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Subunit Organization of Bacterial Malonate Decarboxylases: The Smallest δ Subunit as an Acyl-Carrier Protein

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Abstract : In order to compare molecular structure, malonate decarboxylases from *Acinetobacter calcoaceticus*, *Pseudomonas fluorescens*, and *Pseudomonas putida* aerobically grown on malonate, were purified by the method employing streptomycin sulfate treatment, chromatography with PBE 94 and ω-aminohexyl agarose. Molecular masses were estimated to be 185, 200, and 200 kDa, respectively. All malonate decarboxylases were multimeric enzymes consisting of four different subunits, 2α, 1β, 1γ, and 1δ. The molecular masses of the *Pseudomonas* enzyme subunits were 65 (α), 33 (β), 30 (γ), and 11 kDa (δ); which are very similar to those, 65 (α), 32 (β), 25 (γ), and 11 kDa (δ) of *Acinetobacter* enzyme. The δ-subunit of the active form of the enzymes was acetylated. The acetyl group may form a thioester bond with the thiol group of the prosthetic group covalently linked to the enzyme. It suggests that such molecular organization is common in all malonate decarboxylases.

Key words : acetyl enzyme, *Acinetobacter calcoaceticus*, malonate decarboxylase, *Pseudomonas fluorescens*, *Pseudomonas putida*.

Malonate decarboxylase, which catalyzes the decarboxylation of malonate to acetate and CO_2, has been studied in various bacteria (Takamura and Kitayama, 1981; Hilbi *et al.*, 1992; Janssen and Harfoot, 1992; Kim and Byun, 1994; Dehning and Schink, 1994; Schmid *et al.*, 1996). It includes bacteria grown aerobically on malonate, *Pseudomonas putida* (originally *P. ovalis*), *Acinetobacter calcoaceticus*, and *Klebsiella pneumoniae* and those grown anaerobically on malonate, *Malonomonas rubra, Sporomusa malonica, Rhodobacter capsulatus, Klebsiella oxytoca*, and *Citrobacter diversus*. These enzymes however were purified only from *P. putida, A. calcoaceticus*, and *K. pneumoniae*, and then characterized (Takamura and Kitayama, 1981; Kim and Byun, 1994; Schmid *et al.*, 1996). Malonate decarboxylase from *P. putida* was a soluble cytosolic enzyme, whereas that from *M. rubra* was a membrane bound enzyme.

To make decarboxylation of malonate chemically feasible, malonate should be converted to an activated form. Malonyl-CoA has been suggested to be the activated form in degradation of malonate by *P. fluorescens, P. putida, S. malonica, R. capsulatus, K. oxytoca*, and *C. diversus*. However, a different malonate decarboxylation system has been described recently where the free malonic acid is decarboxylated after enzyme activation through acetylation (Hilbi *et al.*, 1992). *Malonomonas* enzyme was known to be a typical acetyl-enzyme. Malonate decarboxylase, an acetyl-enzyme as an active form, was also studied in *A. calcoaceticus* grown aerobically on malonate but its subunits were reported to be mistakenly three (Kim and Byun, 1994). Very recently the enzyme was also purified from *K. pneumoniae* grown aerobically on malonate and characterized to be an acetyl-enzyme composed of four different subunits (Schmid *et al.*, 1996). The smallest subunit was reported to play an important role as an acyl-carrier protein. These results suggest a common molecular structure of malonate decarboxylase. So, it is concluded that in order to understand the structure of malonate decarboxylase, the enzymes from various sources must be re-investigated. In this paper we present a common molecular structure of malonate decarboxylases purified from *A. calcoaceticus, P. fluorescens*, and *P. putida*.

