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Short communication

Properties of Malonyl-CoA Decarboxylase from *Rhizobium trifolii*

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A novel gene for malonyl-CoA decarboxylase was discovered in the *mat* operon, which encodes a set of genes involved in the malonate metabolism of *Rhizobium trifolii* (An and Kim, 1998). The subunit mass determined by SDS-PAGE was 53 kDa, which correspond to the deduced mass from the sequence data. The molecular mass of the native enzyme determined by field flow fractionation was 208 kDa, indicating that *R. trifolii* malonyl-CoA decarboxylase is homotetrameric. *R. trifolii* malonyl-CoA decarboxylase converted malonyl-CoA to acetyl-CoA with a specific activity of 100 unit/mg protein. Methylmalonyl-CoA was decarboxylated with a specific activity of 0.1 unit/mg protein. *p*-Chloromercuribenzoate inhibited this enzyme activity, suggesting that thiol group(s) is(are) essential for this enzyme catalysis. Database analysis showed that malonyl-CoA decarboxylase from *R. trifolii* shared 32.7% and 28.1% identity in amino acid sequence with those from goose and human, respectively, and it would be located in the cytoplasm. However, there is no sequence homology between this enzyme and that from *Saccharopolyspora erythreus*, suggesting that malonyl-CoA decarboxylases from human, goose, and *R. trifolii* are in the same class, whereas that from *S. erythreus* is in a different class or even a different enzyme, methylmalonyl-CoA decarboxylase. According to the homology analysis, Cys-214 among three cysteine residues in the enzyme was found in the homologous region, suggesting that the cysteine was located at or near the active site and plays a critical role in catalysis.

Keywords: FFF, Inhibitors, Malonyl-CoA decarboxylase, *Rhizobium trifolii.*

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Introduction

Malonyl-CoA decarboxylase activity has been observed in a variety of organisms from microbes to mammals. The enzyme, which catalyzes the decarboxylation of malonyl-CoA to acetyl-CoA, has been well studied in the uropygial gland of goose and rat liver mitochondria (Kim and Kolattukudy, 1978a; 1978b). The role of this decarboxylase in the gland was proposed to ensure that methylmalonyl-CoA is the only chain elongating substrate available to fatty acid synthesis and thus brings about the production of multimethyl branched fatty acids (Kim and Kolattukudy, 1978a). In mitochondria, it has been proposed that malonyl-CoA is nonspecifically formed by propionyl-CoA carboxylase, and the enzyme decarboxylates malonyl-CoA to acetyl-CoA (Kim and Kolattukudy, 1978b). If malonyl-CoA is accumulated, key mitochondrial enzymes such as methylmalonyl-CoA mutase (Babior, 1973) and pyruvate carboxylase (Scrutton and Utter, 1967) could be inhibited. Malonyl-CoA decarboxylase keeps some decarboxylase activity with methylmalonyl-CoA (Hsieh and Kolattukudy, 1994), but it is different from methylmalonyl-CoA decarboxylase (Bott *et al.*, 1997). *Saccharopolyspora erythreus* malonyl-CoA decarboxylase has been purified and characterized, and its gene has been cloned (Hsieh and Kolattukudy, 1994). It has been postulated that the decarboxylase provided propionyl-CoA for erythromycin biosynthesis via decarboxylation of methylmalonyl-CoA derived from succinyl-CoA (Hunaiti and Kolattukudy, 1984; Hsieh and Kolattukudy, 1994), suggesting that this enzyme is methylmalonyl-CoA decarboxylase rather than malonyl-CoA decarboxylase. Malonyl-CoA decarboxylase activity was detected in the cell extract of malonate-grown bacteria such as *Pseudomonas fluorescens* and *Acinetobacter calcoaceticus*, but it has been found that this enzyme activity in those organisms is a partial reaction of malonate decarboxylase (Byun and Kim, 1995). Other methylmalonyl-CoA decarboxylases from *Propionigenium*

modestum were also reported to have malonyl-CoA decarboxylase activity (Bott *et al.,* 1997).

