Identification of the Active Site of Phosphoribosyl-dephospho-coenzyme A Transferase and Relationship of the Enzyme to an Ancient Class of Nucleotidyltransferases†

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ABSTRACT: Malonate decarboxylase from *Klebsiella pneumoniae* contains an acyl carrier protein (MdcC) to which a 2′-(5″-phosphoribosyl)-3′-dephospho-CoA prosthetic group is attached via phosphodiester linkage to serine 25. We have shown in the preceding paper in this issue that the formation of this phosphodiester bond is catalyzed by a phosphoribosyl-dephospho-coenzyme A transferase MdcG with the substrate 2′-(5″-triphosphoribosyl)-3′-dephospho-CoA that is synthesized from ATP and dephospho-coenzyme A by the triphosphoribosyl transferase MdcB. The reaction catalyzed by MdcG is related to nucleotidyltransfer reactions, and the enzyme indeed catalyzes unphysiological nucleotidyltransfer, e.g., adenylyltransfer from ATP to apo acyl carrier protein (ACP). These unspecific side reactions are favored at high Mg^{2+} concentrations. A sequence motif including D134 and D136 of MdcG is a signature of all nucleotidyltransferases. It is known from the well-characterized mammalian DNA polymerase β that this motif is at the active site of the enzyme. Site-directed mutagenesis of D134 and/or D136 of MdcG to alanine abolished the transfer of the prosthetic group to apo ACP, but the binding of triphosphoribosyl-dephospho-CoA to MdcG was not affected. Evidence is presented that similar to MdcG, MadK encoded by the malonate decarboxylase operon of *Malonomonas rubra* and CitX from the operon encoding citrate lyase in *Escherichia coli* are phosphoribosyl-dephospho-CoA transferases catalyzing the attachment of the phosphoribosyl-dephospho-CoA prosthetic group to their specific apo ACPs.

Malonate decarboxylase is the key enzyme of malonate degradation in various aerobic or anaerobic bacteria. The decarboxylase from *Klebsiella pneumoniae* has been well-characterized at the genetic and the protein level and is therefore regarded as the prototype for malonate decarboxylases of aerobic bacteria (*1*−*4*). The nine genes for the malonate decarboxylase are clustered on the chromosome forming the *mdc* operon that is only expressed if malonate is present in the growth medium. Included in this operon are the four structural genes for the malonate decarboxylase *mdcA*, *mdcD*, *mdcE*, and *mdcC*, the *mdcF* gene encoding a malonate transporter and the *mdcR* gene coding for a LysR-type regulator protein. Also included are the genes *mdcB* and *mdcG* that encode enzymes for the biosynthesis of the 2′-(5″-phosphoribosyl)-3′-dephospho-CoA prosthetic group and *mdcH* that encodes a malonyl-CoA:ACP transacylase.[1] This enzyme converts the thiol moiety of the prosthetic group into a malonyl thioester and thereby transforms the inactive into the catalytically active enzyme specimen. Once the malonyl-*S*-ACP has been formed, it is quickly decarboxylated to the acetyl-*S*-ACP species by the decarboxylase subunits MdcDE of the enzyme complex. In the presence of malonate, the ACP transferase subunit MdcA converts acetyl-*S*-ACP back into malonyl-*S*-ACP with the liberation of acetate. Hence, the catalytic activities of the decarboxylase and ACP transferase subunits perform an interconversion between malonyl-ACP and acetyl-ACP that leads to the decarboxylation of free malonate to acetate and CO_2. Bacteria disposing of similar *mdc* operons and hence encoding malonate decarboxylases with an analogous catalytic mechanism are *Pseudomonas putida* (*5*), *P. aeruginosa* (http://www.pseudomonas.com), and *Acinetobacter calcoaceticus* (*6*). A related malonate decarboxylase has also been isolated from *P. fluorescens* (*7*).

Malonomonas rubra is an anaerobic bacterium that grows from the decarboxylation of malonate to acetate and CO_2 and hence utilizes the decarboxylation energy for ATP synthesis. The malonate decarboxylase of *M. rubra* shares basic features for malonate activation with the aerobic decarboxylases (*8*, *9*): It has an acyl carrier protein with a covalently bound phosphoribosyl-dephospho-CoA prosthetic group, and the attached acetyl thioester is converted into the malonyl thioester by an ACP transferase that is related to MdcA. The decarboxylation of the malonyl thioester, however, is more elaborate and involves transfer of the carboxyl

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

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group to a protein-bound biotin moiety and its subsequent decarboxylation coupled to Na^+ ion pumping across the membrane. A *mad* operon encoding this type of a primary sodium pump coupled to malonate decarboxylation is also present in *Rhodobacter capsulatus* (http://rhodol.uchicago.edu).

