The Acyl Carrier Protein of Malonate Decarboxylase of *Malonomonas rubra* Contains 2′-(5″-Phosphoribosyl)-3′-dephosphocoenzyme A as a Prosthetic Group†

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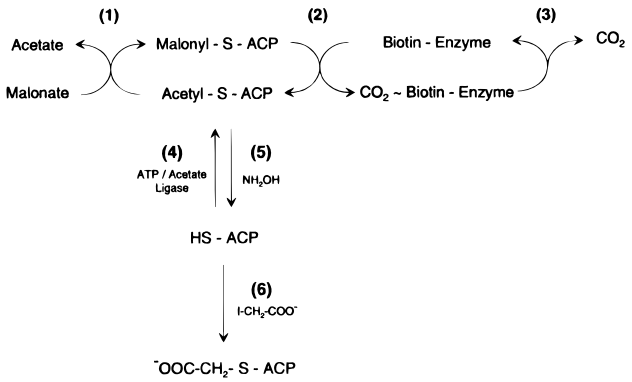
ABSTRACT: Malonate decarboxylase of *Malonomonas rubra* is composed of soluble and membrane-bound components and contains an acetyl residue that is essential for catalytic activity. Upon incubation with hydroxylamine, the acetyl residue is removed, forming an inactive thiol enzyme, which is reactivated by acetylation with ATP, acetate, and a specific ligase. After incubation of the thiol enzyme with iodoacetate in the presence of excess dithioerythritol, the prosthetic group thiol residue was carboxymethylated and reactivation by acetylation was impaired. Radioactive labeling with [1-^{14}C]iodoacetate revealed the site of carboxymethylation on a distinct cytoplasmic protein with the apparent molecular mass of 14 000 Da. The same protein was specifically labeled by enzymic acetylation of the thiol enzyme with [1-^{14}C]acetate and ATP. Malonate decarboxylation by [^{14}C]acetyl malonate decarboxylase resulted in the release of the radioactive acetyl residue from the enzyme, indicating that this acetyl residue is exchanged for a malonyl residue during catalysis. The acyl carrier protein has been purified as its [^{14}C]carboxymethylated derivative to apparent homogeneity. The prosthetic group of the acyl carrier protein was isolated after alkaline hydrolysis, and its chemical structure was identified by high-performance liquid chromatography (HPLC) with the corresponding compound from citrate lyase from *Klebsiella pneumoniae* as reference and by mass spectrometry. Malonate decarboxylase was found to carry the same prosthetic group as citrate lyase, i.e. 2′-(5″-phosphoribosyl)-3′-dephospho-CoA.

Malonomonas rubra, a Gram-negative anaerobic bacterium isolated from marine sediments, is able to grow on malonate which is decarboxylated to acetate (Dehning & Schink, 1989). Malonate decarboxylase, an essential enzyme of substrate degradation and energy conservation, is related to the sodium ion pumping decarboxylases from other sources, e.g. the oxaloacetate decarboxylase from *Klebsiella pneumoniae*, the methylmalonyl-CoA decarboxylase from *Propionigenium modestum* or *Veillonella parvula*, and the glutaconyl-CoA decarboxylase from *Acidaminococcus fermentans* [for a review, see Dimroth (1987)]. These membrane-bound, biotin-containing enzymes are activated by sodium ions and convert the free energy of the decarboxylation reaction into an electrochemical sodium ion gradient ($\Delta\tilde{\mu}Na^+$). Analogous features of the malonate decarboxylase are the participation of a biotin protein and membrane-bound protein components in catalysis and the activation by sodium ions (Hilbi et al., 1992, 1993; Hilbi & Dimroth, 1994). This decarboxylase, however, does not form a stable membrane-bound complex but consists after cell rupture of several water soluble and membrane-bound enzyme components (Hilbi et al., 1992).

Malonate decarboxylase performs a unique mechanism of substrate activation in order to make malonate decarboxylation under physiological conditions (∼30 °C) chemically feasible. The reactions involved in malonate activation and its subsequent decarboxylation are summarized in Scheme 1 [see also Hilbi and Dimroth (1994)]. The catalytically active form of the decarboxylase is generated by acetylation of a SH group of an acyl carrier protein with ATP, acetate, and a specific ligase (step 4, Scheme 1). The acetyl-*S*-acyl carrier protein is then converted to malonyl-*S*-acyl carrier protein by a specific transferase, which has been purified and characterized (step 1, Scheme 1) (Hilbi & Dimroth, 1994). The free carboxyl group of the thus activated malonyl thioester derivative is then transferred to a biotin protein (step 2, Scheme 1), and the acetyl thio ester residue on the acyl carrier protein is regenerated. Decarboxylation of the carboxybiotin derivative with a membrane-bound enzyme completes the decarboxylation of malonate (step 3, Scheme

Scheme 1: Proposed Mechanism of the Malonate Decarboxylase of *M. rubra* (Steps 1−3) and of the Inactivation−Reactivation Pathways of the Enzyme System (Steps 4−6)[a]



[a] ACP is the acyl carrier protein. The enzymes catalyzing the individual steps are as follows: (1) acetyl-*S*-acyl carrier protein: malonate acyl carrier protein-SH transferase, (2) malonyl-*S*-acyl carrier protein carboxytransferase, (3) carboxybiotin decarboxylase, and (4) HS-acyl carrier protein:acetate ligase. Steps 5 and 6 are nonenzymic reactions.

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1). This last step is believed to be coupled to sodium ion pumping.

