# Biosynthesis of Triphosphoribosyl-dephospho-coenzyme A, the Precursor of the Prosthetic Group of Malonate Decarboxylase†

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ABSTRACT: Malonate decarboxylase from *Klebsiella pneumoniae* consists of four subunits MdcA, D, E, and C and catalyzes the cleavage of malonate to acetate and $CO_2$. The smallest subunit MdcC is an acyl carrier protein to which acetyl and malonyl thioester residues are bound via a 2′-(5″-phosphoribosyl)-3′-dephospho-CoA prosthetic group and turn over during the catalytic mechanism. We report here on the biosynthesis of holo acyl carrier protein from the unmodified apoprotein. The prosthetic group biosynthesis starts with the MdcB-catalyzed condensation of dephospho-CoA with ATP to 2′-(5″-triphosphoribosyl)-3′-dephospho-CoA. In this reaction, a new α (1″ → 2′) glycosidic bond between the two ribosyl moieties is formed, and thereby, the adenine moiety of ATP is displaced. MdcB therefore is an ATP:dephospho-CoA 5′-triphosphoribosyl transferase. The second protein involved in holo ACP synthesis is MdcG. This enzyme forms a strong complex with the 2′-(5″-triphosphoribosyl)-3′-dephospho-CoA prosthetic group precursor. This complex, called $MdcG_i$, is readily separated from free MdcG by native polyacrylamide gel electrophoresis. Upon incubation of $MdcG_i$ with apo acyl carrier protein, holo acyl carrier protein is synthesized by forming the phosphodiester bond between the 2′-(5″-phosphoribosyl)-3′-dephospho-CoA prosthetic group and serine 25 of the protein. MdcG corresponds to a 2′-(5″-triphosphoribosyl)-3′-dephospho-CoA:apo ACP 2′-(5″-phosphoribosyl)-3′-dephospho-CoA transferase. In absence of the prosthetic group precursor, MdcG catalyzes at a low rate the adenylylation of apo acyl carrier protein using ATP as substrate. The adenylyl ACP thus formed is an unphysiological side product and is not involved in the biosynthesis of holo ACP. The 2′-(5″-triphosphoribosyl)-3′-dephospho-CoA precursor of the prosthetic group has been purified and its identity confirmed by mass spectrometry and enzymatic analysis.

Many aerobic and a few anaerobic bacteria are known to grow on malonate as the sole carbon and energy source, and aerobic growth on malonate is an important criterion in bacterial diagnostics (*1*). Despite the importance of this metabolic pathway, the enzymic and genetic basis for malonate utilization was resolved only recently (for a review, see ref *2*). The key enzyme is a specific malonate decarboxylase that converts malonate directly into acetate and $CO_2$. Malonate is chemically rather inert, especially at neutral pH, when both carboxylic acid residues are deprotonated. In fact, aqueous solutions of disodium malonate show no decarboxylation within 48 h at 125 °C (*3*). To overcome this difficulty, malonate is activated for C−C bond cleavage by transiently forming a thioester with the enzyme (for a review, see ref *4*).

Malonate decarboxylase from *Klebsiella pneumoniae* is a water-soluble enzyme complex of 142 kDa that consists of four different subunits MdcA (65 kDa), MdcD (34 kDa), MdcE (30 kDa), and MdcC (12 kDa) in an apparent 1:1:1:1 stoichiometry. The smallest subunit MdcC is an acyl carrier protein (ACP)[1] and provides the thiol moiety necessary for substrate activation in the form of a covalently attached 2′-(5″-phosphoribosyl)-3′-dephospho-coenzyme A prosthetic group (*5*). The structure of this compound is shown in Figure 1, panel B, and the catalytic mechanism of the malonate decarboxylase is shown in Figure 1, panel A. To generate a catalytically active malonate decarboxylase, apo ACP has to be converted into holo HS-ACP by attachment of the phosphoribosyl-dephospho-CoA prosthetic group and subsequently, the thiol moiety of this group has to be converted into the acetyl thioester derivative. It has been shown that the latter is a two-step process (*6*). In the first reaction, MdcH catalyzes the malonyl transfer from malonyl-CoA to yield malonyl-*S*-ACP and in the second reaction, malonyl-*S*-ACP is decarboxylated to acetyl-*S*-ACP with the MdcDE subunits of malonate decarboxylase. Once acetylated, the enzyme catalyzes a continuous turnover of malonate to acetate and $CO_2$. The reaction sequence starts with the MdcA-catalyzed transfer of the ACP-SH moiety from acetyl-*S*-ACP to malonate yielding malonyl-*S*-ACP and acetate and is completed with the MdcDE-catalyzed decarboxylation of malonyl-S-ACP, thereby regenerating acetyl-*S*-ACP (see above).

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[1] Abbreviations: ACP, acyl carrier protein; DTT, dithiothreitol; IPTG, isopropyl-β-D-thiogalactopyranoside; NMN, nicotinamide mononucleotide; NTA, nitrilotriacetic acid; PAGE, polyacrylamide gelelectrophoresis; SDS, sodium dodecyl sulfate.

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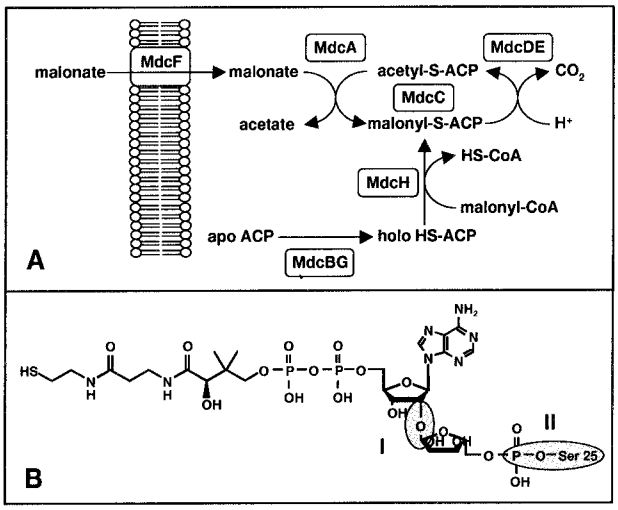


FIGURE 1: (A) Reaction mechanism proposed for malonate decarboxylation in *K. pneumoniae*. MdcA, acetyl-*S*-ACP:malonate ACP transferase; MdcC, acyl carrier protein (ACP); MdcDE, malonyl-*S*-ACP decarboxylase; MdcF, malonate transporter; MdcH, malonyl-CoA:ACP-SH transacylase; MdcBG, proteins described in this work; (B) Structure of the prosthetic group of malonate decarboxylase. Reaction I is necessary for biosynthesis and reaction II is necessary for the attachment of the prosthetic group to serine 25 of apo ACP.

The *mdc* gene cluster from *K. pneumoniae*, consisting of the nine consecutive genes *mdcABCDEFGHR*, has been cloned and characterized recently. Next to the five genes encoding components of the malonate decarboxylase enzyme complex *mdcA*, *mdcC*, *mdcD*, *mdcE*, and *mdcH*, additional genes are present in the cluster that are necessary for the catabolism of malonate (*7*). The *mdcF* gene codes for a malonate uptake system (S. Hoenke, unpublished), and *mdcR* encodes a LysR-type regulator protein, which activates the expression of the *mdc* genes (*7*, *8*). The role of the two remaining genes *mdcB* and *mdcG* was unknown, but it was speculated that their gene products might be involved in the biosynthesis of a functional prosthetic group, because an active holo malonate decarboxylase was synthesized when the *mdc* gene cluster was expressed in *Escherichia coli*. In fact, two reactions have to be catalyzed to obtain holo ACP containing a complete phosphoribosyl-dephospho-CoA prosthetic group: (i) An α (1″ → 2′) glycosidic bond has to be formed between the two ribosyl moieties and (ii) the prosthetic group has to be attached to the ACP via phosphodiester linkage to serine 25 (see Figure 1, panel B).