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Materials and Methods

Materials

A. calcoaceticus var. Kim is a bacterium isolated from

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 Biochemistry and Biotechnology (Taeduck, Korea) and IAM Culture Collection Center (Tokyo, Japan), respectively. Sodium malonate, Mops, *p*-nitroblue tetrazolium, 5-bromo-3-indolyl phosphates, malonyl-CoA, acetyl-CoA, bromoacetate, *N*-ethylmaleimide, and acetic anhydride were purchased from Sigma Chem. Co. (St. Louis, USA). [2-^{14}C]Malonate (56.8 mCi/mmol) was obtained from DuPont NEN, and bromo[1-^{14}C]acetate (55 mCi/mmol) was from Amersham Co. (Amersham, UK). Immobilon nitrocellulose transfer membrane was purchased from Millipore Co. (Massachasett, USA). All other reagents were of analytical grade.

Growth of bacteria

A. calcoaceticus, *P. fluorescens*, and *P. putida* were grown aerobically at 30°C for 8 h, 18 h, and 18 h, respectively on malonate as a sole carbon and energy source. The growth medium contained 0.6% malonic acid, 0.3% KH_2PO_4, 0.3% NH_4Cl, 0.04% $MgSO_4 \cdot 6H_2O$, and 0.01% $FeSO_2 \cdot 7H_2O$. 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

Malonate deacrboxylase activity was determined at 30°C, by the method reported previously (Byun and Kim, 1994).

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°C, 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°C 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 precipitates 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 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°C. The enzyme eluted from PBE 94 was applied again on a ω-aminohexyl agarose column (2.0×4.0 cm) and followed by 20% buffer B washing. 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 different bacteria and were concentrated to about 2~3 mg/ml using PM-30 Amicon ultrafiltration membrane and then stored at 4°C.

Gel Electrophoresis

PAGE on 9% slab gels was performed. In order to find the location of enzyme, some gel was sliced and soaked for 12 h in 0.1 M Mops buffer, pH 6.8, and then the enzyme activity was determined. For subunit analysis, the enzyme was eluted from the gel slice containing malonate decarboxylase, and subjected to SDS/PAGE according to the procedure of Laemmli (1970). The protein on the gel was stained with Coommassie blue R-250 or silver staining reagent.

Immunoblot analysis

Antisera against purified malonate decarboxylases from *A. calcoaceticus* and *P. fluorescens* were prepared in New Zealand White rabbit. IgG fractions were prepared by ammonium sulfate precipitation and DEAE-Sephacel chromatography. Crude extracts and the purified enzymes from three bacteria were subjected to SDS/PAGE, and the proteins were transferred electrophoretically to nitrocellulose filter according to the method of Sambrook *et al.* (1989). Immunodetection was carried out with two antibodies prepared as the first antibodies (1:5000 dilution), and alkaline phosphatase-conjugated goat anti-rabbit antibody as the second antibody.

Inactivation

Malonate decarboxylases were treated with group specific reagents, such as pyridoxal-5′-phosphate, diethylpyrocarbonate, *N*-acetylimidazole, iodoacetamide, bromoacetate, *N*-ethylmaleimide, and 5,5′-dinitrobis(2-nitrobenzoic acid) to find functional group prior to or after activation by malonyl-CoA. Bromo[1-^{14}C] acetate (5 nmol) was used to label the thiol group located on the active site of the enzymes (13 μg). After PAGE, ^{14}C-carboxymethylated enzymes were eluted from the gel. The location of the isotopically labeled polypetide was determined by autoradiography of SDS/PAGE gel. ^{14}C-Carboxymethylated enzyme (0.05 ml, 0.1 mg) labelled with bromo[1-^{14}C]acetate was denatured

(10 min, at 100°C), and the prosthetic group was hydrolysed by NaOH (final concentration of 0.1 M). After the protein was precipitated by adding of 0.03 ml 37% HCl and centrifugation at 15,000×*g* for 30 min, the presence of radioactivity on δ-subunit was checked by autoradiography following SDS/PAGE.