In the accompanying paper in this issue (*4*), the biosynthesis of the phosphoribosyl-dephospho-CoA prosthetic group has been unraveled. First, the triphosphoribosyl transferase MdcB (abbreviated as precursor synthase in this article) catalyzes the synthesis of triphosphoribosyl-dephospho-CoA with ATP and dephospho-CoA as substrates (eq 1). To form the new 1″ → 2′ glycosidic bond between the two ribose moieties, the adenine moiety of ATP is displaced. The prosthetic group precursor is captured by the phosphoribosyl-dephospho-CoA transferase MdcG (holo ACP synthase), forming the tight complex $MdcG_i$ and is transferred to apo ACP yielding holo ACP (eq 2). In absence of its physiological substrate, holo ACP synthase catalyzes at a low rate the adenylylation of apo ACP using ATP as substrate (eq 3). Not only do the various malonate decarboxylases share the phosphoribosyl-dephospho-CoA prosthetic group but citrate lyase (*10*) and citramalate lyase (*11*) also do, and there is evidence that the biosynthesis occurs by the same route in all cases.

$$\text{ATP} + \text{dephospho-CoA} \rightarrow \text{triphosphoribosyl-dephospho-CoA} + \text{adenine} \quad (1)$$

$$\text{triphosphoribosyl-dephospho-CoA} + \text{apo ACP} \rightarrow \text{holo ACP} + PP_i \quad (2)$$

$$\text{ATP} + \text{apo ACP} \rightarrow \text{adenylyl ACP} + PP_i \quad (3)$$

In this paper, the phosphoribosyl-dephospho-CoA transfer reaction of the prosthetic group biosynthesis has been further investigated. Holo ACP synthase shares a motif including two conserved aspartic acid residues with the large family of nucleotidyltransferases. These aspartic acid residues are known to be at the active site of these enzymes, and accordingly, mutagenesis of the aspartic acid residues in holo ACP synthase destroyed its activity. We have used the mechanism of the nucleotidyltransferases to propose a mechanism for the MdcG-catalyzed phosphoribosyl-dephospho-CoA transfer reaction leading to holo ACP synthesis.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions. Escherichia 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 pET16 derivatives, 50 μg/mL kanamycin for pET24 derivatives). Cell extract was prepared as described (*3*).

Recombinant DNA Work. For routine work with DNA, established protocols were used (*12*). Oligonucleotides utilized for mutagenesis were custom-synthesized by Microsynth (Balgach, Switzerland). All PCR-derived inserts were sequenced using the Dye-Dideoxy Terminator Cycle Sequencing Kit on an ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems). The construction of plasmids $pET16C_{His}$, $pET16C_{His}B$, $pET124G_{His}$, and $pET124B_{His}$ has been described in the preceding paper in this issue (*4*).

The *madK* gene, coding for a protein of previously unknown function from the *mad* gene cluster from *M. rubra* has been cloned recently (*13*). Plasmid pET24KBioL contains in addition to *madK* the genes *madF*, encoding a small biotin protein of *M. rubra*, and the biotin ligase gene *birA* from *E. coli*. To allow coexpression of the *madK* gene with *mdcC* and *mdcB*, the gene was cloned into pET124b, a plasmid with a compatible origin of replication. For that means, pET24KBioL was digested with *Nde*I and *Bam*HI and the resulting 1-kb fragment, containing both *madK* and *madF*, was cloned into the *Nde*I and *Bam*HI restriction sites of pET124b, yielding pET124KBio.

The *citX* gene from *E. coli* has been cloned recently yielding pET124-citX (*14*). To allow affinity purification of the *citX* gene product, a linker encoding a His_{10} tag was inserted after the initiation codon to pET124-citX. This linker was prepared by annealing the 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 pET124-citX, the linker was phosphorylated with polynucleotide kinase. The resulting plasmid $pET124\text{-}citX_{His}$ encodes citrate lyase holo ACP synthase with an N-terminal His_{10} tag (MH_{10}).