In this work, we investigated the biosynthesis of the prosthetic group of malonate decarboxylase from *K. pneumoniae*. We show that holo ACP synthesis is a two-step reaction catalyzed by MdcB and MdcG and involves the formation of an activated precursor of the prosthetic group. In a first step, the ATP:dephospho-CoA 5′-triphosphoribosyl transferase MdcB (abbreviated as precursor synthase) catalyzes the formation of the glycosidic bond between the ribosyl moieties from ATP and dephospho-CoA, displacing the adenine moiety of ATP. The triphosphoribosyl-dephospho-CoA precursor of the prosthetic group binds strongly, but noncovalently to the MdcG protein and is then transferred by this 2′-(5″-triphosphoribosyl)-3′-dephospho-CoA:apo ACP 2′-(5″-phosphoribosyl)-3′-dephospho-CoA transferase (abbreviated as holo ACP synthase) to apo ACP, yielding the holo ACP-SH specimen.

## EXPERIMENTAL PROCEDURES

*Bacterial Strains and Growth Conditions. E. coli* DH5α (Bethesda Research Laboratories) and *E. coli* BL21(DE3)-pLysS (Novagen) were used in this study. All strains were grown at 37 °C in Luria−Bertani medium supplemented with the appropriate antibiotics (100 μg/mL ampicillin for pBluescript and pET16 derivatives, 50 μg/mL kanamycin for pET24 derivatives). Cell extract was prepared as described (*6*).

*Recombinant DNA Techniques.* For routine work with DNA, established protocols were used (*9*). Oligonucleotides utilized for cloning were custom synthesized by Microsynth (Balgach, Switzerland). All inserts derived from polymerase chain reaction (PCR), and the ligation sites were controlled by DNA sequencing according to the dideoxy-nucleotide chain termination method (*10*) using the Dye-Dideoxy Terminator Cycle Sequencing Kit on an ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems).

*Construction of Prosthetic Group Deficient Deletion Mutants.* To construct a *mdcB*-deficient malonate decarboxylase expression vector, plasmid pHS15 (*7*) was digested with *Eco*47III. By that means, a 750-bp DNA fragment was removed coding for amino acids 55−305 of MdcB and amino acids 1−24 of MdcC. To restore the 5′-end of the *mdcC* gene, a DNA fragment was generated by PCR using the oligonucleotide primers mb-a (CTAACAATAG CGCTACG-TAC AAGGCGCTTG TGAGGTGCT) and mc-z (TATT-AGGATC CTAGCTGCCG TCGTCCAGC). The resulting fragment covered 159 bp of *mdcB*, and the whole *mdcC* gene and contained a newly introduced *Eco*47III site at the mb-a generated end. The DNA was digested with *Eco*47III, and the resulting 230-bp fragment ligated into the *Eco*47III digested pHS15, yielding pHS15Δ*B*.

For the deletion of *mdcG*, a truncated pHS15 derivative was constructed to introduce appropriate single restriction sites. For that reason, pHS15 was digested with *Apa*I, the resulting 5.5-kb vector fragment gel purified and religated. The resulting plasmid pKS-GH contained a 2.6-kb fragment of the *mdc* operon with the complete *mdcG* and *mdcH* genes. Plasmid pKS-GH was subsequently digested with *Hin*cII and *Pfl*23II, generating a 3.5-kb blunt end vector fragment and two *Hin*cII/*Pfl*23II fragments of 380 bp and 1.7 kb. The larger fragment was treated with T4 DNA polymerase to fill in the *Pfl*23II end and ligated back into the 3.5-kb *Hin*cII fragment. The resulting plasmid pKS-H contained a 380-bp deletion in *mdcG*. The truncated *mdcG* gene was then isolated from pKS-H by digestion with *Sac*I and ligated into a *Sac*I digested and dephosphorylated pHS15 plasmid, yielding pHS15ΔG, in which 61% of the *mdcG* gene was deleted.

*Construction of Single Gene Expression Plasmids.* For the investigation of prosthetic group biosynthesis, *mdcB*, *mdcC*, and *mdcG* were cloned separately into pET16b and pET124b. Plasmid pET124b is a pET24 derivative bearing a p15A origin of replication from pACYC184 (New England Biolabs), permitting simultaneous coexistence in *E. coli* with pET16b derivatives containing a ColE1 origin of replication (*11*).

The coding region of *mdcC* was amplified from plasmid pSH41 (*7*) using primers mc-c (CATCGTCGCA TATG-GAACAG ATTACATTGT CATTTC) and mc-y (TCCA-GTCGGA TCCTTACGCA TGAGCGGCCT CCTC) that

introduced a *Nde*I restriction site at the initiation codon and a *Bam*HI site after the stop codon. The PCR product was digested with *Nde*I and *Bam*HI and cloned into the expression vector pET16b. The resulting plasmid pET16$C_{His}$ encodes the ACP with an N-terminally fused stretch of 21 amino acids, including a $His_{10}$-tag ($MGH_{10}$SSGHIEGRH) to allow purification by $Ni^{2+}$-NTA chromatography. To construct the expression plasmid pET124G, the coding region of *mdcG* was amplified using primers mg-c (GATTCGTCAT ATGTCAGCCA CGCCGCGTCC C) and mg-z (TTATGGCGGG ATCCTTATTC CTCCCTGTGC CACGG). The *Nde*I and *Bam*HI digested PCR fragment was cloned into pET124b treated with the same enzymes, yielding plasmid pET124G. Likewise, the coding region of *mdcB* was amplified by PCR using primers mb-b (TGACCAACCA TATGAAAAAT CTCTCCCCCC TGCAC) and mb-z (GCGATCGAGG ATCCTTAGCCG GCAACTCTGTC GAGAAAC). After digestion with *Nde*I and *Bam*HI, *mdcB* was cloned into pET124b cut with the same enzymes, yielding pET124B.

To facilitate purification of the precursor synthase and the holo ACP synthase by $Ni^{2+}$-NTA chromatography, a DNA linker encoding a $His_{10}$ tag was inserted immediately after the initiation codon to pET124B and pET124G. This linker was prepared by annealing oligonucleotide $His_{10}$for (TATGCACCAT CACCATCACC ATCACCATCA CCA) with the complementary oligonucleotide $His_{10}$rev (TATGGTGATG GTGATGGTGA TGGTGATGGT GCA), which resulted in the formation of *Nde*I compatible overhangs at both ends. Prior to ligation into *Nde*I digested and dephosphorylated pET124G and pET124B, respectively, the linker was phosphorylated with polynucleotide kinase. The resulting plasmids pET124$G_{His}$ and pET124$B_{His}$ encode the respective enzymes with an N-terminal $His_{10}$ tag ($MH_{10}$).

To permit simultaneous expression of all three *mdc* genes together, *mdcB* was cloned behind *mdcC* into pET16$C_{His}$. DNA from plasmid pET16$C_{His}$ was linearized with *Bam*HI, and the ends were filled in with T4 DNA polymerase. Plasmid pET124B was digested with *Xba*I and *Sac*I, and the ends were filled in with T4 DNA polymerase. The 880-bp fragment, which included the ribosomal binding site as well as the *mdcB* gene, was gel-purified and ligated into the linearized pET16$C_{His}$ DNA, resulting in pET16$C_{His}$B.

*Detection of Malonate Decarboxylase Proteins and Activity of the Enzyme Complex.* Malonate decarboxylase activity of whole cells was assayed using Ewing's modified malonate broth (*12*). Five milliliters of malonate broth was inoculated with a single colony of a malonate decarboxylase expressing *E. coli* clone and incubated 25 h at 37 °C. Degradation of malonate led to a rise of pH that was measured with a pH meter or determined qualitatively with bromthymol blue (25 mg/L). Both mutant strains revealed a negative phenotype. The pH of the medium increased only slightly to 6.9, whereas *E. coli* pHS15 (wild-type strain) was malonate-positive (pH 7.9).