A very similar substrate activation mechanism is known for citrate lyase from *K. pneumoniae* (Buckel et al., 1971; Dimroth, 1988) and citramalate lyase from *Clostridium tetanomorphum* (Buckel & Bobi, 1976). These enzymes contain an acyl carrier protein subunit of about 10 000 Da (Dimroth & Eggerer, 1975; Buckel & Bobi, 1976) that is acetylated at its 2′-(5″-phosphoribosyl)-3′-dephospho-CoA prosthetic group which is covalently attached by a phospho diester bond to serine 14 of the protein (Robinson et al., 1976; Oppenheimer et al., 1979; Dimroth, 1976; Dimroth & Loyal, 1977; Beyreuther et al., 1978). During catalysis, the acetyl residue is exchanged by a citryl or citramalyl group and regenerated upon cleavage of the thus activated substrate derivatives (Dimroth et al., 1977a,b).

Removal of the acetyl thio ester residues from the lyases or from malonate decarboxylase by treatment with e.g. hydroxylamine has been shown to inactivate each of these enzymes (Buckel et al., 1971; Hilbi et al., 1992). Enzymic acetylation with ATP, acetate, and a specific ligase or chemical acetylation with acetic anhydride reactivated the deacetylated enzyme specimens (Buckel et al., 1971; Hilbi et al., 1992; Schmellenkamp & Eggerer, 1974). Upon treatment of the deacetylated lyases with iodoacetate in the presence of excess dithioerythritol, the prosthetic group of the respective enzyme became carboxymethylated (Buckel & Bobi, 1976; Dimroth & Eggerer, 1975). Since these derivatives contained a stable thio ether, the lyases were irreversibly inhibited.

The remarkable similarities between malonate decarboxylase and the lyases prompted us to investigate whether the analogies could be extended to the specific reaction with iodoacetate and to the structure of the thiol cofactor. We show here that malonate decarboxylase is irreversibly inhibited by iodoacetate and that this reaction with radiolabeled iodoacetate can be used to identify the acyl carrier protein component of the enzyme. The lyases and malonate decarboxylase from *M. rubra* harbor the identical thiol prosthetic group bound covalently to an acyl carrier protein.

MATERIALS AND METHODS

Materials. *S*-(Carboxymethyl)cysteamine was prepared according to De Marco et al. (1964). 3′-Dephospho-CoA was prepared from CoA-SH by treatment with alkaline phosphatase (Boehringer Mannheim). *M. rubra* was a gift from B. Schink (Konstanz), and the bacteria were grown in a 300 l fermenter (Hilbi et al., 1992). Citrate lyase from *K. pneumoniae* was isolated as described (Dimroth et al., 1977b).

General Procedures. Cell rupture, deacetylation of malonate decarboxylase in cell extract, reacetylation, and determination of malonate decarboxylase activity were described elsewhere (Hilbi et al., 1992). Protein concentrations were determined by the method of Bradford (1976), using bovine serum albumin as a standard. SDS−PAGE[1] was performed using the procedure of Schaegger and von Jagow (1987). In order to separate samples containing [^{14}C]-acetyl-acyl carrier protein, 2-mercaptoethanol was omitted in the loading buffer. Autoradiography was performed according to Sambrook et al. (1989).

Carboxymethylation of Deacetylated Malonate Decarboxylase with Iodoacetate and [1-^{14}C]Iodoacetate. Cell free extract of *M. rubra* containing deacetyl malonate decarboxylase [in 50 mM potassium phosphate buffer (pH 7.5) containing 50 mM NaCl and 1 mM $MgCl_2$] was incubated with 20 mM DTE (15−30 min, pH 7.5, room temperature) in order to completely reduce the sulfhydryl residue of the prosthetic group of the acyl carrier protein. Following the addition of 0.1 or 1 mM iodoacetate, the incubation was continued for another 30−60 min. The control was kept under the same conditions without addition of iodoacetate. Malonate decarboxylase activity was measured after enzymic acetylation with ATP and acetate.

[^{14}C]Carboxymethylation was performed as described above with 0.1 mM [1-^{14}C]iodoacetate (10 mM in 50 mM Tris/HCl, pH 8.0, 5.9 mCi/mmol). The mixture was then ultracentrifuged (30 min, 200 000*g*), and the supernatant was used without further treatment for SDS−PAGE and purification of the acyl carrier protein.

Labeling of the Acyl Carrier Protein with [1-^{14}C]Acetate and Release of the [1-^{14}C]Acetyl Residue from the Enzyme by Incubation with Malonate or Succinate. Cell extract containing the deacetylated malonate decarboxylase was incubated for 3 min at 30 °C with 5.0 mM ATP and 2.6 mM [1-^{14}C]acetate (21.3 mM in ethanol, 47.0 mCi/mmol).

The exchange reaction was started by the addition of 100 mM malonate or 100 mM succinate. Samples of 10 μL were precipitated with 30 μL of 20% trichloroacetic acid. To each sample was added 0.4 mg of serum albumin as a carrier. The protein precipitates were centrifuged and washed four times with 0.5 mL of 12% trichloroacetic acid. Subsequently, the pellet was dissolved in 0.1 mL of 0.1 M NaOH and counted after addition of 4 mL of Scintigel and 0.1 mL of 0.1 M HCl.

Prior to SDS−PAGE of [1-^{14}C]acetyl-acyl carrier protein, membrane components were removed by ultracentrifugation.