Results and Discussion

Purification

Malonate decarboxylases were purified from *A. calcoaceticus*, *P. fluorescens* and *P. putida* by the same method reported previously (Kim and Byun, 1994). Table 1 summarizes the purification procedure. All enzymes were rapidly inactivated during purification but activity was recovered by treatment with malonyl-CoA or acetic anhydride. Following streptomycin sulfate treatment, chromatography with PBE 94 and ω-aminohexyl agarose, apparently pure enzymes were obtained. Specific activity of *P. putida* enzyme was twice as high as that of *A. calcoacetcus* and about five times that of *P. fluorescens*. Each purified enzyme showed a single protein band on PAGE gel and enzyme activity was found to be located on the protein (data not shown), indicating the tight association of subunits like the enzyme from *K. pneumoniae* (Schmid *et al.*, 1996). The subunits of *M. rubra* malonate decarboxylase were known to be dispersed in membrane and cytoplasm (Hilbi and Dimroth, 1994).

Molecular mass and oligomeric structure

The molecular mass of malonate decarboxylases from *A. calcoaceticus* was reported to be 185 kDa. Those of the enzymes from *P. fluorescens* and *P. putida* were estimated to be identically 200 kDa by a pore gradient PAGE, and 220 kDa by a gel filtration with Protein Pak 300 SW gel filtration column (0.75×30 cm) connected on HPLC. This molecular size is a little larger than that (185 kDa) of *Acinetobacter* enzyme but it is considerably larger than that (142 kDa) of *Klebsiella* enzyme (Schmid *et al.*, 1996). SDS/PAGE of the purified enzymes showed four protein bands, with molecular mass of 65 (α), 33 (β), 30 (γ), and 11 (δ) kDa for two *Pseudomonas* enzymes, and with molecular mass of 65 (α), 32 (β), 25 (γ), and 11 (δ) kDa for *Acinetobacter* enzyme which is different from the previous report (Takamura and Kitayama, 1981; Kim and Byun, 1994) (Fig. 1A). These subunit compositions were very similar with those (α,β,γ and δ with molecular size of 65, 34, 30, and 12 kDa) of the enzyme from *K. pneumoniae* (Schmid *et al.*, 1996). However, the stoichiometry of the subunits of these enzymes (2:1:1:1), estimated from the amount of Coomassie blue R-250 dye bound to each subunit protein on SDS/PAGE using densitometry, was different from that of *K. pneumoniae* enzyme (1:1:1:1). It seems that this difference was derived from the different estimation of molecular mass of the malonate decarboxylase complex, or the different nature of proteins for staining. Interestingly the δ-subunit of the enzyme from *K. pneumoniae* was reported undetectable by silver staining (Schmid *et al.*, 1996), contrary to that of *Acinetobacter* enzyme which was intensively stained by silver nitrate (Fig. 1B) rather than Coomassie blue.

Table 1. Purification of malonate decarboxylases from *A. calcoaceticus*, *P. fluorescens*, and *P. putida*. The enzyme activity was determined by spectrometric method. Malonyl-CoA (3 nmol/assay) was added for reactivation of the enzyme prior to the additiion of malonate.

Step	Total protein (mg)	Total unit (μmol/min)	Specific activity (U/mg)	Recovery (%)	Purification fold
A. calcoaceticus					
Crude extract	1092.0	476.0	0.44	100.0	1.00
Streptomycin sulfate	815.0	374.1	0.46	78.6	1.05
PBE 94	106.4	165.7	1.56	34.8	3.98
ω-Aminohexyl agarose	10.0	101.2	10.02	21.3	22.98
P. fluorescens					
Crude extract	902.0	501.8	0.56	100.0	1.00
Streptomycin sulfate	750.0	485.0	0.65	76.7	1.15
PBE 94	10.3	26.7	2.58	5.0	4.61
ω-Aminohexyl agarose	3.8	18.4	4.48	3.7	8.64
P. putida					
Crude extract	649.6	1516.0	2.33	100.0	1.00
Streptomycin sulfate	586.1	1277.8	2.18	84.3	0.94
PBE 94	147.5	1085.4	7.36	71.6	3.16
ω-Aminohexyl agarose	8.8	207.4	23.56	13.7	10.13