Malonate decarboxylase was partially purified by anion exchange chromatography. *E. coli* cell extract was prepared from clones pHS15, pHS15ΔB, and pHS15ΔG. Nineteen milliliters of extract was centrifuged at 200000*g* for 1 h, and the supernatant was pumped onto a Fractogel TSK-DEAE (Merck) column connected to a FPLC apparatus (Amersham-Pharmacia Biotech). The enzyme was eluted with a gradient of buffer I (50 mM potassium phosphate, pH 7.5) to buffer II (50 mM potassium phosphate, 1 M NaCl, pH 7.5). The subunits of the enzyme were visualized by Western blotting. For this purpose, 20 μg of partially purified decarboxylase was subjected to SDS−PAGE on a 12% gel (*13*). The subunits were subsequently electroblotted onto a nitrocellulose membrane (*14*) and stained using polyclonal antibodies against the purified enzyme complex (rabbit, 1:1000 diluted serum) and an alkaline phosphatase conjugated secondary antibody (goat anti-rabbit IgG, BioRad). Activity of partially purified malonate decarboxylase was determined by $CO_2$ formation from malonate with a gas chromatograph as described (*5*).

*Purification of Mdc Proteins by $Ni^{2+}$-NTA Chromatography.* Apo ACP was synthesized by expression of plasmid pET16$C_{His}$ in *E. coli*, adenylyl ACP was obtained by coexpression of pET16$C_{His}$ with pET124G, and holo ACP was formed by the simultaneous expression of pET16$C_{His}$B and pET124G. All His-tagged ACP derivatives were purified by $Ni^{2+}$-NTA chromatography. A total of 3.5 mL of cell extract was loaded onto a column containing 1 mL of bed volume of His-bind resin (Novagen) loaded with 3.5 mL of 50 mM $NiSO_4$ and equilibrated with 3.5 vol of wash buffer (20 mM Tris/HCl, 500 mM NaCl, 10 mM imidazole, pH 7.9). The column was washed with 3.5 vol of wash buffer containing 10 mM imidazole, 3.5 vol of wash buffer containing 60 mM imidazole, and with 7 vol of wash buffer containing 100 mM imidazole. All bound protein complexes were eluted with 7 vol of wash buffer containing 250 mM imidazole and concentrated by centrifugation through a Ultrafree-4 centrifugal filter unit (Biomax-5 membrane, Millipore) at 4 °C and 6000*g*. Purified protein solutions were stabilized through the addition of $NaN_3$ (0.02% final concentration). From a 2-L LB culture, approximately 1.5 mg of pure ACP was obtained.

Holo ACP synthase was synthesized in *E. coli* by expression of plasmid pET124$G_{His}$. A total of 3.5 mL of cell extract was loaded onto a His-bind column, washed with 3.5 vol each of wash buffer containing 10, 60, or 100 mM imidazole and eluted with 7 vol of buffer containing 200 mM imidazole. The eluted protein was concentrated by centrifugation using a Biomax-10 membrane (Millipore). The final yield was 32 mg of pure enzyme from a 2-L culture.

Precursor synthase was synthesized by expression of pET124$B_{His}$ in *E. coli*. $MdcB_{His}$-containing cell extract was loaded onto the His-bind column, which was subsequently washed with 3.5 vol each of wash buffer containing 10, 60, or 100 mM imidazole and 7 vol of wash buffer containing 150 mM imidazole. The protein was eluted with wash buffer containing 250 mM imidazole. Because $MdcB_{His}$ aggregated during concentration, the diluted elution fraction was used directly for the experiments. Purified His-tagged precursor synthase lost activity within a few days at 4 °C, but the stability was improved after addition of 50% glycerol (v/v) and storage at −20 °C. A 2-L expression culture yielded approximately 3.5 mg of pure enzyme.

*In Vitro Assay for the Analysis of the Prosthetic Group Biosynthesis Pathway.* The biosynthesis of the prosthetic group was investigated in an in vitro assay followed by native or SDS−PAGE. The reaction mixtures usually contained in a final volume of 16 μL of 50 mM potassium phosphate (pH 7.0), 63 mM $MgCl_2$, 10 nmol each of ATP and dephospho-CoA, 3−5 μg of purified apo ACP, 6−25 μg of

pure holo ACP synthase, and either 1 $\mu$g of purified precursor synthase modified with a His-tag or 20−40 $\mu$g of *E. coli* cell extract containing the unmodified enzyme. After incubation for 10−15 min at room temperature, the reactions were diluted with 4 $\mu$L of native sample buffer (final concentration 100 mM Tris, pH 6.8, 10% glycerol, trace bromphenol blue) or 8 $\mu$L of SDS sample buffer (63 mM Tris/HCl, pH 6.8, 10% glycerol, 2% SDS, trace bromphenol blue) and analyzed by gel electrophoresis. Apo ACP was usually separated from adenylyl ACP and holo ACP on a 14% (*13*) or 16% SDS−polyacrylamide gel (*15*). The holo ACP synthase MdcG was separated from $MdcG_i$ and from the MdcG/holo ACP protein complex on an 8% gel by native PAGE, which was performed essentially as described (*15*), but SDS was omitted from all solutions.

For radioactive labeling experiments the ATP of the reaction mixtures was replaced by 10 nmol of $\alpha[^{32}P]$ or $\gamma[^{32}P]$ ATP(300−500 nCi). After electrophoresis was performed, the gel was stained for 5 min with Coomassie Brilliant Blue, dried, and exposed to a PhosphoImager screen (Molecular Dynamics). The presence of the adenine moiety originating from ATP was assayed with 1 nmol of adenine[8-$^{14}$C] labeled ATP (60 nCi) and 1 nmol of dephospho-CoA. The gel was soaked for 30 min sequentially in ethanol/acetic acid/glycerol/$H_2O$ (20:1:1:28), water, and 1 M sodium salicylic acid (pH 6.0), dried, and exposed to an X-ray film.

A protein-free triphosphoribosyl-dephospho-CoA preparation also containing ATP and dephospho-CoA was prepared in vitro by incubating His-tagged precursor synthase with ATP and dephospho-CoA. The enzyme was subsequently removed by applying the sample to a $Ni^{2+}$-NTA column, and low molecular compounds were eluted with 10 mM imidazole. The absence of protein contamination was checked by UV spectroscopy and SDS−PAGE.

*Isolation of the Phosphoribosyl-dephospho-CoA Prosthetic Group from Citrate Lyase and of Covalent Adducts from Malonate Decarboxylase Holo and Adenylyl ACP*. The phosphoribosyl-dephospho-CoA prosthetic group of citrate lyase from *K. pneumoniae* was isolated by mild alkaline hydrolysis (*16*, *17*). A total of 5 mg of lyophilized citrate lyase was dissolved in 500 $\mu$L of 0.1 mM NaOH and kept 30 min at 65 °C under argon. Afterward, the protein was precipitated by adjusting the pH to 2.2 with HCl, and the supernatant was used for further analysis.

The prosthetic group of malonate decarboxylase holo ACP and the AMP moiety from adenylyl ACP were isolated accordingly. Cell extract of an *E. coli* strain containing plasmids $pET16C_{His}B$ and pET124G was prepared and MdcG/holo ACP complex purified by $Ni^{2+}$-NTA chromatography as described above. A total of 3.6 mg of purified protein in 300 $\mu$L was brought to 100 mM NaOH and kept 30 min at 65 °C under argon. The protein was precipitated by the addition of HCl to a final pH of 2.2, and the supernatant analyzed by HPLC.