Purification of the Acyl Carrier Protein. [1-^{14}C]Carboxymethylated cytoplasm prepared as described above (10 mL) was loaded onto a Fractogel TSK-DEAE column (1.6 × 12 cm) connected to an FPLC apparatus (Pharmacia). The column was equilibrated with buffer I [50 mM potassium phosphate (pH 7.5)], and the following gradient of buffer II [50 mM potassium phosphate and 1 M NaCl (pH 7.5)] was applied at 1.0 mL/min: 0−50 mL, 0% buffer II; 50−150 mL, 0−40% buffer II. The radioactive protein eluted in a sharp peak at about 150 mM NaCl. The pooled fractions were adjusted to 0.5 M ammonium sulfate, centrifuged (10 min, 20 000*g*), and pumped onto a Fractogel EMD-Propyl column (1.0 × 14 cm) equilibrated with buffer III [50 mM potassium phosphate and 0.5 M ammonium sulfate (pH 7.5)]. Under these conditions, the radioactive protein did not bind to the column and was eluted with 40 mL of buffer III. Pooled fractions containing the radioactivity were adjusted to 1.5 M ammonium sulfate, centrifuged (10 min, 20 000*g*), and again loaded onto the Fractogel EMD-Propyl column (1.0 × 14 cm) equilibrated with buffer IV [50 mM potassium phosphate and 1.5 M ammonium sulfate (pH 7.5)]. The protein was eluted with a stepwise gradient of buffer I at 2.5 mL/min: 0−40 mL, 0% buffer I; 40−45 mL, 0−50%

[1] Abbreviations: DTE, dithioerythritol; FPLC, fast protein liquid chromatography; HPLC, high-performance liquid chromatography; MALDI, matrix-assisted laser desorption ionization; PAGE, polyacrylamide gel electrophoresis; PVDF, poly (vinylidene difluoride); SDS, sodium dodecyl sulfate; TLC, thin layer chromatography

buffer I; 45−80 mL, 50% buffer I. The radioactive fractions which eluted at 750 mM ammonium sulfate were pooled, concentrated by ultrafiltration to about 1 mL (PM-10 membrane, Amicon), and chromatographed on a Superdex 75 preparatory grade column (1.6 × 60 cm) equilibrated with buffer V [50 mM potassium phosphate and 150 mM NaCl (pH 7.5)] at 0.5 mL/min. The labeled protein eluted after about 60 mL of buffer V and was concentrated to 0.5 mL by ultrafiltration (PM-10 membrane, Amicon). After dilution with 4.5 mL of water, the sample was pumped onto a Mono Q column (0.5 × 5 cm) equilibrated with buffer I and was eluted in a linear gradient of buffer II (0−10 mL, 0% buffer II; 10−35 mL, 0−20% buffer II). Radioactive fractions which eluted at about 100 mM NaCl were pooled and concentrated by ultrafiltration (Centricon 10, Amicon).

N-Terminal Sequence of the Acyl Carrier Protein and Attachment Site of the Prosthetic Group. Purified (partially) acyl carrier protein was separated by SDS−PAGE, electroblotted onto a PVDF membrane, and stained with 0.1% Coomassie Brilliant Blue, and the 14 kDa band was cut out. The following N-terminal sequence was determined by Edman degragation (Dr. P. James, Protein Chemistry Facility, ETH Zürich): MEGML^{5}NELNF^{10}KFKSE^{15}NPVDV^{20}-VLPKH^{25}Y.

Purified (partially) acyl carrier protein was treated with 0.1 M NaOH (65 °C, 30 min) to cleave the prosthetic group from the protein. Afterward, the solution was adjusted to pH 8, 10 mM sodium borohydride was added, and incubation was continued for 60 min at 30 °C. A total of 16 μg of enriched acyl carrier protein devoid of the prosthetic group was subjected to N-terminal sequencing by Edman degragation after SDS−PAGE and electroblotting onto a PVDF membrane.

Identification of the Thiol Moiety of the Prosthetic Group by TLC. The acyl carrier protein in the cytoplasm (200 μL) was specifically [1-^{14}C]carboxymethylated as described above. Excess [1-^{14}C]iodoacetate was removed by gel filtration (PD-10 column, Pharmacia, 1.5 × 5 cm) with 0.1% NH_4HCO_3 solution as eluent. The fractions containing protein were freeze-dried under vacuum, and an acid hydrolysis of the peptide bonds was performed (Moore & Stein, 1963). A two-dimensional thin layer chromatogram of the hydrolysate mixed with authentic *S*-(carboxymethyl)-cysteine and *S*-(carboxymethyl)cysteamine was performed (eluent A, 4:1 phenol/water; eluent B, 12:3:5 *n*-butanol/acetic acid/water). Amino groups were visualized with ninhydrin, and the chromatogram was exposed to an X-ray film for 2 weeks: *S*-(carboxymethyl)cysteamine, R_f (A) = 0.70 and R_f (B) = 0.38; *S*-(carboxymethyl)cysteine, R_f (A) = 0.37 and R_f (B) = 0.19.

Isolation of the Prosthetic Group of the Acyl Carrier Protein from M. rubra. The prosthetic group was isolated according to a procedure described by Singh et al. 1977 using an acyl carrier protein preparation enriched by chromatography over the first two columns as described above. The protein in 1.0 mL concentrated eluate was denatured by addition of 50 mM NaOH and heating (30 min, 65 °C). Afterward, the protein was precipitated by adjusting to pH 2.2 with hydrochloric acid, and the supernatant was examined for radioactivity. Since almost none of the enzyme-bound radioactivity was liberated, probably because the base treatment was performed in a buffered solution in which the base strength was not strong enough, the prosthetic group was cleaved from the denatured protein by resuspension of the precipitate in 200 μL of 0.1 M NaOH (65 °C, 30 min). The solution was adjusted to pH 2.6, diluted with 10 mL of 3 mM HCl (pH 2.6), and loaded onto a Mono Q HR column (0.5 × 5 cm) equilibrated with 3 mM HCl. The column was washed with 10 mL of 3 mM HCl, and the prosthetic group was eluted with a linear gradient of LiCl (0 to 100 mM in 30 mL) in 3 mM HCl. Fractions containing both the radioactivity and material absorbing at 260 nm eluted at about 50 mM LiCl. The fractions were pooled and used for the characterization of the prosthetic group.

Isolation of the Prosthetic Group of Citrate Lyase of K. pneumoniae. Citrate lyase (9.6 mg) in 1.0 mL of 0.1 M Tris/HCl (pH 8.0) was [^{14}C]carboxymethylated by incubation with 20 mM DTE (15 min, room temperature) followed by treatment with 0.1 mM [1-^{14}C]iodoacetate (5.9 mCi/mmol) for 60 min, prior to the isolation of the prosthetic group (Singh et al., 1977).

