-S-mal} \rightarrow \text{E-S-ac} + CO_2$$

In CoA transferase of malonate decarboxylase system from *M. rubra*, it has been reported that the maximum velocity of CoA transfer from acetyl-CoA to malonate is about 200 times lower than that of CoA transfer from malonyl-CoA to acetate (Hilbi and Dimroth, 1994). The results described above might explain the large difference between two CoA transfer rates by the enzyme. Malonyl-CoA production from malonate and acetyl-CoA might be inhibited by cyclic decarboxylation of malonate.

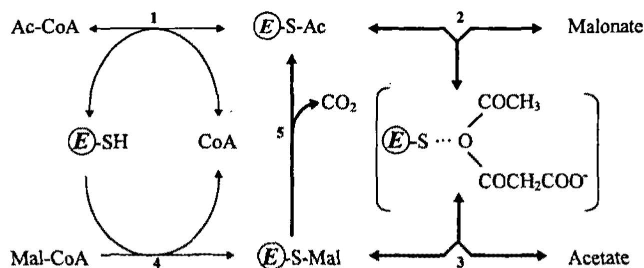


Fig. 4. The proposed mechanism of malonate decarboxylase for its multicatalytic activity. Bold line (➔) indicates the pathway of malonate decarboxylation.

In summary the results indicate that CoA transfer reactions and malonyl-CoA decarboxylation as well as malonate decarboxylation are processed by the cyclic acyl enzyme reaction mechanism (Fig. 4), and three activities by the enzyme are tightly related with each other. That is, malonate is decarboxylated through the reaction 1→2→3→5 or 4→5→2→3→5. CoA is transfered through the reaction 4→3→2→1 for malonyl-CoA: acetate CoA transfer, and 1→2→3→5→1 for acetyl-CoA: malonate CoA transfer. Malonyl-CoA is directly decarboxylated through the reaction 4→5→1.

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