Recently, we discovered a gene for malonyl-CoA decarboxylase in the *mat* operon that encodes a set of genes involved in the malonate metabolism of *R. trifolii* (Lee and Kim, 1993a; 1993b; An and Kim, 1998). We reported that the K_m and V_{max} of the *R. trifolii* enzyme for malonyl-CoA were 0.47 mM and 52 μmol/min/mg, respectively (An and Kim, 1998). It was also reported that the K_m and V_{max} of goose enzyme were 0.10 mM and 80 μmol/min/mg, respectively (Kolattukudy *et al.*, 1981), and those of *S. erythreus* enzyme were 0.14 mM and 0.25 μmol/min/mg, respectively (Hunaiti and Kolattukudy, 1984). In summary, the catalytic efficiency of *S. erythreus* enzyme was considerably lower than that of *R. trifolii* and goose enzyme, indicating that *S. erythreus* enzyme may be a different class of enzyme, such as methylmalonyl-CoA decarboxylase.

In this paper, we present properties of this newly discovered first bacterial malonyl-CoA decarboxylase from *R. trifolii*.

Materials and Methods

Materials Malonyl-CoA, methylmalonyl-CoA, acetyl-CoA, potassium arsenate, *p*-chloromercuribenzoic acid (PCMB), *N*-ethylmaleimide (NEM), iodoacetamide, malate dehydrogenase, and citrate synthase were purchased from Sigma Chemical Co. (St. Louis, USA). Phosphotransacetylase was purchased from Boehringer Mannheim (Indianapolis, USA). All other chemicals and reagents were of the highest grade commercially available.

Expression and affinity purification of malonyl-CoA decarboxylase The expression and purification of GST-fused malonyl-CoA decarboxylase were carried out according to the manufacturer's instructions (Pharmacia, UK). A forty milliliter culture of *E. coli* containing the *R. trifolii* malonyl-CoA decarboxylase gene was induced by the addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG), and the cells were harvested and sonicated. Bacterial cell lysate was loaded on a Gluthathione Sepharose 4B RediPack. Fusion protein bound to the matrix was cleaved with thrombin for 18 h at 22°C. The supernatant was assayed for enzyme activity.

Determination of malonyl-CoA decarboxylase activity Malonyl-CoA decarboxylase activity was assayed by measuring the rate of production of acetyl-CoA spectrophotometrically by coupling the decarboxylase with malate dehydrogenase and citrate synthase (Kolattukudy *et al.*, 1981). The reaction mixture contained 40 mM sodium phosphate (pH 7.0), 0.2 mM dithiothreitol, 4 mM malate, 0.2 mM NAD^+, 0.2 mM malonyl-CoA, 20 unit malate dehydrogenase, 4 unit citrate synthase, and malonyl-CoA decarboxylase preparation in a total volume of 1.0 ml. The reaction was initiated by the addition of the decarboxylase, and the increase in absorbance at 340 nm was measured.

Determination of methylmalonyl-CoA decarboxylase activity The methylmalonyl-CoA decarboxylase activity was also determined by spectrophotometric measurement (Hilpert and Dimroth, 1983). Propionyl-CoA generated by methylmalonyl-CoA decarboxylase was hydrolyzed by phosphotransacetylase in the presence of arsenate, and the decrease of the thioester absorbance at 232 nm was measured. The measured reaction mixture (1.0 ml) containing 10 mM potassium arsenate, pH 7.0, 3 mM NaCl, 0.2 mM methylmalonyl-CoA, and 20 units phosphotransacetylase (Boehringer Mannheim) was incubated at 30°C.

Determination of molecular mass Molecular mass of the enzyme was determined by the field flow fractionation (FFF) method (Giddings, 1993) by using a Model F-1000 Universal Fractionator purchased from the FFFractionation, LLC (Salt Lake City, USA). Eluted samples were monitored using a M-720 UV detector purchased from the Young-In Scientific Inc. (Seoul, Korea). A specific carrier solution was used to maintain the same condition as the stock solution. Malonyl-CoA decarboxylase was dissolved at pH 7.3, 50 mM potassium phosphate buffer. The final concentration of the enzyme was 3 mg/ml.