Separation of the Prosthetic Group by HPLC. The analyses by HPLC of the prosthetic groups of the acyl carrier proteins from malonate decarboxylase and citrate lyase were performed by the method described for the analysis of CoA derivatives (Hilbi & Dimroth, 1994; Hoffmann et al., 1989). A Hypersil ODS column (250 mm × 4 mm, 5 μm particle size, Hewlett-Packard) was equilibrated with buffer A [0.2 M potassium phosphate (pH 5.0)] at a flow rate of 1 mL/min, and the compounds were eluted in a linear gradient (20 to 50% within 9.5 min) of buffer B [0.2 M potassium phosphate and 20% acetonitrile (pH 5.0)] and detected at 254 nm. Fractions of 250 μL were collected and analyzed for radioactivity by liquid scintillation. The retention times of peaks containing the radioactivity were compared to those of CoA-SH, 3′-dephospho-CoA-SH, and the respective S-carboxymethylated derivatives. The retention times (in minutes) were as follows: CoA-SH (7.6), 3′-dephospho-CoA-SH (9.9), *S*-(carboxymethyl)-CoA (5.3), *S*-(carboxymethyl)-3′-dephospho-CoA (6.5), and prosthetic groups (6.0).

Identification of the Prosthetic Group by Mass Spectrometry. The prosthetic group of the acyl carrier protein was cleaved and isolated by FPLC as described above. Radioactive fractions were pooled and evaporated to dryness. The colorless solid was dissolved in a minimum of methanol, and 10 volumes of acetone were added to precipitate the prosthetic group (Singh et al., 1977). After centrifugation for 5 min at 15 800*g*, the supernatant was removed and the pellet (∼5 μg) was evaporated to dryness. This isolate was used for mass spectrometry (MALDI, Bruker Reflex). Peaks were detected at *m*/*z* 959.0, 964.2, 970.1, 976.7, and 981.6.

RESULTS

Identification of the Acyl Carrier Protein of the Malonate Decarboxylase. In analogy to the experiments with citrate lyase from *K. pneumoniae* (Dimroth & Eggerer, 1975), we treated crude cell extract of *M. rubra* with iodoacetate in the presence of excess DTE to avoid unspecific carboxymethylation reactions. Under these conditions, it has been reported for citramalate lyase that the sulfhydryl group of the prosthetic group reacts 4 orders of magnitude faster than other mercaptans with iodoacetate (Buckel & Bobi, 1976). Incubation of deacetyl malonate decarboxylase with 0.1 or 1.0 mM iodoacetate in the presence of 20 mM DTE impaired the reactivation by enzymic acetylation to 38 or 70%,

Table 1: Effect of Iodoacetate Treatment of Deacetylated Malonate Decarboxylase in Crude Cell Extracts on Its Enzymic Acetylation[a]

addition to deacetylated malonate decarboxylase	specific activity (U/mg)	degree of reactivation (%)
none	2.47	
20 mM DTE	2.06	100
20 mM DTE + 0.1 mM iodoacetate	1.27	62
20 mM DTE + 1 mM iodoacetate	0.67	30

[a] The enzyme was incubated with the compounds indicated, and malonate decarboxylase activity was determined after reactivation with ATP and acetate.

respectively, compared to the control experiment with no addition of iodoacetate (Table 1). The irreversible inactivation of the deacetyl malonate decarboxylase by iodoacetate indicates that this compound alkylates the sulfhydryl residue of the prosthetic group, thus preventing its acetylation and therefore the reactivation of the enzyme. The activity obtained after acetylation of the enzyme in the presence of 20 mM DTE as the only additive was only 83% of that in the absence of mercaptans. The lower activity probably results from the transesterification of a part of the acetyl residues from the prosthetic group to the added thiol compound. In control experiments, it was shown that incubation of cell extract with 20 mM DTE and 1 mM iodoacetate did not result in a significant inactivation of the ligase responsible for the enzymic acetylation reaction.

In order to determine the site of the iodoacetate reaction, the experiment was repeated with 0.1 mM [1-^{14}C]iodoacetate in the presence of 20 mM DTE. Only a single band with the apparent molecular weight of 14 000 was visible after SDS gel electrophoresis and autoradiography (Figure 1A). Hence, the reaction with [1-^{14}C]iodoacetate led to the specific labeling of a small protein, referred to as the acyl carrier protein of the malonate decarboxylase since the carboxymethylation of a specific thiol group prevented the subsequent acetylation with concomitant reactivation of the enzyme.

The function of this protein that specifically reacts with iodoacetate as acyl carrier was confirmed by labeling with radioactive acetate. After acetylation of deacetyl malonate decarboxylase with [1-^{14}C]acetate, ATP, and the ligase (step 4, Scheme 1), the 14 000 Da protein was strongly labeled (Figure 1B). Hence, the sites for enzymic acetylation and carboxymethylation are probably identical.