Site-Directed Mutagenesis of Plasmid pET124G$_{His}$. Both catalytic aspartic acid residues 134 and 136 of $MdcG_{His}$ were changed into alanine individually or in combination by site-directed mutagenesis using the PCR based overlap extension method (*15*). In a first step, the 5′-end of the *mdcG* coding region was amplified by *Pfu* polymerase (Stratagene) using $pET124G_{His}$ as template and T7 promotor primer and the respective mutagenic primer ($D134A_{rev}$, $D136A_{rev}$, $D134/136A_{rev}$). Each primer contained up to three point mutations to change the codon for aspartic acid (GAT) to alanine (GCT or GCG) and to introduce a new *Eco*47III restriction site (AGCGCT). Accordingly, the 3′-end of *mdcG* was amplified using T7 terminator primer and mutagenic primers $D134A_{for}$, $D136A_{for}$, $D134/136A_{for}$. The resulting PCR products were gel-purified, and the corresponding fragments were used as templates in a second PCR reaction. In this extension reaction, the PCR fragments annealed by their complementary ends, yielding a 0.9-kb product that was further amplified using T7 promotor and T7 terminator primer. This fragment, containing the whole *mdcG* gene with the desired mutations, was subsequently digested with *Fsp*I and *Hin*dIII, and the resulting 426-bp fragment was gel-purified and ligated into a *Fsp*I/*Hin*dIII treated $pET124G_{His}$ fragment. By that means, the desired point mutations were introduced yielding $pET124G_{His}$ derivatives D134A, D136A, and D134/136A.

Synthesis and Purification of Prosthetic Group Biosynthetic Enzymes. Expression and purification of His-tagged *mdc* apo ACP, precursor synthase, and holo ACP synthase from the malonate decarboxylase gene cluster from *K. pneumoniae* is described in detail in the preceding paper in this issue (*4*). Citrate lyase holo ACP synthase was synthesized in *E. coli* by expression of $pET124\text{-}citX_{His}$, mutated malonate decarboxylase holo ACP synthase species by expression of $pET124G_{His}$ derivatives D134A, D136A, and D134/136A. The recombinant His-tagged enzymes were purified by Ni^{2+}-NTA chromatography as described (*4*).

In Vitro Assay for Prosthetic Group Biosynthesis. Biosynthesis and transfer of the prosthetic group was investigated in an in vitro assay using purified proteins. In the reaction mixture, the following components were usually present in a final volume of 16 μL of potassium phosphate buffer (50 mM, pH 7.0): $MgCl_2$ (0−63 mM), nucleosidetriphosphate (10 nmol of ATP, dATP, GTP, UTP, or CTP), dephosphocoenzyme A (10 nmol), apo ACP (0−5 μg), precursor synthase (0.5 μg), and holo ACP synthase (10 μg). Occasionally, purified 2′-(5″-triphosphoribosyl)-3′-dephospho-CoA (150 pmol) was used instead of ATP and dephospho-CoA. The absence of ATP contamination in the used nucleosidetriphosphates (Roche Diagnostics) was controlled by reversed phase HPLC with a gradient as described (*4*). The reactions were diluted after different incubation times at room temperature with 4 μL of native sample buffer (final concentration 100 mM Tris/HCl, pH 6.8, 10% glycerol, trace bromphenol blue) or 8 μL of SDS sample buffer (63 mM Tris/HCl, pH 6.8, 10% glycerol, 2% SDS, trace bromphenol blue) and analyzed by gel electrophoresis. The different ACP derivatives were usually separated on a 14% SDS−polyacrylamide gel (*16*). Free holo ACP synthase was separated from the enzyme binding the prosthetic group precursor ($MdcG_i$) or holo ACP (MdcG/holo ACP complex) on an 8% gel by native PAGE, which was performed as described (*4*).

For radioactive labeling experiments, 10 nmol of the respective α[^{32}P]-labeled nucleosidetriphosphate (300−500 nCi) was present in the in vitro prosthetic group biosynthesis assay. After electrophoresis, the gel was stained for 5 min in Coomassie Brilliant Blue, dried, and exposed to an X-ray film or a PhosphoImager screen (Molecular Dynamics).