For synthesis of adenylyl ACP, *E. coli* cells harboring both plasmids $pET16C_{His}$ and pET124G were grown, and cell extract was prepared as described above. The adenylyl ACP synthesized by these cells accumulated partly in solution, partly in inclusion bodies. Soluble adenylyl ACP was purified by $Ni^{2+}$-NTA chromatography as described above. The inclusion bodies were harvested by centrifugation at 20000*g* for 15 min and washed twice with lysis buffer containing 0.1% Triton X-100. The pellet was suspended in 3 mL of renaturation buffer (50 mM potassium phosphate, 6 M guanidine hydrochloride, 4 mM DTT, pH 7.0). After 40 h, undissolved material was removed by centrifugation at 200000*g* for 30 min at 4 °C, and the solubilized adenylyl ACP was purified through $Ni^{2+}$-NTA chromatography. To remove salts, the preparation was loaded onto a gel filtration column (PD10, Amersham-Pharmacia Biotech) and eluted with buffer I (50 mM potassium phosphate, pH 7.5). The pooled adenylyl ACP preparations (3 mg of protein) were combined with 100 mM NaOH in 5 mL and heated to 65 °C for 30 min to eliminate the adenylyl moiety from the protein. The protein was precipitated by addition of HCl (final pH 2.0) and removed by centrifugation (5 min, 15000*g*). The supernatant was concentrated 5-fold in a vacuum centrifuge and analyzed by HPLC as described below.

*Analysis of Prosthetic Group Derivatives by HPLC*. Nucleotide and CoA derivatives were analyzed by reversed phase HPLC on a Hypersil ODS column (250 × 4 mm, 5 $\mu$m particle size, Hewlett-Packard) equilibrated with buffer A (0.2 M potassium phosphate, pH 5.0). Samples were diluted with buffer A prior to injection and were eluted with a linear gradient (0 to 60% usually within 18 min) of buffer B (0.2 M potassium phosphate, pH 5.0, and 20% acetonitrile) and monitored at 254 nm. The retention times of isolated prosthetic group derivatives were compared with those of the following standards: NMN 3.3 min, ATP 5.2 min, ADP 6.1 min, AMP 7.6 min, CoA 13.2 min, dephospho-CoA 15.2 min.

*Identification of the Hydrolysis Product from Adenylyl ACP as 5′-AMP*. The retention time of the alkaline hydrolysis product from adenylyl ACP (7.6 min) was identical to 5′-AMP. To confirm this identity, the material eluting at 7.6 min from the HPLC was evaporated to dryness, redissolved in $H_2O$ and subjected to AMP analysis with the coupled spectrophotometric assay with myokinase, pyruvate kinase, and lactate dehydrogenase (*18*). Myokinase is absolutely specific for adenosine nucleotides. The reaction mixture contained, in a final volume of 200 $\mu$L, 100 mM sodium phosphate (pH 7.3), 100 mM KCl, 10 mM $MgCl_2$, 500 $\mu$M phosphoenolpyruvate, 100 $\mu$M ATP, 50 $\mu$M NADH, 11 U lactate dehydrogenase, 4 U pyruvate kinase, 1.4 U myokinase, and variable concentrations of the isolated hydrolysis product. The AMP-dependent oxidation of NADH was measured in a spectrophotometer at 340 nm ($\epsilon = 6.3$ $mM^{-1}$ $cm^{-1}$).

*Isolation of the Prosthetic Group Precursor Bound Noncovalently to $MdcG_i$*. The $MdcG_i$ complex consisting of holo ACP synthase and the postulated precursor of the prosthetic group was synthesized in vitro. The incubation mixture contained in 800 $\mu$L at 25 °C 50 mM potassium phosphate buffer, pH 7.0, 25 mM $MgCl_2$, 0.625 mM ATP, 0.625 mM dephospho-CoA, 2.6 mg of purified holo ACP synthase ($MdcG_{His}$) and *E. coli* cell extract (2 mg of protein) containing unmodified precursor synthase (MdcB). After 30 min, the mixture was applied to a $Ni^{2+}$-NTA column and holo ACP synthase with the tightly bound prosthetic group precursor ($MdcG_i$) was purified as described for the free enzyme, except that 5 mM $MgCl_2$ was added to all wash buffers. Purified $MdcG_i$ could be stored at 4 °C for several days, but prolonged storage resulted in the decay of the

complex, yielding free holo ACP synthase. This decay was due to degradation of the low molecular weight compound bound to the protein, as was shown by HPLC analysis. The precursor of the prosthetic group was liberated from $MdcG_i$ by denaturing the protein at 80 °C (3 min). The mixture was centrifuged, and the concentration of triphosphoribosyl-dephospho-CoA estimated by comparing HPLC peak areas with those of CoA standards.

*Conversion of Triphosphoribosyl-dephospho-CoA to Ribosyl-dephospho-CoA.* To secure the structure of the prosthetic group precursor, isolated as described above, terminal phosphate residues of triphosphoribosyl-dephospho-CoA were hydrolyzed by alkaline phosphatase, and the cleavage products were identified by HPLC using phosphoribosyl-dephospho-CoA and ribosyl-dephospho-CoA as standards. The hydrolysis mixtures contained in 20 $\mu$L of 50 mM Tris/HCl buffer, pH 8.5, 0.1 mM EDTA, 650 pmol of the triphosphoribosyl-dephospho-CoA isolated and 1 U alkaline phosphatase from calf intestine (Roche Diagnostics). The ribosyl-dephospho-CoA standard was prepared by an identical alkaline phosphatase treatment of authentic phosphoribosyl-dephospho-CoA, isolated from citrate lyase. After 5 min, phosphate hydrolysis was complete, and the phosphatase was precipitated with 1 $\mu$L of 2 M HCl and removed by centrifugation. HPLC was performed after dilution with 0.5 mL of buffer A.

*Mass Spectrometry of Triphosphoribosyl-dephospho-CoA.* For mass spectrometry, the triphosphoribosyl-dephospho-CoA from 1 mg of $MdcG_i$ was purified by HPLC using the same gradient as described above. The substance eluting in a single peak at 13.7 min was collected and the absence of imidazole was checked by UV−Vis spectrometry. The mass of the prosthetic group intermediate was determined by MALDI-TOF mass spectrometry (Bruker Biflex III mass spectrometer) in delayed extraction reflected positive mode. A total of 1 $\mu$L of the sample (70 pmol) was mixed with 1 $\mu$L of a 0.5 M solution of 2, 4, 6-trihydroxyacetophenon in ethanol, and 1 $\mu$L of the mixture was transferred to the target and dried at room temperature. The accelerating voltage was 19 kV, and the reflector voltage was set to 20 kV.

## RESULTS

*Identification of the Gene Products Responsible for the Biosynthesis of the Phosphoribosyl-dephospho-CoA Prosthetic Group of Malonate Decarboxylase.* We have recently cloned the *mdc* gene cluster from *K. pneumoniae* on a single plasmid, designated pHS15. *E. coli* transformed with this plasmid acquires the ability to grow aerobically on malonate as sole carbon and energy source (*7*). The isolated recombinant enzyme was indistinguishable from wild-type malonate decarboxylase (*5*, *6*). The functions of seven proteins encoded by the *mdc* genes have been identified, but the function of the gene products from the two remaining genes *mdcB* and *mdcG* was obscure. However, on the basis of the fact that the recombinant enzyme contained a functional prosthetic group it was hypothesized that these proteins were responsible for biosynthesis and attachment of the phosphoribosyl-dephospho-CoA compound to the apo ACP (Figure 1, panel A).