Purification of the Acyl Carrier Protein and Participation in Malonate Decarboxylation. The anticipated reaction mechanism of malonate decarboxylase predicted that the acetyl thio ester residue of the acyl carrier protein turns over during catalysis (see Scheme 1). The [1-^{14}C]acetyl residue should therefore be released from the protein upon incubation with malonate, yielding malonyl-acyl carrier protein, the actual substrate of decarboxylation. This was indeed the case (Figure 2). However, if the [^{14}C]acetyl-labeled enzyme was incubated with succinate instead of malonate, a significant release of enzyme-bound radioactivity was not observed. Hence, the release of the [^{14}C]acetate is specific for malonate, indicating the participation of the [^{14}C]acetyl-acyl carrier protein in malonate decarboxylation. Moreover, the transferase catalyzing the exchange reaction (step 1, Scheme 1) (Hilbi & Dimroth, 1994) is specific for malonate and does not accept a related dicarboxylate. In summary, these results

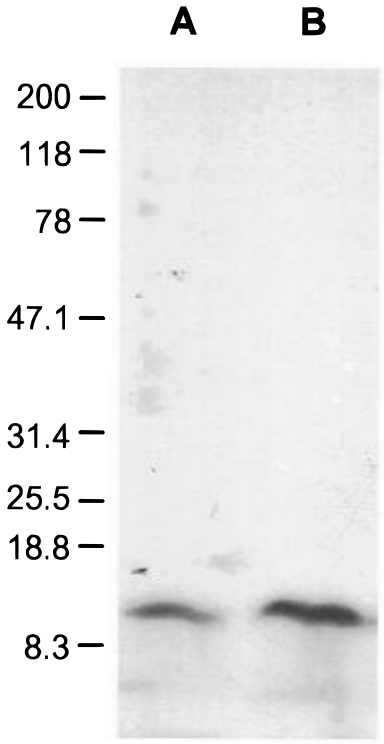


FIGURE 1: Autoradiographs of the ^{14}C-labeled acyl carrier protein separated by SDS−PAGE. (A) The deacetylated malonate decarboxylase in the cytoplasm was incubated with 0.1 mM [1-^{14}C]-iodoacetate in the presence of 20 mM DTE. (B) The deacetylated enzyme in the cytoplasm was incubated with 2.6 mM [1-^{14}C]acetate and 5.0 mM ATP. For details, see Materials and Methods.

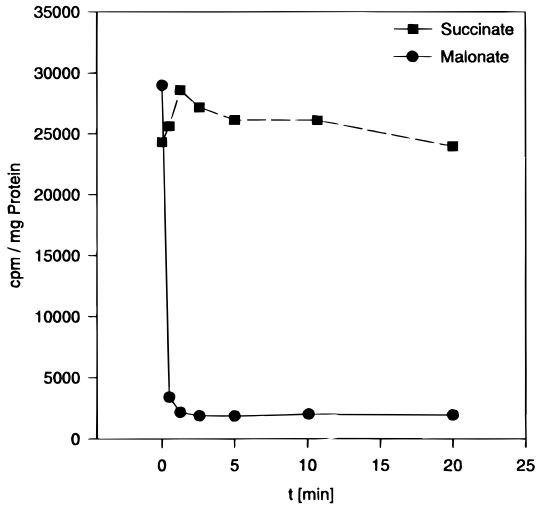


FIGURE 2: Removal of enzyme-bound [^{14}C]acetate during malonate decarboxylation. The deacetylated malonate decarboxylase in cell extract was enzymatically ^{14}C-acetylated by incubation with [1-^{14}C]-acetate and ATP. Following the addition of malonate (●) or succinate (■), samples were precipitated after the indicated times and the residual enzyme-bound radioactivity was determined (see Materials and Methods).

indicate that a protein of 14 000 Da has the function of an acyl carrier protein in the malonate decarboxylation reaction which proceeds via malonyl-*S*-acyl carrier protein.

After specific labeling of the acyl carrier protein with [1-^{14}C]iodoacetate, the thus modified polypeptide could be purified by following the radioactivity in the chromatographic fractions. Pure [^{14}C]carboxymethylated acyl carrier protein was obtained after five chromatographic steps (see Figure 3). The final purification factor of 44 implies that about

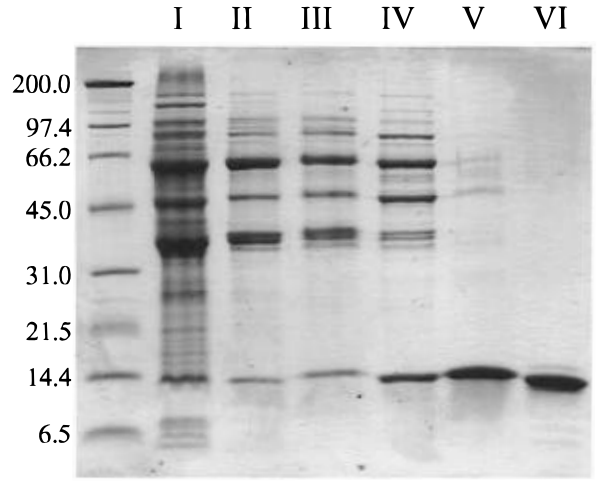


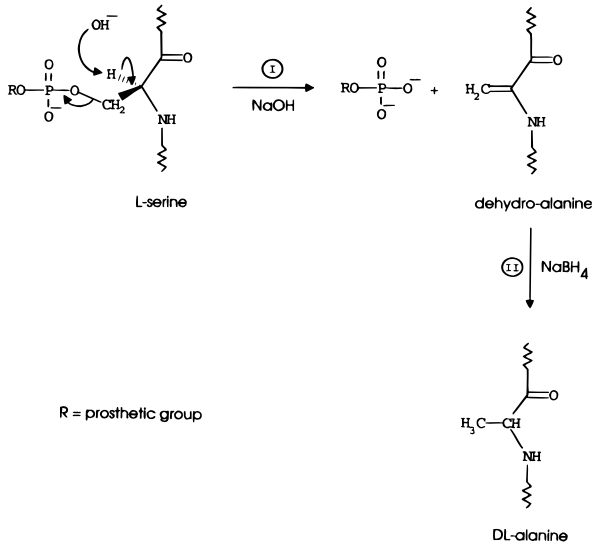
FIGURE 3: SDS gel electropherogram of [1-^{14}C]carboxymethylated acyl carrier protein at different steps of purification. The lanes of a 10% polyacrylamide gel contained (from left to right) 10 μg of cell extract (I) and 5 μg of each of the pooled fractions containing the enzyme-bound radioactivity after the following columns: Fractogel TSK-DEAE (II), Fractogel EMD-Propyl, first and second run (III and IV), Superdex 75 (V), and Mono Q (VI). The lane on the left shows protein standards with molecular masses of 200, 97.4, 66.2, 45.0, 31.0, 21.5, 14.4, and 6.5 kDa.