Purification of the 2′-(5″-Triphosphoribosyl)-3′-dephospho-CoA Prosthetic Group Precursor. $MdcG_i$ was purified and triphosphoribosyl-dephospho-CoA was isolated from the complex as described in the preceding paper (*4*). The prosthetic group precursor was further purified by ion exchange chromatography. After heat-induced denaturation (3 min) of $MdcG_i$, 100 μL of supernatant was diluted to 200 μL with H_2O, applied on a Dowex 50 cation exchange column (H^+ form) to remove contaminating cations. The precursor was eluted with 800 μL of H_2O, evaporated to dryness, and dissolved in 100 μL of H_2O. The final sodium content (15 mM) of the sample was determined by atomic absorption spectrometry (Shimadzu AA-646 spectrophotometer), and the concentration of the precursor was determined by UV−Vis spectrometry, assuming an extinction coefficient of the prosthetic group precursor similar to that of CoA (17; $\epsilon = 16.8$ mM^{-1} cm^{-1}). The absorption maximum was 258 nm, and the concentration obtained was 150 μM.

In Vivo Cross Reactivity of Holo ACP Synthases. It was tested whether the holo ACP synthases MadK and CitX could functionally replace MdcG during in vivo *mdc* holo ACP synthesis. The biosynthesis of *mdc* holo ACP was assayed by coexpression of plasmids pET124G, pET124KBio, or pET124-citX with $pET16C_{His}B$ in *E. coli*, whereas the formation of adenylylated *mdc* ACP was tested by coexpression of the respective plasmids with $pET16C_{His}$. The different *mdc* ACP derivatives were purified by affinity chromatography as described (*4*).

Protein Sequence Analysis and Databank Searches. Proteins distantly related to holo ACP synthase MdcG were identified by screening the GenBank database with the pattern-hit initiated BLAST program (PHI-BLAST, see ref *18*). This method searches in the databank for a specified protein motif and constructs local alignments of the surrounding sequences to a query protein sequence. We used the following consensus sequence of the holo ACP synthase active site G−[GS]−x(5,16)−S−D−[LI]−D−L as pattern and the protein sequence of MdcG from *K. pneumoniae* as template. To identify even more distantly related members of the nucleotidyltransferase superfamily, the more relaxed pattern G−[GS]−x(5,16)−[GSH]−D−[LIMV]−D−[LITFV]−[LIANV]−[LITFV] was used. Multiple alignments were constructed with the software package of the Genetic Computer Group of the University of Wisconsin (UWGCG).

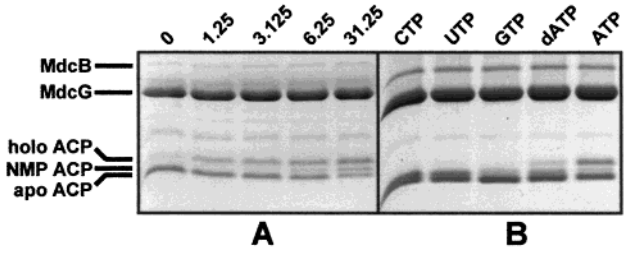

FIGURE 1: (A) Magnesium dependency of prosthetic group transferase and adenylyltransferase activities of holo ACP synthase. Purified apo ACP (0.25 nmol) was incubated with holo ACP synthase (0.4 nmol) and precursor synthase (30 pmol) for 10 min in a buffer containing 10 nmol of dephospho-CoA, 10 nmol of ATP, and variable concentrations of $MgCl_2$ (0−31.25 mM) (B) Nucleotide specificity of prosthetic group biosynthesis. Purified apo ACP (0.4 nmol) was incubated with holo ACP synthase (0.8 nmol) and precursor synthase (30 pmol) for 15 min in a buffer containing 31 mM $MgCl_2$, 10 nmol of dephospho-CoA, and 10 nmol of CTP, UTP, GTP, dATP, and ATP. Abbreviation: NMP ACP, ACP with covalently bound nucleotidemonophosphate.