To test this hypothesis, we deleted *mdcB* or *mdcG* from plasmid pHS15, yielding pHS15ΔB and pHS15ΔG, respectively. *E. coli* transformed with these plasmids (strain ΔB and ΔG, respectively) was unable to degrade malonate. This defect could be attributed to the lack of malonate decarboxylase activity. Partially purified wild-type malonate decarboxylase had a specific activity of 2.9 U/mg of protein, while the enzymes purified by the same procedure from the ΔB or ΔG strains were completely inactive. The purification of inactive malonate decarboxylase specimens was confirmed by Western blotting using polyclonal antibodies against malonate decarboxylase. As shown in Figure 2, the ACP transferase (MdcA) and the decarboxylase subunits (MdcD and MdcE) were stained equally intensive in the wild type, the ΔB-, or the ΔG-derived enzyme. In contrast, the smallest subunit MdcC (ACP) was stained well in the wild-type enzyme but only weakly in the mutant decarboxylases. It is also apparent that the mutant acyl carrier proteins are with 11 kDa (strain ΔG) or 11.4 kDa (strain ΔB) smaller than the wild-type protein (12 kDa). As the molecular mass of the prosthetic group is 900 Da, the ACP specimen from strain ΔB might contain an incomplete prosthetic group. It will be shown below that the 11-kDa ACP specimen is indeed apo ACP and that the 11.4-kDa ACP specimen is adenylyl ACP. Holo ACP formation therefore apparently depends on both genes *mdcB* and *mdcG*. The gene product of *mdcG* could be a holo ACP synthase involved in the synthesis of the phosphodiester bond between the prosthetic group and the ACP, whereas the protein encoded by *mdcB* could be a precursor synthase involved in the synthesis of the glycosidic bond between the two ribose moieties (see Figure 1, panel B).

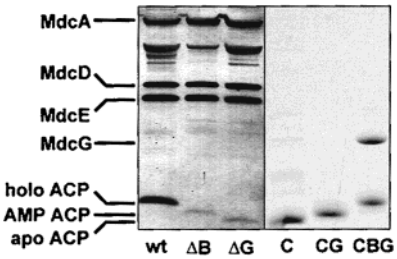


FIGURE 2: In vivo synthesis of three different ACP derivatives of malonate decarboxylase. (Left) Western blot of partially purified malonate decarboxylase from the wild-type *E. coli* strain (wt), from a *mdcB* deficient strain (ΔB), and from a *mdcG* deficient strain (ΔG). The blot was probed with antibody specific for malonate decarboxylase from *K. pneumoniae*. Please note that the bands appearing between the MdcA and MdcD subunits are due to unspecific cross reaction of the antiserum with impurities. (Right) SDS−PAGE of purified ACP (0.4 nmol) after simultaneous expression of different *mdc* genes. C, apo ACP purified from a $pET16C_{His}$ expressing *E. coli* strain; CG, adenylyl ACP (AMP ACP) purified from a $pET16C_{His}$ and pET124G coexpressing *E. coli* strain; CBG, protein complex of holo ACP and MdcG purified from a $pET16C_{His}B$ and pET124G coexpressing *E. coli* strain.

*Synthesis of Different ACP Species by Expression of the mdcC Gene in Various Combinations with the mdcB and mdcG Genes.* To facilitate further investigations of prosthetic group biosynthesis, the *mdcC*, *mdcB*, and *mdcG* genes were cloned in compatible plasmids that allowed synthesis of different His-tagged ACP derivatives in *E. coli* and subsequent purification by affinity chromatography. Expression of plasmid $pET16C_{His}$ in *E. coli* yielded apo ACP, coexpression of plasmids $pET16C_{His}$ and pET124G produced adenylyl ACP, and coexpression of plasmids $pET16C_{His}B$ and pET124G yielded holo ACP. Upon purification of this

holo ACP by affinity chromatography, an additional protein of 24 kDa was copurified in an apparent 1:1 stoichiometry (Figure 2). The N-terminal amino acid sequence of this protein was 100% identical to that of the holo ACP synthase MdcG (SATPR′PHDLV′WLNHA). Coexpression of *mdcC*, *mdcB*, and *mdcG* therefore results in the formation of a stable complex between holo ACP synthase and holo ACP, which is not dissociated during purification.

To ensure that holo ACP synthesized by the expression system described above contained the entire 2′-(5″-phosphoribosyl)-3′-dephospho-CoA prosthetic group, the phosphodiester bond to the protein was hydrolyzed, and the cleavage products were analyzed by HPLC. The main peak (31% relative quantity) eluted at 15.0 min, exactly as authentic 2′-(5″-phosphoribosyl)-3′-dephospho-CoA isolated from citrate lyase. Upon mixing, both prosthetic groups coeluted at 15.0 min, indicating that the majority of the ACP synthesized in *E. coli* contained the identical 2′-(5″-phosphoribosyl)-3′-dephospho-CoA prosthetic group as the ACP of wild-type malonate decarboxylase. The substances eluting from HPLC at 7.4, 10.1, and 13.2 min (minor signals) and at 17.9 min (30% relative quantity) were not identified. They could be the degradation or oxidation products of the prosthetic group.

The ACP specimen derived from the coexpression of plasmids pET16C$_{His}$ and pET124G was identified as adenylyl ACP by alkaline hydrolysis and HPLC. The major peak eluted at 7.6 min, exactly as 5′-AMP and when mixed, the two substances eluted together. Several other peaks with retention times between 1.5 and 5 min were not identified. The hydrolysis product eluting at 7.6 min was further characterized by its UV spectrum that was identical to that of 5′-AMP ($\lambda_{max}$ = 259 nm). Its identity with 5′-AMP was confirmed with a coupled enzymic assay with myokinase. This enzyme is absolutely specific for adenine nucleotides and converts 5′-AMP plus ATP into ADP which is determined in a coupled spectrophotometric assay with pyruvate kinase and lactate dehydrogenase. The NADH oxidations resulting from different additions of the isolated cleavage product and from additions of the same amount of 5′-AMP were identical. Hence, holo ACP is synthesized in *E. coli* if the *mdcC* gene is expressed together with *mdcB* and *mdcG*, whereas the expression of *mdcC* and *mdcG* leads to the synthesis of adenylyl ACP, and with *mdcC* alone, apo ACP is synthesized.

*In vitro Synthesis of Different ACP Specimens.* The identification of an adenylyl ACP species indicated that the substrates for the prosthetic group biosynthesis were an AMP donor such as ATP or NADH and dephospho-CoA. To test this assumption, purified apo ACP and holo ACP synthase were mixed with cell extract of *E. coli* containing unmodified precursor synthase (or with purified His-tagged enzyme) and incubated with dephospho-CoA and ATP or NADH in a buffer containing $Mg^{2+}$. The formation of holo ACP was subsequently determined by SDS−PAGE. It was observed only in the presence of ATP, but not with NADH, which is therefore not the donor of the phosphoribosyl moiety of the prosthetic group.

Further insight into the prosthetic group biosynthesis was obtained by radioactive labeling studies with α[$^{32}$P] ATP or γ[$^{32}$P] ATP. In the complete in vitro system for holo ACP synthesis, incorporation of the radioactive label into holo ACP was observed from α[$^{32}$P] ATP, but not from γ[$^{32}$P] ATP (see Figure 6, panel B). No radioactivity was incorporated in either precursor synthase or holo ACP synthase. This result is in accord with the suppositions that ATP is the source for the phosphoribosyl moiety that connects the prosthetic group with serine 25 of the protein and that the ribose moiety of the prosthetic group that derives from ATP is bound by its 5′-phosphate residue to the ACP moiety. If either precursor synthase or dephospho-CoA was omitted, adenylyl ACP was formed which again was labeled with α[$^{32}$P] ATP but not with γ[$^{32}$P] ATP as substrate. No turnover of apo ACP was observed in absence of either MdcG or ATP.