In the FFF channel, the retention of the sample is measured by the retention ratio *R*.

$$R = \frac{t^0}{t_r} = \frac{V^0}{V_r} = 6\left(\frac{l}{w}\right)\left[\coth\left(\frac{w}{2l}\right) - \frac{2l}{w}\right] = 6\lambda \quad (1)$$

where t^0 is the void time, t_r is retention time of the sample component, V^0 is the channel void volume, V_r is the retention volume, l is the mean layer thickness of the particles, and w is the channel thickness. The retention parameter λ is defined by

$$\lambda = \frac{bLkT}{3\pi\eta\ wdV_c} = \frac{V^0kT}{3\pi\eta\ w^2dV_c} \quad (2)$$

where k is the Boltzmann's constant, T is the absolute temperature, d is the hydrodynamic diameter of the sample particle, η is the viscosity of the carrier liquid, V_c is the cross flow rate, b is the breadth of the channel, and L is the length of the channel.

Using Eq. (2) the particle diameter is determined from λ which is determined by measuring the retention time t_r (or the retention volume V_r) of the sample. Molecular weight of malonyl-CoA decarboxylase is determined from the calibration curve of hydrodynamic diameters versus molecular weight of standard proteins.

Inactivation of malonyl-CoA decarboxylase The inactivation of the enzyme by PCMB was performed at 25°C for 10 min in the dark by addition of reagent into the enzyme solution in 0.14 mM NaCl, 0.27 mM KCl, 0.1 mM Na_2HPO_4 and 0.18 mM KH_2PO_4 (pH 7.3). The final concentrations of PCMB and the enzyme were 0.1 mM and 10 μM, respectively. The inactivation of the enzymes by NEM and iodoacetamide was performed at 25°C for 10 min by addition of reagents into the enzyme solution in 0.14 mM NaCl, 0.27 mM KCl, 0.1 mM Na_2HPO_4 and 0.18 mM KH_2PO_4 (pH 7.3). The final concentrations of NEM, iodoacetamide, and

the enzyme were 1 mM, 1 mM, and 10 μM, respectively. After the inactivation of the enzyme with chemical reagents, aliquots were withdrawn for the assay of activity.

Results and Discussion

Molecular mass Malonyl-CoA decarboxylase has a calculated molecular mass of 51 kDa and consists of 462 amino acids (An and Kim, 1998). As shown in Fig. 1A, the FFF fractograms of standard proteins with malonyl-CoA decarboxylase showed a linear plot of logMW *vs* log λ (Fig. 1B). The molecular mass of the native enzyme was determined to be 208 kDa, and the linearity of calibration curve is about $R^2 = 0.97$. The subunit molecular mass was determined to be 53 kDa by SDS-PAGE (An and Kim, 1998), suggesting that this enzyme is homotetrameric. The enzyme from goose was reported to be homotetrameric, whereas that from *S. erythreus* was homodimer (Hunaiti and Kolattukudy, 1984).

Decarboxylation of methylmalonyl-CoA *R. trifolii* malonyl-CoA decarboxylase converted malonyl-CoA to acetyl-CoA with a specific activity of 100 unit/mg protein. Methylmalonyl-CoA was decarboxylated with a specific activity of 0.1 unit/mg protein. Goose malonyl-CoA decarboxylase converted malonyl-CoA to acetyl-CoA with a specific activity of 17.1 unit/mg protein, and methylmalonyl-CoA was decarboxylated with a specific activity of 0.086 unit/mg protein (Kim and Kolattukudy, 1978a). Methylmalonyl-CoA decarboxylase activity of the enzymes from *R. trifolii* and goose were 200- to 1000- fold lower than their imalonyl-CoA decarboxylase activity, respectively. Methylmalonyl-CoA decarboxylase of *P. modestum* converted malonyl-CoA to acetyl-CoA with a specific activity of 7 unit/mg protein. However, it decarboxylated methylmalonyl-CoA with a specific activity of 25 unit/mg protein. In summary, it was suggested that the malonyl-CoA decarboxylases from *R. trifolii* and goose were used to malonyl-CoA as a primary substrate.