RESULTS

Effect of Mg^{2+} Concentration and NTP Variation on Holo ACP and Nucleotidyl ACP Synthesis. It has been shown in the accompanying paper (*4*) that the holo ACP synthase MdcG may also act at slow rates as an adenylyltransferase. Furthermore, triphosphoribosyl-dephospho-CoA is formed from ATP and dephospho-CoA by the precursor synthase MdcB. We investigated the effect of magnesium concentration on the MdcG-catalyzed transfer reactions. For this purpose, the in vitro system for holo ACP synthesis consisting of purified apo ACP, precursor synthase, holo ACP synthase, dephospho-CoA, and ATP was employed at different Mg^{2+} concentrations. The results of Figure 1, panel A show an analysis of the samples by SDS−PAGE and indicate that holo ACP formation occurs already below 1 mM Mg^{2+} and reaches its maximum between 1 and 3 mM Mg^{2+}, whereas efficient adenylylation seems to occur only at Mg^{2+} concentrations above 5 mM. It should be noted that the concentration of intracellular free Mg^{2+} in bacteria is generally between 1 and 2 mM (*19*) and that in our in vitro assay holo ACP synthase is generally present at a 15-fold molar excess as compared to precursor synthase. Therefore, under physiological Mg^{2+} concentrations with an equimolar ratio of both enzymes, the adenylyltransferase activity of holo ACP synthase is virtually absent. Indeed, only holo ACP is synthesized during simultaneous expression of *mdcC*, *mdcB*, and *mdcG* in the cell (see Figure 3, lane 3).

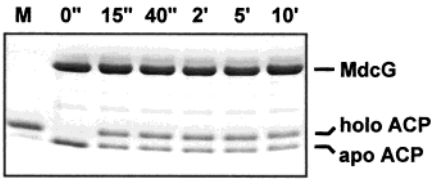

FIGURE 2: Rapid holo ACP synthesis with purified triphosphoribosyl-dephospho-CoA. Apo ACP (2.2 nmol) was incubated with holo ACP synthase (2.4 nmol) and purified triphosphoribosyl-dephospho-CoA (0.9 nmol) in 60 μL of potassium phosphate buffer (50 mM, pH 7.0) in the presence of 10 mM $MgCl_2$. After the indicated times, samples of 10 μL were taken, and the reaction was stopped by the addition of SDS sample buffer. M, mixture of holo ACP and apo ACP purified from an *mdcB*, *madK*, and *mdcC* expressing *E. coli* strain (see text).

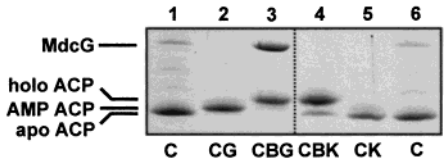

FIGURE 3: *M. rubra* holo ACP synthase MadK functionally replaces the *K. pneumoniae* enzyme during in vivo holo ACP biosynthesis. Lane 1 and 6, apo ACP purified from a pET16C_{His} expressing *E. coli* strain; lane 2, adenylyl ACP purified from a pET16C_{His} and pET124G coexpressing *E. coli* strain; lane 3, protein complex of holo ACP and MdcG purified from a pET16C_{His}B and pET124G coexpressing *E. coli* strain; lane 4, mixture of apo and holo ACP purified from a pET16C_{His}B and pET124KBio coexpressing *E. coli* strain. lane 5, apo ACP purified from a pET16C_{His} and pET124KBio coexpressing *E. coli* strain. A total of 5 μg of each purified ACP preparation was loaded onto a 16% SDS−PAGE with 6 M urea and stained with Coomassie Brilliant Blue.

Furthermore, we studied whether ATP can be replaced by other nucleosidetriphosphates in the precursor synthase reaction. For this purpose, ATP was replaced in the in vitro system in individual samples by 2′-deoxy ATP (dATP), GTP, CTP, and UTP. Figure 1, panel B, shows that apo ACP is converted into holo ACP only with ATP and to a minor extent with dATP. The use of α[^{32}P]-labeled nucleotides yielded the same result (data not shown). These results indicate that the adenine moiety is obligatory as a leaving group during formation of triphosphoribosyl-dephospho-CoA. However, the unphysiological nucleotidyltransfer to apo ACP by holo ACP synthase was observed with all tested nucleotides and is therefore not restricted to the previously described transfer of the AMP moiety.