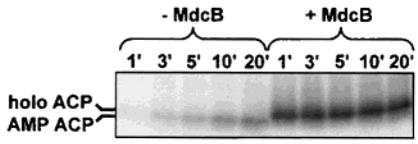


FIGURE 3: Comparison of the kinetics of adenylyl ACP (AMP ACP) and holo ACP formation as catalyzed in the absence or presence of precursor synthase, respectively. Purified apo ACP (3.7 nmol) was incubated with holo ACP synthase (0.8 nmol) and with *E. coli* control cytoplasm (−MdcB) or precursor synthase expressing *E. coli* cytoplasm (+MdcB) in a buffer containing α[$^{32}$P]-labeled ATP (40 nmol, 1.23 μCi) and dephospho CoA (40 nmol). After 1, 3, 5, 10, and 20 min, samples equaling 0.6 nmol of ACP were added to SDS sample buffer and separated on a 16% SDS gel. The gel was dried and exposed to a PhosphoImager plate for 14 h.

To assess whether adenylyl ACP is a physiological intermediate in the prosthetic group biosynthesis, the kinetics of holo ACP and adenylyl ACP formation were compared (Figure 3). In the complete assay mixture including precursor synthase, incorporation of radioactivity from α[$^{32}$P] ATP into holo ACP was complete within 1 min with no further increase during the following 20-min incubation. The rate of α[$^{32}$P] adenylyl ACP formation in the absence of precursor synthase, however, was significantly slower: Incorporation of radioactivity increased continuously over the entire 20-min incubation period and did not even then reach the same level as observed with the complete system. We conclude, therefore, that adenylyl ACP is not an intermediate in the biosynthesis of the prosthetic group but rather an unphysiological side product generated in the absence of precursor synthase. Holo ACP synthase is apparently not completely specific for its physiological substrate triphosphoribosyl-dephospho-CoA (see below) but catalyzes at a slow rate an adenylyl transfer from ATP to serine 25 of the apo ACP.

*Formation of a Tight Complex between Holo ACP Synthase and the Prosthetic Group Precursor.* It has been shown above that holo ACP synthase forms a stable complex with holo ACP but not with adenylyl ACP (Figure 2), indicating that this enzyme binds the prosthetic group more tightly than the AMP moiety. We reasoned therefore that holo ACP synthase should make a tight complex with the prosthetic group precursor as well and that this complex might have an increased mobility in native PAGE due to the multiple negatively charged phosphate residues. The results of Figure 4 show a significant increase of the mobility of holo ACP synthase after its incubation with ATP, dephospho-CoA, and precursor synthase but not if either dephospho-CoA or precursor synthase was omitted. The shifted band became labeled if α[$^{32}$P] ATP or γ[$^{32}$P] ATP was included in the complete assay mixtures (compare Figure 6, panel A). Hence, the compound bound to MdcG contained all three phosphate

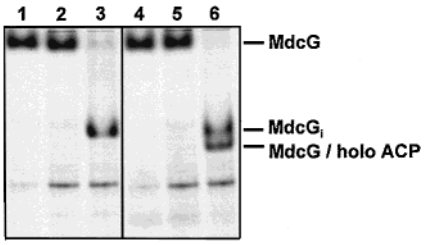


FIGURE 4: Complex formation of holo ACP synthase with triphosphoribosyl-dephospho-CoA and with holo ACP as detected on native PAGE. Lanes 1−3, purified holo ACP synthase (1 nmol) was incubated with *E. coli* cytoplasm containing precursor synthase (lanes 2 and 3) or *E. coli* control cytoplasm (lane 1) for 5 min with ATP (10 nmol) in the presence (lanes 1 and 3) or absence (lane 2) of dephospho-CoA (10 nmol). After termination of the reaction by adding native sample buffer, the proteins were subjected to native PAGE on a 8% gel and stained with Coomassie Brilliant Blue. Lanes 4−6, as above, with additional presence of apo ACP (0.4 nmol) in each assay. MdcG, free holo ACP synthase; MdcG$_i$, complex of holo ACP synthase with triphosphoribosyl-dephospho-CoA; MdcG/holo ACP, complex of holo ACP synthase with holo ACP.

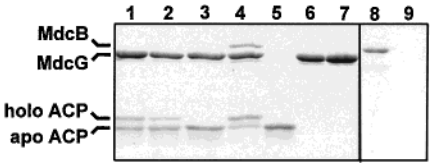


FIGURE 5: In vitro holo ACP biosynthesis is catalyzed by precursor synthase and holo ACP synthase in two separate reactions. A total of 0.2 nmol of apo ACP was incubated for 15 min with either 0.2 nmol of MdcG$_i$ complex (lane 1), 0.2 nmol holo ACP synthase plus 8 $\mu$L of protein-free triphosphoribosyl-dephospho-CoA preparation (lane 2), 0.2 nmol of holo ACP synthase plus 8 $\mu$L of control imidazole buffer (lane 3), 0.2 nmol of holo ACP synthase plus 30 pmol of precursor synthase in the presence of 5 nmol of ATP and dephospho-CoA (lane 4). Control apo ACP (lane 5), holo ACP synthase (lane 6), MdcG$_i$ complex (lane 7), precursor synthase (lane 8), protein-free triphosphoribosyl-dephospho-CoA preparation (lane 9).

residues of ATP, i.e., it probably represents triphosphoribosyl-dephospho-CoA. The complex of holo ACP synthase (MdcG) with the prosthetic group precursor was termed MdcG$_i$ (intermediate). If apo ACP was present in the reaction assay in addition to MdcG$_i$ the formation of a second protein complex was detected (Figure 4, lane 6). Analysis of MdcG$_i$ and the second protein complex by SDS−PAGE revealed that the only protein component present in MdcG$_i$ was holo ACP synthase (see also Figure 5, lanes 6 and 7) and that the second protein complex consisted of holo ACP synthase and holo ACP (data not shown).

If MdcG$_i$, prepared as described above, was separated from precursor synthase and low molecular weight substrates by affinity chromatography and incubated with apo ACP, holo ACP was synthesized (Figure 5, lane 1). The same result was obtained if a protein-free triphosphoribosyl-dephospho-CoA preparation, prepared as described above, was incubated with apo ACP and holo ACP synthase (lane 2). Holo ACP synthase alone could not substitute for MdcG$_i$ or triphosphoribosyl-dephospho-CoA in this reaction (lane 3), as expected, but holo ACP synthase plus precursor synthase, ATP, and dephospho-CoA also converted the apo ACP form into holo ACP (lane 4). This experiment clearly shows that a prosthetic group precursor synthesized solely by the precursor synthase MdcB is present in the MdcG$_i$ complex

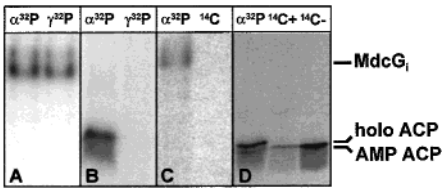


FIGURE 6: The prosthetic group precursor contains the α and $\gamma$ phosphate groups but not the adenine moiety of ATP. (A) A total of 1 nmol purified holo ACP synthase was incubated with *E. coli* extract containing precursor synthase for 8 min in a buffer containing 10 nmol of dephospho-CoA and 10 nmol of either α[$^{32}$P] or $\gamma$[$^{32}$P] labeled ATP (0.3 $\mu$Ci), followed by native PAGE and autoradiography. (B) Like panel A, but 0.4 nmol of apo ACP was additionally present in the assay. The proteins were separated by SDS−PAGE. (C) A total of 0.5 nmol of purified holo ACP synthase was incubated with precursor synthase-containing *E. coli* extract for 15 min in a buffer containing 1 nmol of dephospho-CoA and 1 nmol of either α[$^{32}$P] (47 nCi) or adenine-[$^{14}$C] labeled ATP (60 nCi), followed by native PAGE and autoradiography for 12 days. (D) A total of 0.5 nmol purified holo ACP synthase was incubated with 0.2 nmol of purified apo ACP in the presence (+) or absence (−) of precursor synthase-containing *E. coli* extract as in panel C. The proteins were separated by SDS−PAGE.

and is transferred to apo ACP without participation of precursor synthase.