Immunological properties

Antibody prepared against malonate decarboxylase from *P. fluorescens* showed a cross reactivity against α-subunit of the enzyme from *P. putida*. However it did not show reactivity against the enzyme from *A. calcoaceticus* (Fig. 2C). This result was also confirmed by antibody prepared against the enzyme purified from *Acinetobacter* (Fig. 2B). The enzymes from two different sources are immunologically different, suggesting a very distinct amino acid sequence difference between them.

Thiol group for acetylation

The purified enzymes from three different bacteria were pretreated with various modifying reagents to analyze functional amino acids for decarboxylation of malonate. As shown in Table 2, the treatment of the enzyme with most thiol group-directed reagents completely prevented its reactivation by malonyl-CoA. However, acetyl enzyme, prepared by preincubation with malonyl-CoA were fully protected from the inactivation. These results indicated that malonate decarboxylases from *Pseudomonas* had a catalytically essential thiol group for decarboxylation of malonate, which is similar to the case of *A. calcoaceticus*, *M. rubra* and *K. pneumoniae*, reported previously (Hilbi *et al.*, 1992; Kim and Byun, 1994; Schmid *et al.*, 1996). This thiol group in *Klebsiella* enzyme is known not to be on cysteine but on a prosthetic group, 2′-(5″-phosphoribosyl)-3′-dephospho-CoA, linked to serine residue of the enzyme (Schmid *et al.*, 1996). When the en-

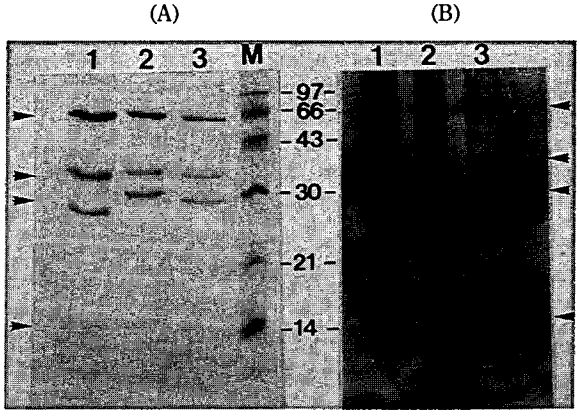

Fig. 1. SDS/PAGE of three malonate decarboxylases which were stained by Coomassie brilliant blue (A) or silver nitrate (B). All three malonate decarboxylases showed four polypeptides with molecular masses 65, 32, 25, and 11 kDa for *A. calcoaceticus* enzyme (lane 1); 65, 33, 30, and 11 kDa for *P. fluorescens* enzyme (lane 2) and *P. putida* enzyme (lane 3). Five micrograms of the enzyme were loaded onto each lane.

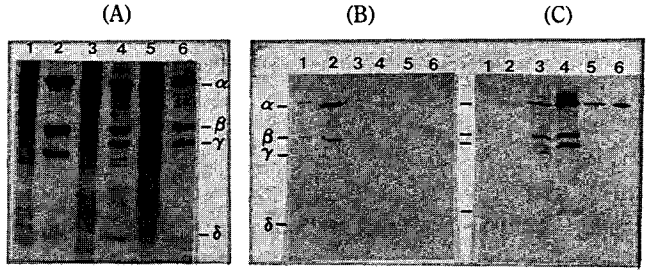

Fig. 2. The immunological analysis of the enzymes from *A. calcoaceticus*, *P. fluorescens*, and *P. putida*. SDS/PAGE of three enzymes were performed (A), followed by immunoblotting with antibodies prepared against *Acinetobacter* enzyme (B) and *P. fluorescens* enzyme (C). The crude extracts from *A. calcoaceticus* (lane 1), *P. fluorescens* (lane 3) and *P. putida* (lane 5), and the purified enzymes from *A. calcoaceticus* (lane 2), *P. fluorescens* (lane 4), and *P. putida* (lane 6) were used.