Catalytic Activity of Holo ACP Synthase with 2′-(5″-Triphosphoribosyl)-3′-dephospho-CoA. In Figure 2, the activity of holo ACP synthase with its true substrate triphosphoribosyl-dephospho-CoA is shown. Rapid conversion of apo ACP into holo ACP is observed upon incubation with holo ACP synthase and the isolated prosthetic group precursor. Holo ACP formation was complete within 15 s and did not increase any further during the subsequent 10-min incubation. Please note that apo ACP was in excess over triphosphoribosyl-dephospho-CoA, and therefore, only a fraction of it could be converted into holo ACP. It was also shown that the presence of 1 mM externally added magnesium was sufficient for complete prosthetic group transfer to ACP (data not shown).

Identification of MadK as Holo ACP Synthase in the M. rubra mad Gene Cluster. As mentioned in the introduction, malonate decarboxylases from *M. rubra* and *K. pneumoniae* contain the same prosthetic group. However, a gene corresponding to *mdcG* coding for a holo ACP synthase has not been identified in the *M. rubra mad* gene cluster (*8*). Careful visual comparison of the MdcG protein sequence with that of Mad proteins with unknown function revealed a conserved stretch of amino acids in MadK, containing a prominent SDLDL motif. However, the overall sequence similarity with MdcG is rather low (21% identical residues), therefore failing to occur in previous databank searches (*2*, *9*). No function has been assigned to the MadK protein yet, and an earlier speculation that this enzyme could be involved in the biotinylation of the small biotin carrier protein MadF in *M. rubra* could not be verified (*13*).

We investigated whether the gene products of *mdcG* and *madK*, despite their low sequence similarity, are functionally equivalent. For this purpose, the *mdcC* and *mdcB* genes from *K. pneumoniae* were coexpressed in *E. coli* with the putative *mdcG*-homologue *madK* from *M. rubra*. It was found that about 90% of the ACP synthesized by these cells was in the holo form, and therefore, the MadK protein acts as a holo ACP synthase even with apo ACP from *K. pneumoniae*, which has only 24% identity with the ACP (MadE) from *M. rubra* (Figure 3, lane 4). This low similarity between the acyl carrier proteins may explain why holo ACP from *K. pneumoniae* forms a stable complex with MdcG (Figure 3, lane 3) but not with MadK. It is also noteworthy that the unphysiological adenylylation of ACP that occurs upon coexpression of *mdcC* and *mdcG* is hardly observed upon coexpression of *mdcC* and *madK*.

To investigate whether *mdc* holo ACP synthesized in the presence of the *mad* holo ACP synthase retained the ability to form a strong complex with *mdc* holo ACP synthase, the holo ACP was bound to a Ni^{2+}-NTA column via its His_{10} tag. Contaminating proteins were removed by washing, and the cytoplasmic fraction of *E. coli* containing untagged *mdc* holo ACP synthase was applied to the column. After washing, the ACP fraction was eluted by increasing the imidazole concentration. Holo ACP synthase eluted in the same fraction showing that the two proteins form a strong complex. No binding of holo ACP synthase was observed, however, in similar experiments with adenylyl ACP or apo ACP (data not shown).

Identification of the Catalytic Center of Holo ACP Synthase. The presence of two conserved aspartic acid residues in holo ACP synthases from *K. pneumoniae* and *M. rubra* was a hint that these amino acids could be of functional significance. We therefore mutated the respective aspartic acid residues 134 and 136 in the *Klebsiella* enzyme to alanine, both separately and in combination. The mutant proteins were purified and used in the in vitro prosthetic group synthesis assay. In all three cases, no formation of holo ACP was detectable on SDS−PAGE (Figure 4, panel A), however, when radioactive α[^{32}P] ATP was used, a very low content of holo ACP was evident (Figure 4, panel B). The formation of the $MdcG_i$ complex consisting of holo ACP synthase and triphosphoribosyl-dephospho-CoA, however, was found with wild-type or mutant enzymes (Figure 4, panel C), and these complexes became labeled if α[^{32}P] ATP was used as a substrate for the precursor synthesis (not shown). It can therefore be concluded that residues D134 and D136 are involved in prosthetic group transfer but not in the