Thus far we have shown that MdcG$_i$ contains the α and $\gamma$ phosphate groups of ATP and that holo ACP contains the α but not the $\gamma$ phosphate group of ATP (Figure 6, panels A and B). To make the glycosidic linkage between the ribose moiety derived from ATP and that derived from dephospho-CoA, the adenine portion of ATP must be displaced. To test this supposition, similar labeling experiments as described above with α[$^{32}$P] ATP or $\gamma$[$^{32}$P] ATP were performed with [$^{14}$C]adenine labeled ATP. The results of Figure 6 (panels C and D) show that radioactivity from the [$^{14}$C]adenine ATP is neither incorporated into MdcG$_i$ nor into holo ACP. A small amount of adenylyl ACP, which was formed as a side product under our assay conditions, however, retained the [$^{14}$C]adenine moiety of [$^{14}$C]adenine ATP, as expected. If precursor synthase was omitted from the reaction mixture, the labeling of the [$^{14}$C]adenylyl ACP was significantly increased, which is in accord with the view that the triphosphoribosyl-dephospho-CoA precursor formed by precursor synthase is the preferred substrate for holo ACP synthase, while holo ACP synthase in the absence of precursor synthase can only form adenylyl ACP with the inferior substrate ATP.

*Identification of 2′-(5″-Triphosphoribosyl)-3′-dephospho-CoA as Cofactor Bound to MdcG$_i$.* To prove that the MdcG$_i$ bound cofactor was 2′-(5″-triphosphoribosyl)-3′-dephospho-CoA, MdcG$_i$ was synthesized in vitro and purified by affinity chromatography. The MdcG$_i$ complex was destroyed by heating to 80 °C, and the protein precipitate was removed by centrifugation. The supernatant was analyzed by reversed phase HPLC, and the prosthetic group precursor eluted in a single peak at 13.7 min. By comparing the peak area with those of CoA standards, from 6 $\mu$g of MdcG$_i$ (245 pmol) 228 pmol of the chromophore was recovered, corresponding to a yield of 93%. As the MdcG$_i$ preparations always contain a small amount of free holo ACP synthase (Figure 4, lane 3), the precursor was recovered quantitatively and binds in a stoichiometry of 1:1 to MdcG$_i$. The isolated precursor still contained the $\gamma$ phosphate group of ATP, as shown by

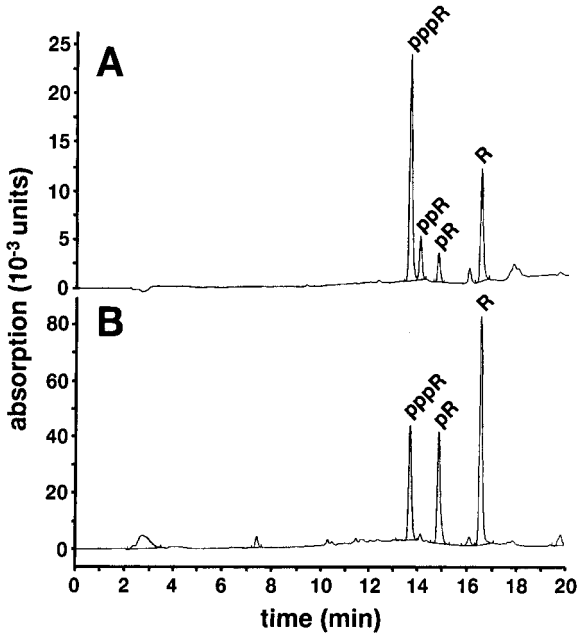


FIGURE 7: (A) Enzymatic dephosporylation of triphosphoribosyl-dephospho-CoA with alkaline phosphatase. Approximately 650 pmol triphosphoribosyl-dephospho-CoA was incubated for 50 s with 0.5 U of alkaline phosphatase. The reaction was stopped by addition of HCl, and the reaction products were separated by reversed phase HPLC. (B) Comparison of native and dephosphorylated forms of prosthetic group precursor with 2′-(5″-phosphoribosyl)-3′-dephospho-CoA and ribosyl-3′-dephospho-CoA. Approximately 650 pmol each of triphosphoribosyl-dephospho-CoA and phosphoribosyl-dephospho-CoA was mixed with completely dephosphorylated triphosphoribosyl-dephospho-CoA and with ribosyl-dephospho-CoA and separated by HPLC; pppR, 2′-(5″-triphosphoribosyl)-3′-dephospho-CoA; ppR, 2′-(5″-diphosphoribosyl)-3′-dephospho-CoA; pR, 2′-(5″-phosphoribosyl)-3′-dephospho-CoA; R, 2′-ribosyl-3′-dephospho-CoA.

comigration of the chromophore and radioactivity if $MdcG_i$ had been labeled with $\gamma[^{32}P]$ ATP (data not shown).

For further structural analyses, the isolated precursor was dephosphorylated with alkaline phosphatase and subjected to reversed phase HPLC. The results of Figure 7 show that upon dephosphorylation the retention time shifted from 13.7 min (precursor) to 16.6 min, which was the only product observed after prolonged incubation with the phosphatase (not shown). The dephosphorylated product obtained from the prosthetic group precursor was identical to that obtained by alkaline phosphatase treatment of authentic phosphoribosyl-dephospho-CoA and hence represents 2′-ribosyl-3′-dephospho-CoA. The minor products with retention times 14.1 and 14.9 min probably represent diphosphoribosyl-dephospho-CoA and phosphoribosyl-dephospho-CoA. The latter of these has the same retention time as authentic phosphoribosyl-dephospho-CoA, and both intermediates are completely converted into the fully dephosphorylated species by prolonged incubation with the phosphatase.

The anticipated chemical structure was finally confirmed by mass spectrometry. The precursor was prepared as above and desalted by reversed phase HPLC. The purified preparation (30 $\mu$M in 0.2 M potassium phosphate, pH 5.0) was analyzed by MALDI-TOF mass spectrometry (Figure 8). The molecular ion peak ($MH^+$) found in this spectrum was *m*/*z* 1060.10, which is the correct value for the anticipated structure ($C_{26}H_{46}O_{26}N_7P_5S$; monoisotopic mass *m*/*z* = 1060.10).

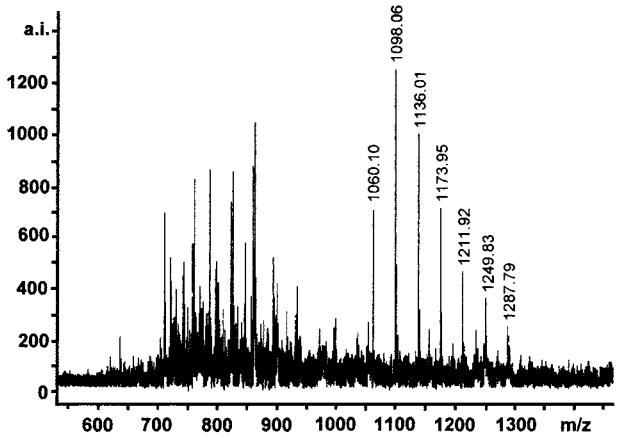


FIGURE 8: MALDI-TOF mass spectrum of purified prosthetic group precursor of malonate decarboxylase from *K. pneumoniae* in 0.2 M potassium phosphate buffer (pH 5.0). The calculated values for 2′-(5″-triphosphoribosyl)-3′-dephospho-CoA are *m*/*z* 1060.10 for the molecular ion peak ($MH^+$) and 1098.05, 1136.01, 1173.96, 1211.92, 1249.88, and 1287.83 for one to six protons exchanged by potassium. The signals from *m*/*z* 600 to 1000 result mainly from potassium phosphate clusters of the buffer.