2.5% of the cytoplasmic protein comprises the acyl carrier protein (cf. Table 2). The UV spectrum of the purified protein showed its maximum at 262 nm (21.9 mM^{-1} cm^{-1}), typical for an adenine-containing protein (Dimroth, 1975) which is evidence for an adenine-containing prosthetic group (data not shown). In addition, the UV spectrum showed a shoulder at 288 nm, which results from tryptophan residues in the protein (one of these has been found in the N-terminal region,Trp-26).

N-Terminal Sequencing and Attachment Site of the Prosthetic Group. In citrate lyase and in citramalate lyase, the prosthetic group is linked to the hydroxyl side chain of a specific serine in the N-terminal region (Ser-14) by a phospho diester linkage (Beyreuther et al., 1978; Dimroth, 1988) which can be cleaved under alkaline conditions. Sequencing of the malonate decarboxylase acyl carrier protein by Edman degragation revealed no homologies to the lyases' acyl carrier, but it contained a serine residue at the same position (Ser-14). This finding together with the fact that the prosthetic group of the decarboxylase's protein can be cleaved in an analogues reaction (see below) prompted us to investigate whether Ser-14 represents the binding site of the prosthetic group. Following alkaline treatment, the putative dehydroalanine was reduced with borohydride to the corresponding alanine (cf. Scheme 2) and the N-terminal sequence of the thus treated acyl carrier protein was determined. Ser-14 was not modified to alanine by the treatment described. Therefore, if the hydrolysis follows the same mechanism as in citrate lyase and citramalate lyase, Ser-14 of the malonate decarboxylase acyl carrier protein is not the attachment site for the prosthetic group.

The Prosthetic Group of the Acyl Carrier Protein of the Malonate Decarboxylase. The catalytic mechanism of malonate decarboxylase is very similar to that of citrate lyase, involving an acetylation and an acyl carrier protein transferase reaction (steps 4 and 1, Scheme 1). The activated substrates thus obtained are malonyl-*S*-acyl carrier protein or citryl-*S*-acyl carrier protein, respectively. Additionally,

Scheme 2: Cleavage of the Prosthetic Group from the Acyl Carrier Protein in a β-Elimination Type Reaction upon Alkaline Treatment (I) and Conversion of the Intermediate Dehydroalanine to the Corresponding Alanine (II)



both acyl carrier protein transferases accept the corresponding CoA derivatives as alternative substrates, i.e. acetyl-CoA, malonyl-CoA, or citryl-CoA (Hilbi & Dimroth, 1994; Dimroth et al., 1977b). These mechanistic homologies might extend to the structure of the prosthetic group of the acyl carrier protein which, in the case of citrate lyase, is a covalently bound 2′-(5″-phosphoribosyl)-3′-dephospho-CoA (Figure 4) (Robinson et al., 1976).

In order to identify the catalytic essential thiol as part of a cysteamine residue, two-dimensional TLC of a total hydrolysate of [^{14}C]carboxymethylated cytoplasm was performed. Almost all the radioactivity was found in one spot which cochromatographed with authentic *S*-(carboxymethyl)-cysteamine, and only a little was found in *S*-(carboxymethyl)-cysteine (data not shown). Since the labeling with [1-^{14}C]-iodoacetate of cytoplasmic proteins was specific for the acyl carrier protein (Figure 1A), the minor spot may be explained by an increased sensitivity of the TLC assay. Here, after hydrolysis of the cytoplasmic proteins, cysteine residues from different proteins which have been unspecifically carboxymethylated to a small extent accumulated in one spot. This result indicates that the catalytically essential thiol group is part of a cysteamine moiety as found for the prosthetic groups of citrate lyase and citramalate lyase.

For further analysis of its structure, the prosthetic group of malonate decarboxylase was isolated as the [^{14}C]carboxymethylated derivative as described for citrate lyase (Singh et al., 1977). After hydrolysis with 100 mM sodium hydroxide at 65 °C (see Scheme 2) (Dimroth, 1976), the prosthetic group could be isolated by chromatography on a Mono Q column (Singh et al., 1977). As a reference, the [^{14}C]carboxymethylated prosthetic group of citrate lyase was isolated under identical conditions. The two prosthetic groups had the same UV absorption spectrum deriving from the adenine moiety. In the subsequent HPLC analysis, the two compounds containing the radioactivity and absorbing at 254 nm eluted with the same retention times and, when cochromatographed, eluted in one peak. Since the reference sample isolated from citrate lyase contained the radioactivity as well as the UV-absorbing chromophore, it could be

Table 2: Purification of the [^{14}C]Carboxymethyl-Acyl Carrier Protein of the Malonate Decarboxylase[a]

	enzyme-bound radioactivity (cpm)	protein (mg)	specific radioactivity (cpm/μg)	yield (%)	purification (-fold)
inactive extract	533 750	66.0	8.1	100	1
cytoplasm	437 920	51.1	8.6	82	1.1
Fractogel TSK-DEAE	274 600	12.5	22	51	2.5
Fractogel EMD-Propyl I	212 700	8.6	25	40	3
Fractogel EMD-propyl II	140 520	3.1	46	26	5
Superdex 75	77 580	0.2	244	15	30
Mono Q	21 614	0.1	354	4	44

[a] During purification, the protein was tracked by its radioactive label

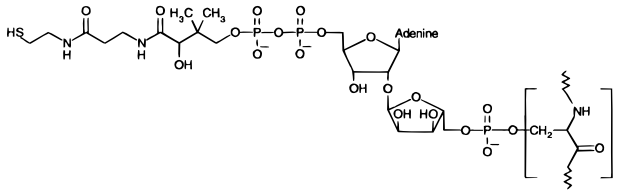


FIGURE 4: Structure of the prosthetic group, 2′-(5″-phosphoribosyl)-3′-dephospho-CoA.