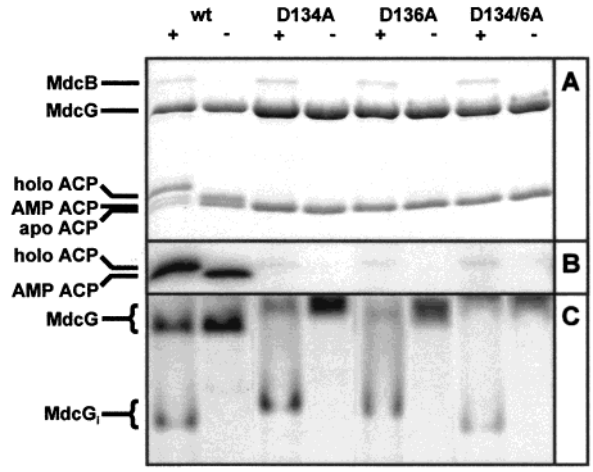

FIGURE 4: Mutagenesis of invariant aspartates in position 134 and 136 of holo ACP synthase. (A) Apo ACP (0.3 nmol) was incubated with wild-type or mutant holo ACP synthase (0.4 nmol) in the presence (+) or absence (−) of 30 pmol of precursor synthase for 15 min in a buffer containing 31 mM $MgCl_2$ and 10 nmol each of ATP and dephospho CoA. (B) Same as in panel A, but 0.51 μCi $\alpha[^{32}P]$ ATP was present in the assay. The proteins were separated by SDS−PAGE, and the incorporation of radioactivity into protein was assayed by exposition of the dried gel for 14 h to a PhosphoImager plate. (C) Wild-type or mutant holo ACP synthase (0.8 nmol) was incubated in the presence (+) or absence (−) of 30 pmol of precursor synthase as above and subjected to native polyacrylamide gelelectrophoresis. Abbreviations: wt, wild-type holo ACP synthase; D134A, mutant holo ACP synthase with aspartate 134 changed to alanine; D136A, mutant holo ACP synthase with aspartate 136 changed to alanine; D134/6A, double mutant holo ACP synthase with both aspartates changed to alanine; AMP ACP, adenylylated ACP; $MdcG_i$, complex of holo ACP synthase with triphosphoribosyl-dephospho-CoA.

binding of the prosthetic group precursor to holo ACP synthase.

Holo ACP Synthase Belongs to an Ancient Nucleotidyltransferase Family. Visual inspection of sequences from proteins with known nucleotidyltransferase function revealed the presence of the S−D−[LI]−D−L motif also in the adenylyltransferase GlnE which is involved in regulation of glutamine synthetase by covalent adenylylation and desadenylylation. A multiple alignment of the protein sequences of all known malonate decarboxylase holo ACP synthases with GlnE from *E. coli* (GenBank accession P30870) and *H. influenzae* (P44419) revealed a conserved sequence G−[GS]−x(5,16)−S−D−[LI]−D−L. This consensus sequence was used as pattern in a databank search with PHI-BLAST (*18*) with the sequence of MdcG from *K. pneumoniae* as query. The search identified local homology of the targeted proteins with MdcG, albeit with high E-values (1.5 for GlnE from *E. coli*, 5.8 for GlnE from *H. influenzae*, 7.2 for MadK from *M. rubra*). Most interestingly, this search also retrieved two other related nucleotidyltransferases, namely, uridylyltransferase/uridylyl-removing enzyme GlnD (best homology in *K. pneumoniae*; E-value 0.78) and streptomycin adenylyltransferase (best homology in *Salmonella typhimurium*; E-value 0.98). A multiple alignment of the conserved amino acid stretch of the most related proteins is depicted in Figure 5. Uridylyltransferase/uridylyl-removing enzyme is the key regulating enzyme of the nitrogen assimilation regulatory cascade that controls the activity and biosynthesis of glutamine synthetase by uridylylation and deuridylylation of the signal transduction protein P_{II} (*20*).