Since the compound was purified in a potassium phosphate buffer, the six acidic protons of the phosphate residues could be exchanged for potassium ions. The masses of *m*/*z* 1098.06, 1136.01, 1173.95, 1211.92, 1249.83, and 1287.79 are readily explained by the exchange of one to six protons for potassium. The formation of alkali ion adducts during MALDI-TOF mass spectrometry has already been observed with the prosthetic group isolated from malonate decarboxylase from *M. rubra* (*19*).

## DISCUSSION

Malonate decarboxylase from *K. pneumoniae* belongs to a class of enzymes with a covalently linked CoA derivative as prosthetic group. Other well-known enzymes of this type are citrate lyase (*20*) or citramalate lyase (*21*). The structure of this particular cofactor was shown to be 2′-(5″-phosphoribosyl)-3′-dephospho-CoA (*22*). In this communication, we have unraveled the biosynthetic pathway that leads to the biosynthesis of the prosthetic group and its covalent attachment to the ACP subunit. We demonstrate that two enzymes encoded by *mdcB* and *mdcG* are necessary for holo ACP synthesis and that the prosthetic group is synthesized with ATP and dephospho-CoA as substrates. This is in contrast to an earlier speculation that the phosphoribosyl moiety of the prosthetic group of citrate lyase derives from $NAD^+$, in analogy to the formation of poly-ADP ribose (*22*). Figure 9, panel A summarizes the prosthetic group biosynthetic reactions. In the first step, the gene product encoded by *mdcB* catalyzes the condensation of ATP and dephospho-CoA to 2′-(5″-triphosphoribosyl)-3′-dephospho-CoA with the liberation of adenine. The MdcB protein is therefore an ATP: dephospho-CoA 5′-triphosphoribosyl transferase (precursor synthase). To our knowledge, this is the first reaction in which a 1 → 2 glycosidic bond between two ribose residues is formed with ATP under the replacement of adenine. The prosthetic group precursor binds by a strong, but noncovalent, interaction to the enzyme encoded by *mdcG*, yielding $MdcG_i$. It is possible that this binding is necessary to protect the prosthetic group precursor in vivo from degradation. The second reaction is the transfer of the prosthetic group from

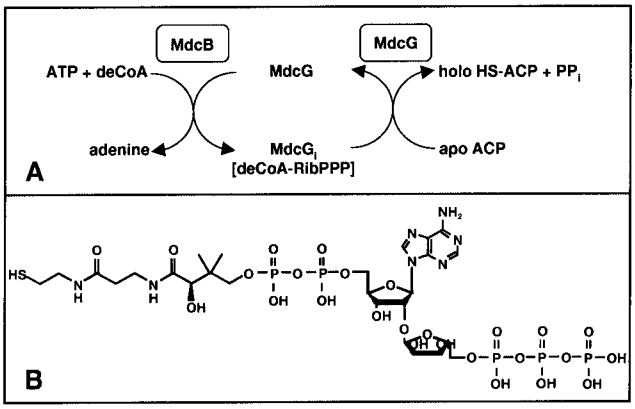


FIGURE 9: (A) Proposed mechanism for the biosynthesis of holo ACP of malonate decarboxylase. MdcB, ATP:dephospho-CoA 5′-triphosphoribosyl transferase; MdcG, 2′-(5″-triphosphoribosyl)-3′-dephospho-CoA:apo ACP 2′-(5″-phosphoribosyl)-3′-dephospho-CoA transferase; MdcG$_i$, complex between 2′-(5″-triphosphoribosyl)-3′-dephospho-CoA (deCoA-RibPPP) and MdcG. (B) Structure of the 2′-(5″-triphosphoribosyl)-3′-dephospho-CoA precursor of the prosthetic group.

MdcG$_i$ to the apo ACP, forming holo ACP and presumably pyrophosphate and regenerating free MdcG. The MdcG protein can therefore be described as a 2′-(5″-triphosphoribosyl)-3′-dephospho-CoA:apo ACP 2′-(5″-phosphoribosyl)-3′-dephospho-CoA transferase (holo ACP synthase). Although the two reactions are catalyzed by the individual enzymes in vitro, it cannot be excluded that in vivo holo ACP synthase forms a prosthetic group biosynthetic complex with precursor synthase and/or with ACP. It is interesting in this context that holo ACP synthase forms a stable protein complex with holo ACP. The physiological role for this tight binding between these proteins is completely unknown. It may protect the prosthetic group from degradation reactions before the malonate decarboxylase complex is assembled, or it may help to assemble the complete complex. During assembly, holo ACP synthase must be released to make the prosthetic group accessible for acetylation and for the interaction with the other subunits of the complex, and indeed, isolated malonate decarboxylase does not contain holo ACP synthase. The prosthetic group precursor was clearly identified as 2′-(5″-triphosphoribosyl)-3′-dephospho-CoA (for the structure, see Figure 9, panel B) by enzymic and mass spectrometric analyses. This is the first report describing this novel protein cofactor.

The biosynthesis of the phosphoribosyl-dephospho-CoA prosthetic group reported here appears to follow the same route in various malonate decarboxylases and citrate lyases. This can be deduced from the presence of precursor synthases in bacterial malonate decarboxylase or citrate lyase encoding operons. The degree of identity between the precursor synthase MdcB of *K. pneumoniae* and the homologues from the *mdc* operons from *Pseudomonas putida* (*23*) or *Acinetobacter calcoaceticus* (*24*) are 56 and 35%, respectively. Other relatives to the *K. pneumoniae* MdcB protein are MadG from the *mad* gene cluster encoding membrane-associated malonate decarboxylase from *Malonomonas rubra* (*25*; 29%), CitG from the citrate lyase operon of *K. pneumoniae* (*26*; 39% identity), *E. coli* (*27*; 35%), *Haemophilus influenzae* (*28*; 35%), *H. ducreyi* (*29*; 36%), *Leuconostoc mesenteroides* (*30*; 35%), and *Weissella paramesenteroides* (*31*; 33%). Enzymes related to the holo ACP synthase MdcG from *K. pneumoniae* are present in the *mdc* operon from *P. putida* (44%) and *A. calcoaceticus* (29%). In some of the other operons, genes with an unidentified function are present which may encode MdcG-like enzymes, but their gene products reveal only an unsatisfactory similarity with MdcG. This is not surprising because the holo ACP synthase analogue of citrate lyase has to recognize besides triphosphoribosyl-dephospho-CoA the ACP moiety from citrate lyase which is structurally not related to the ACP of malonate decarboxylase. The holo ACP synthase MdcG and its related enzymes from the *mad* and *cit* operon are described in more detail in the succeeding article (*32*).

Parallel to our work on malonate decarboxylase, it has been shown that the synthesis of holo ACP from *E. coli* citrate lyase depends on two proteins that are encoded by *citG* and *citX* in the *E. coli cit* gene cluster (*11*). In the citrate lyase operons from *H. influenzae*, *L. mesenteroides*, and *W. paramesenteroides*, the two genes are fused, forming a bifunctional enzyme CitG, where the holo ACP synthase activity is supposed to reside on the N-terminal domain and the precursor synthase activity on the C-terminal domain. On the basis of the presence of related prosthetic group biosynthetic enzymes in all reported operons of enzymes containing a phosphoribosyl-dephospho-CoA prosthetic group, we conclude that the mechanism for prosthetic group biosynthesis of malonate decarboxylase from *K. pneumoniae* via triphosphoribosyl-dephospho-CoA as described in this publication is universally true for all enzymes containing this particular prosthetic group.

## ACKNOWLEDGMENT

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