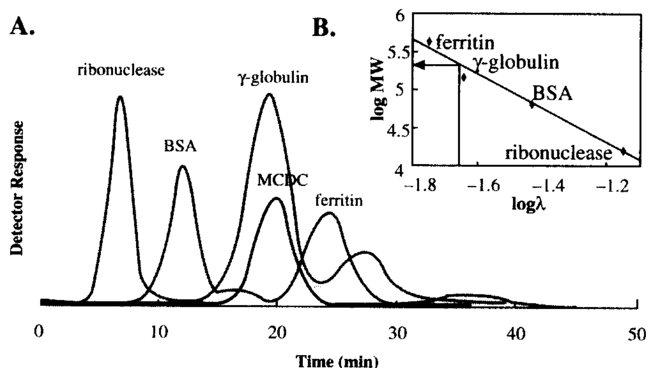

Fig. 1. A. Fractograms of the malonyl-CoA decarboxylase with protein standards. **B.** Calibration curve of the malonyl-CoA decarboxylase with protein standards. The carrier is phosphate buffer saline pH 7.3, flow conditions are the channel flow rate of V_{dot} = 0.50 ml/min and cross-flow rate of V_c = 2.55 ml/min (± 0.05 ml).

Inhibitors Malonyl-CoA decarboxylase from *R. trifolii* was severely inhibited by the thiol-directed reagent, *p*-chloromercuribenzoate (PCMB), although it was slightly inhibited by iodoacetamide and *N*-ethylmaleimide, indicating that thiol group(s) is(are) involved in the enzyme catalysis. Thiol-directed reagents were also known to inhibit malonyl-CoA decarboxylase from goose (Kim and Kolattukudy, 1978a). However, the enzyme from *S. erythreus* was not inactivated by thiol-directed reagents (Hunaiti and Kolattukudy, 1984) suggesting that the active site of *R. trifolii* malonyl-CoA decarboxylase is different from that of the *S. erythreus* enzyme.

Sequence homology and prediction of active site According to the database analysis, malonyl-CoA decarboxylase from *R. trifolii* shared 32.7% and 28.1% identity with those from goose (Jang *et al.*, 1989) and human (Gao *et al.*, 1999), respectively (Fig. 2). However, it showed no sequence homology with *S. erythreus* malonyl-CoA decarboxylase (Hsieh and Kolattukudy, 1994). These results suggest that the enzyme from human, goose, and *R. trifolii* are in the same class, whereas that from *S. erythreus* is in a different class or is even a different enzyme, possibly methylmalonyl-CoA decarboxylase. In goose, the mitochondrial and cytoplasmic forms of malonyl-CoA decarboxylase are transcribed from alternative, in-frame initiation codons separated by 146 nucleotides in exon 1 (Courchesne-Smith *et al.*, 1992). In summary, malonyl-CoA decarboxylase from goose includes an 18 amino acid *N*-terminal motif, which forms an amphipathic alpha helix constituting the mitochondrial targeting sequence (Fig. 2). On the other hand, human malonyl-CoA decarboxylase does not have mitochondrial targeting sequence. Interestingly, a canonical peroxisomal-matrix targeting sequence (SKL) at the C-terminus of the protein (Kozak, 1996) is strictly conserved in human and goose. However, bacterial malonyl-CoA decarboxylase from *R. trifolii* has neither an SKL sequence near the C-terminus nor N-terminal targeting sequences. In bacteria, several periplasmic proteins were also reported to have the targeting sequence (SKL) at the C-terminus (Sha *et al.*, 1994; Herourt *et al.*, 1996). Furthermore, the sequence analysis of the enzyme from *R. trifolii* by using PSORT, which is a computer program for the prediction of protein localization sites in cells, predicted that the enzyme is located in the bacterial cytoplasm. Studies with sulfhydryl modifying reagents described above suggested that one or more cysteine