Table 2. Effect of chemical reagents on malonate decarboxylase. The purified deacetyl enzymes were incubated at 30°C for 30 min with the reagents listed. After reactivation of these unmodified enzymes with 3 nmol malonyl-CoA, the enzyme activities were measured

Reagents	Concentration (mM)	Specific activity, U/mg (%)		
		A. calcoaceticus	*P. fluorescens*	*P. putida*
Control		10.0(100.0)	4.9(100.0)	25.7(100.0)
Pyridoxal phosphate	1.0	8.2(82.5)	5.5(112.0)	23.6(92.0)
Diethylpyrocarbonate	1.0	9.2(91.5)	5.6(114.0)	22.6(88.0)
N-Acetylimidazole	1.0	9.5(95.4)	5.7(117.0)	24.6(95.6)
Iodoacetamide	0.5	5.8(58.1)	N.D.	N.D.
Malonyl-CoA/Iodoacetamide[a]	0.5	8.9(89.1)	N.D.	N.D.
Bromoacetate	0.5	0.9(9.3)	0.1(2.0)	0.4(1.5)
Malonyl-CoA/Bromoacetate	0.5	9.8(98.0)	4.0(85.2)	23.7(92.2)
N-Ethylmaleimide (NEM)	0.5	0.1(1.4)	0.2(3.6)	0.2(0.8)
Malonyl-CoA/NEM[a]	0.5	11.0(110.0)	4.5(92.5)	28.4(105.5)
DTNB	0.5	1.5(14.5)	N.D.	N.D.
Malonyl-CoA/DTNB[a]	0.5	13.2(132.2)	N.D.	N.D.

DTNB : 5,5′-dinitro-bis(2-nitrobenzoic acid), N.D. : not determined.

[a] The purified enzymes were activated by 10 μM of malonyl-CoA prior to the modification by thiol-directed reagents.

Table 3. Reactivation of deacetyl malonate decarboxylase. The purified deacetyl enzymes were incubated at 30°C for 5 min with the compounds listed. The enzyme activities were measured by tracer assay using [1-^{14}C]malonate (Byun and Kim, 1994)

Addition to the (inactivated) deacetyl enzyme	Malonate decarboxylase activity U/mg (%)		
	A. calcoaceticus	*P. fluorescens*	*P. putida*
None	1.8(19.2)	0.3(7.7)	2.0(7.8)
10 mM CoA	2.4(25.5)	0.4(9.1)	2.0(8.2)
1 mM ATP/2 mM acetate	0.9(20.2)	0.4(9.5)	2.5(10.0)
10 mM malonyl-CoA	9.4(100.0)	4.4(100.0)	25.0(100.0)
10 mM acetyl-CoA	8.1(86.2)	2.3(52.8)	20.6(82.3)
10 mM methylmalonyl-CoA	7.9(84.0)	2.2(50.0)	23.7(94.9)
10 mM succinyl-CoA	5.7(60.6)	1.5(34.1)	N.D.
10 mM propyonyl-CoA	3.8(40.4)	2.8(63.6)	8.6(34.5)
0.5 mM acetic anhydride	5.2(55.3)	4.0(90.9)	21.1(84.3)

N.D. : not determined.

zymes labeled with bromo[1-^{14}C]acetate were treated with alkali and they were run on SDS/PAGE gel, the [^{14}C]carboxymethyl group disappeared (data not shown). The result that [^{14}C]carboxymethyl group linked by a thioester of a prosthetic group is labile by alkaline treatment, indicates that all malonate decarboxylase may have the same prosthetic group suggested in *K. pneumoniae* enzyme (Schmid *et al.*, 1996).