concluded that the 3′-dephospho-CoA moiety was intact. However, it was uncertain whether the second ribose was still attached. Hence, the following CoA derivatives were prepared and separated by HPLC. Coenzyme A was dephosphorylated at its 3′-position with alkaline phosphatase, and the S-carboxymethylated derivatives of CoA and 3′-dephospho-CoA were synthesized. None of the investigated substances had the same chromatographic properties as the prosthetic groups which were identified by their radioactive label (Figure 5). *S*-(Carboxymethyl)-3′-dephospho-CoA, which is the product from a hydrolysis of the glycosidic linkage between the two sugar compounds, was separated from the isolated cofactors. From these results, one may conclude that the second ribose was not cleaved and that the substance isolated from citrate lyase, which was used as a reference, was the intact prosthetic group (shown in Figure 4) in the S-carboxymethylated form. The identical chromatographic behavior of the prosthetic groups of the malonate decarboxylase and citrate lyase acyl carrier proteins on an anion exchange column (Mono Q) at pH 2.6 and on a reversed phase column strongly suggests identical chemical structures for the two compounds. The anticipated chemical structure of the isolate from malonate decarboxylase was confirmed by mass spectrometry (MALDI, Figure 6). The molecular ion peak found in this spectrum was *m*/*z* 959.0 (±0.5 mass units) which is the correct value for the anticipated structure ($C_{28}H_{46}N_7O_{22}P_3S$; MH^+, *m*/*z* 958.7). Since the compound was isolated with a LiCl gradient on an anion exchange column, the five acidic protons on the phosphate residues and the carboxymethyl moiety could be exchanged for lithium ions. The masses of *m*/*z* 964.2, 970.1, 976.7, and 981.6 are readily explained by the exchange of one to four protons for lithium. In summary, the structure of the prosthetic group of the acyl carrier protein of *M. rubra* is 2′-(5″-phosphoribosyl)-3′-dephospho-CoA which is the same as the respective compound of citrate lyase from *K. pneumoniae*.

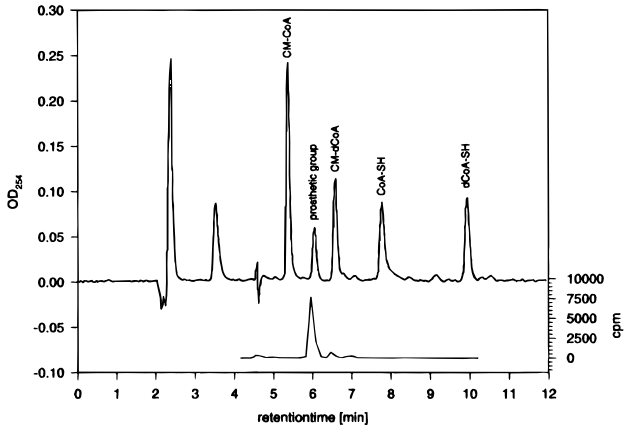


FIGURE 5: HPLC chromatogram of the prosthetic group, isolated from *M. rubra*, and four CoA derivatives: CoA-SH, coenzyme A; dCoA-SH, 3′-dephosphocoenzyme A; CM-CoA, *S*-(carboxymethyl)coenzyme A; and CM-dCoA, *S*-(carboxymethyl)-3′-dephosphocoenzyme A. The left scale is UV absorption at 254 nm; the right scale is radioactivity of the collected fractions. Only the prosthetic group is radioactively labeled.

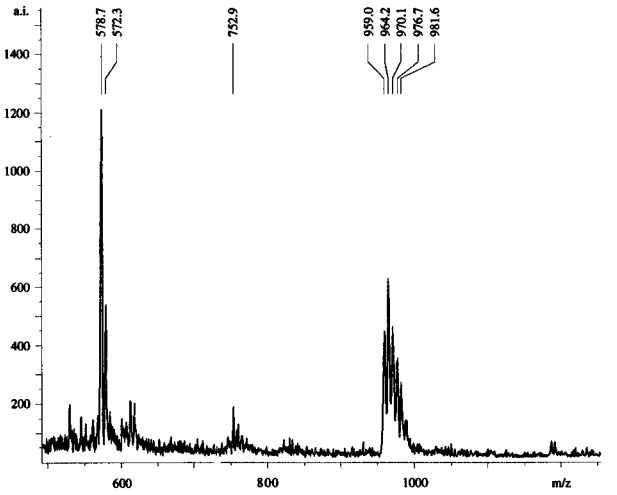


FIGURE 6: Mass spectrum (MALDI) of the isolated prosthetic group from the malonate decarboxylase of *M. rubra*. The calculated values for the anticipated structure ($C_{28}H_{46}N_7O_{22}P_3S$) are *m*/*z* 958.7 for the molecular ion peak (MH^+) and *m*/*z* 964.6, 969.6, 975.5, and 981.4 for the compounds with one to four protons exchanged for lithium ions. Masses are determined with an accuracy of ±0.5 mass units.

DISCUSSION

Malonate decarboxylase is the key enzyme for energy conservation in *M. rubra*, an anaerobic bacterium living from the degradation of malonate to acetate and CO_2 (Dehning & Schink, 1989). It has been shown previously that cytoplasmic and membrane-bound proteins are required to accomplish the decarboxylation of malonate (Hilbi et al., 1992). Furthermore, the reaction was dependent on Na^+ ions which suggested that the decarboxylation energy was directly converted into an electrochemical gradient of Na^+ ions which could then be converted into other forms of biological energy,

e.g. ATP (Hilbi & Dimroth, 1994). In this respect, the malonate decarboxylase belongs to the family of Na^+-translocating decarboxylases comprising oxaloacetate decarboxylase, methylmalonyl-CoA decarboxylase, and glutaconyl-CoA decarboxylase (Dimroth, 1987).