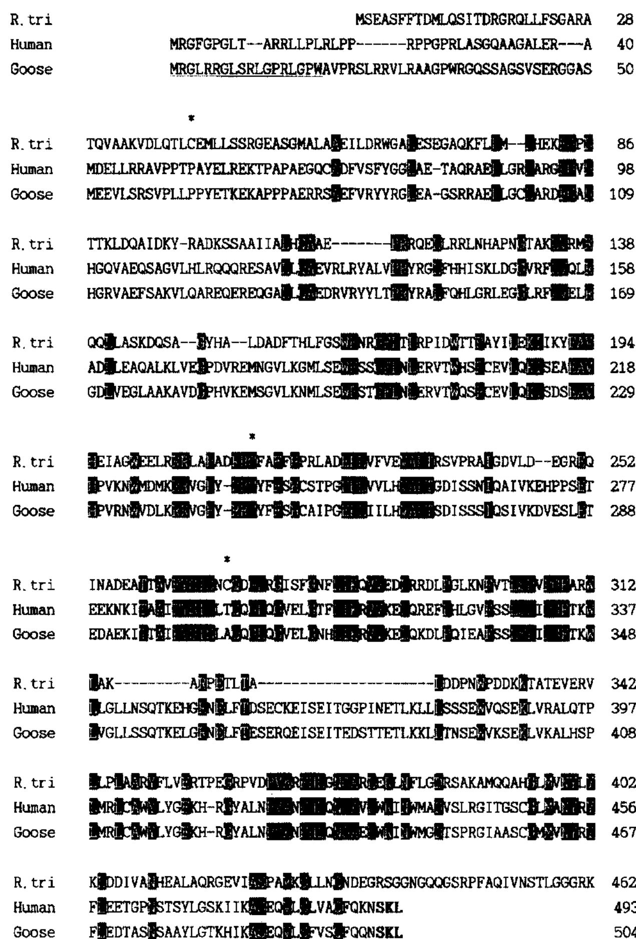

Fig. 2. Sequence alignment of the deduced amino acid sequences of the *R. trifolii* malonyl-CoA decarboxylase with the homologous sequence. An asterisk indicates cysteines in *R. trifolii* malonyl-CoA decarboxylase (*). The 18 amino acids (underlined) that comprise the mitochondrial targeting sequence in the goose and the conserved canonical peroxisomal targeting sequence are indicated in bold type.

residue(s) is(are) essential for malonyl-CoA decarboxylase activity (Table 1). There are three cysteines (Cys-41, Cys-214, and Cys-269) in *R. trifolii* malonyl-CoA decarboxylase (Fig. 2). However, Cys-214 is found in the conserved region, 212-Arg-Arg-Cys-214, in goose and human enzymes, suggesting that Cys-214 is a critical component of the active site of malonyl-CoA decarboxylase. Recently, it was reported that a residue Cys-82 of human *S*-adenosylmethionine decarboxylase (Xiong *et al.*, 1999) may be the proton donor of the decarboxylation reaction, whereas a residue Cys-221 of yeast pyruvate decarboxylase (Baburina *et al.*, 1998) may be involved in distortion of domain interactions.

In summary, we suggest that malonyl-CoA decarboxylase from *R. trifolii* is in the same class of thiol-specific enzymes from human and goose, whereas that

Table 1. Effect of inhibitors on *R. trifolii* malonyl-CoA decarboxylase activity.

Inhibitors	Concentration (mM)	Relative rate (%)
p-Chloromercuribenzoate	0.1	4.5
N-ethylmaleimide	1.0	53
Iodoacetamide	1.0	84

from *S. erythreus* may be classified into different enzymes such as a methylmalonyl-CoA decarboxylase because of the dissimilarities in amino acid sequence and active site.

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