Acetyl enzymes

Catalytically inactive malonate decarboxylases from *Pseudomonas* were reactivated by incubation with various acyl-CoAs or acetic anhydride (Table 3). The best reactivation was achieved with 1 μM malonyl-CoA which is about a 2-fold molar excess of the enzyme concentration. But the reactivation of the enzyme by acetic anhydride was obtained with 20 μM acetic anhydride which is about a 40-fold molar excess of the enzyme. After dialysis of the malonyl-CoA-treated enzyme, malonate decarboxylases retained full activity, indicating that the activation of the enzymes was by acylation. Acyl enzymes, prepared by the preincubation of the purified *Pseudomonas* enzymes with malonyl-CoA, decarboxylated [2-^{14}C]malonate, formed [^{14}C]acetyl enzymes which were isolated by gel filtration with Sephadex G-25 (1.0×30 cm) (data not shown). The radioactivity of the labeled enzymes was completely liberated from the protein, upon the addition of malonate. From labeling the *Pseudomonas* enzymes with [1-^{14}C] acetyl-CoA, the molar ratio of ^{14}C-acetyl group to enzyme was determined to be 0.89 and 0.71, indicating that one acetyl group is bound to one molecule of enzyme. By the results described above, two *Pseudomonas malonate* decarboxylases were also proved to be acetyl enzymes in addition to those from *A. calcoaceticus* and *K. pneumoniae* (Kim and Byun, 1994; Schmid *et al.*, 1996). In order to find the location of acetyl group on the enzyme, the enzymes labeled with bromo[1-^{14}C]acetate were run on SDS/PAGE gel, and the gel was dried and exposed to X-ray film. Three malonate decarboxylases were uniformly labelled (Fig. 3A), however only δ-subunits of the enzymes were labeled (Fig. 3B) similar to an acyl carrier δ-subunit which was reported in *K. pneumoniae* enzyme.

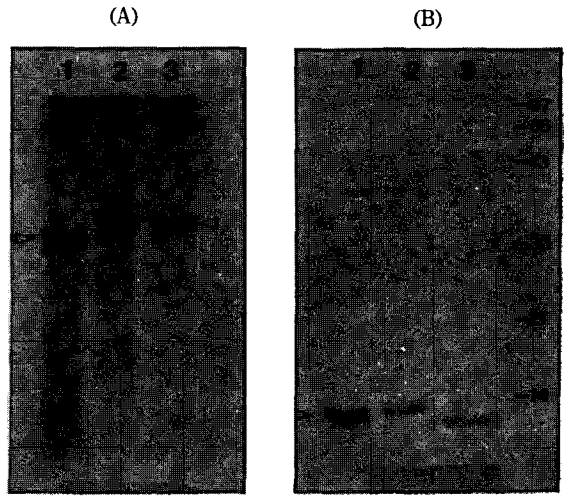

Fig. 3. The identification of acyl-carrier protein for malonate decarboxylases. Purified deacyl enzymes (13 μg) from *A. calcoaceticus* (lane 1), *P. fluorescens* (lane 2), and *P. putida* (lane 3) were treated with 5 nmol of bromo[1-^{14}C]acetate. Following PAGE (A), SDS/PAGE (B) of these labelled enzymes which were eluted from (A), were performed. The gels were dried and exposed to X-ray film.

In summary, evidence presented above strongly indicates that all malonate decarboxylases from *A. calcoaceticus, K. pneumoniae, P. putida* and *P. fluorescens*, are multimeric enzymes consisting of four subunits with the smallest subunit, δ, being an acyl carrier protein. An active form of malonate decarboxylase is an acetyl enzyme which an acetylgroup is linked to a thiol group of the δ-subunit. The thiol group was found not to be on cysteine but may be 2′-(5″-phosphoribosyl)-3′-dephospho-CoA linked covalently to seryl residue of the enzyme according to a previous report (Schmid *et al.*, 1996). This concludes that such molecular organization may be common in all malonate decarboxylases.

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