Distinct features between these enzymes and malonate decarboxylase, however, are the number of proteins involved in the catalysis, the physical organization of the catalysts, and the catalytic mechanism. The typical Na^+-translocating decarboxylases consist of three to five subunits that form a tightly membrane-bound complex, and these enzymes catalyze the decarboxylation of dicarboxylic acids which are activated by either a keto group or a coenzyme A thio ester residue in the position β to the carboxylate (Dimroth, 1987). In contrast, a tight membrane-bound complex was not found for the malonate decarboxylase, and this catalysis rather involves several water soluble and membrane-bound proteins. Part of these differences in the oligomeric organization is readily explained by the need to activate the C−C bond of the free malonic acid for cleavage. This problem is elegantly solved by formation of the activated malonyl thio ester on the enzyme. We have shown here that an acyl carrier protein subunit with an M_r 14 000 is the chemical equivalent to coenzyme A for the formation of an activated malonyl thio ester. Acetyl-*S*-acyl carrier protein is formed by acetylation of the catalytically competent SH group of this protein involving acetate, ATP, and a specific ligase. The transferase subunit (Hilbi & Dimroth, 1994) then transfers the acyl carrier protein-SH moiety to malonate, yielding the activated malonyl-*S*-acyl carrier protein specimen which in turn delivers its carboxyl group to the biotin prosthetic group of a protein with an M_r of 120 000 and thereby regenerates the acetyl-*S*-acyl carrier protein. The transferase subunit and the acyl carrier protein comprise comparable amounts of the cellular protein [transferase is 4% (Hilbi & Dimroth, 1994) and acyl carrier protein is 2.5%] which is in accord with the described function of these proteins in the reaction mechanism.

The activation for cleavage of a C−C bond next to an acetyl thio ester moiety is shared with citrate lyase and citramalate lyase (Dimroth, 1988). Like malonate decarboxylase, these lyases dispose of an acyl carrier protein component that becomes posttranslationally acetylated at a specific SH group with acetate, ATP, and a specific ligase. After taking over citrate or citramalate, respectively, with liberation of acetate, the activated acyl-*S*-acyl carrier protein derivatives undergo C−C bond cleavage, liberating oxaloacetate or pyruvate, respectively, thereby regenerating the acetyl-*S*-acyl carrier protein derivatives.

The relationship in the activation mechanism for C−C bond cleavage includes acyl carrier protein-SH transferases that catalyze the formation of the respective acyl-*S*-acyl carrier protein derivatives from acetyl-*S*-acyl carrier protein and each specific substrate. All three acyl carrier protein-SH-transferases are not completely specific for the acyl carrier protein derivatives but can also catalyze a CoA transfer from acetyl-*S*-CoA to each specific carboxylic acid substrate (Dimroth, 1988; Hilbi & Dimroth, 1994). The chemical reason for this remarkable property is certainly the structural similarity of the phosphoribosyldephospho-CoA prosthetic group that is covalently bound to each of these acyl carrier proteins with coenzyme A. A prosthetic group with this structure that may be regarded as a protein-bound coenzyme A is therefore not unique to citrate lyase and citramalate lyase but also occurs in malonate decarboxylase. It may in fact be even more widely distributed in nature, participating in other C−C bond cleavage reactions with nonactivated carboxylic acids.

In contrast to the identical prosthetic groups, the primary structure of the acyl carrier proteins is distinct. Whereas in the acyl carrier proteins of citrate lyase and citramalate lyase stretches of identical amino acids are clearly evident and the attachment site of the prosthetic group is identical (Ser-14), the N-terminal sequence of the acyl carrier protein from malonate decarboxylase is not related to that of the lyases. Incidentally, this acyl carrier protein also has a serine residue at position 14, but this has been shown not to be the attachment site for the prosthetic group. Since the prosthetic group is liberated from the different acyl carrier proteins under similar conditions of alkaline hydrolysis, a phospho diester linkage to a serine residue is the likely connection in malonate decarboxylase as well. The exact attachment site, however, remains to be determined.

The chemical interconversions of the different acyl carrier proteins at the sulfur atom of their prosthetic group are remarkably similar. The catalytically inactive SH compounds that are formed by desacetylation with hydroxylamine or mercaptans can be reconverted into the acetyl thio ester derivatives with acetic anhydride in the presence of excess mercaptans. This acetylation has been shown for the lyases to be an enzyme-catalyzed reaction in which acetic anhydride acts as an intermediate analogue of a mixed anhydride of acetate with citrate or citramalate, respectively, the anticipated intermediate of the acyl carrier protein-SH transferase reaction (Buckel, 1976; Dimroth, 1988). Another remarkable reaction is the specific alkylation of the prosthetic SH group of the acyl carrier proteins from the different enzymes upon incubation with iodoacetate in the presence of excess mercaptans. This reaction, therefore, probably involves recognition of iodoacetate as an acetate analogue by the acyl carrier protein-SH transferase bringing the prosthetic SH group of the acyl carrier protein into close contact with the iodoacetate and thereby facilitating its alkylation.

Recently, soluble malonate decarboxylases were isolated from *Acinetobacter calcoaceticus* (Kim & Byun, 1994) and from *K. pneumoniae* (Schmid et al., 1996). The *Klebsiella* enzyme which has been more thoroughly characterized consists of four different subunits, one of them being an acyl carrier protein harboring 2′-(5″-phosphoribosyl)-3′-dephospho-CoA as a prosthetic group. The catalysis resembles that of the *M. rubra* malonate decarboxylase performing the turnover of the acetyl and malonyl thio ester residues on this prosthetic group. It is distinct, however, because the carboxyl group of the malonyl-*S*-enzyme is not delivered to biotin but is directly released as CO_2 with H^+ as the second substrate.

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