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Kp-MdcG     GVTGSTGYALATEIPVLHAASDLDLLIR
Pp-MdcG     GPTGGVGYQLATGMEVVHAGSDLDLLLR
Pa-MdcG     GVTGGAGFELASGVAVLHPDSDLDLLLR
Ac-MdcG     YVYGSYAYEYLTQEAYVRATSDLDLVLY
Mr-MadK     GIWGSAALELATCLPYTTDQSDLDLNTD
Rc-MadK     GLIGSAALQTVTGLPYLRPDSDLDLVVR
Kp-GlnD     VAVGGYG------RGELHPLSDIDLLIL
Ec-GlnE1    GKLGGG---------ELNFSSDIDLIFA
Ec-GlnE2    GKLGGW---------ELGYSSDLDLIFL
St-AadA     HLYGSAV------DGGLKPYSDIDLLVT
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FIGURE 5: Comparison of the conserved active site sequence of holo ACP synthases with other nucleotidyltransferases. Residues obeying the G−[GS]−x(5,16)−S−D−[LI]−D−L motif are underlayed in black; the two invariant carboxylic acids are marked with a star. Abbreviations (accession numbers in parentheses): Kp-MdcG, holo ACP synthase from *Klebsiella pneumoniae* (U95087); Pp-MdcG, holo ACP synthase from *Pseudomonas putida* (AB017138); Pa-MdcG, holo ACP synthase from *Pseudomonas aeruginosa* (RPA04684); Mr-MadK, holo ACP synthase from *Malonomonas rubra* (U87980); Rc-MadK, holo ACP synthase from *Rhodobacter capsulatus* (RRC02283); Kp-GlnD, uridylyltransferase from *Klebsiella pneumoniae* (P41393); Ec-GlnE1/2, adenylyltransferase domain 1/2 from *Escherichia coli* (P30870); St-AadA, streptomycin nucleotidyltransferase from *Salmonella typhimurium* (AF071555).

Streptomycin adenylyltransferase belongs to the ANT(3″)-Ia class (*21*) of aminoglycoside-modifying enzymes and inactivates streptomycin by adenylylation of the 3″-hydroxyl moiety of this particular antibiotic.

GlnE, GlnD, as well as ANT(3″)-Ia enzymes are all members of an ancient superfamily of nucleotidyltransferases (*22*, *23*), which share a consensus sequence G−[GS]−x(5,-16)−[GSH]−D−[LIMV]−D−[LITFV]−[LIANV]−[LIT-FV] in their active site. Other members of this superfamily are DNA polymerase β from the eukaryotic DNA polymerase X family, poly(A) polymerase, terminal deoxynucleotidyltransferase, and tRNA adenylyltransferase, which indeed were retrieved when the database was searched with this more relaxed motif. The fact that the holo ACP synthase MdcG contains an active site similar to members of the nucleotidyltransferase family with two conserved essential aspartic acid residues and has an unspecific nucleotidyltransferase activity in the presence of high concentration of Mg^{2+} ions identifies this enzyme as a new member of the nucleotidyltransferase superfamily.

MdcG Cannot be Functionally Replaced by CitX from E. coli, the Citrate Lyase Holo ACP Synthase. Parallel work on citrate lyase of *E. coli* which also contains a phosphoribosyl-dephospho-CoA prosthetic group has shown that the prosthetic group biosynthesis of this enzyme depends on the gene products of *citG* and *citX* (*14*). CitG is homologous to the precursor synthase MdcB (39% identical residues), but the sequence of CitX reveals only an unsatisfactory similarity with MdcG (below 20%). Although CitX does not contain the nucleotidyltransferase signature, this enzyme contains a conserved stretch of amino acids L−W−D−[LI]−D−V, including two aspartic acid residues at positions 122 and 124. If holo ACP synthase was replaced by CitX in our in vivo system, all ACP remained in the apo form, and therefore, CitX is unable to catalyze the phosphoribosyl-dephospho-CoA transfer to the apo ACP of malonate decarboxylase. Considering the low sequence identity between the gene products of *citX* and *mdcG* and between the two different

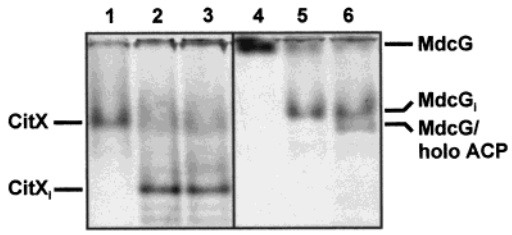

FIGURE 6: Complex formation of citrate lyase and malonate decarboxylase holo ACP synthases with triphosphoribosyl-dephospho-CoA. A total of 0.6 nmol of citrate lyase holo ACP synthase (CitX; lanes 1−3) or malonate decarboxylase holo ACP synthase (MdcG; lanes 4−6) was incubated with precursor synthase (30 pmol) and 10 nmol each of ATP and dephospho CoA for 10 min in a buffer containing 62 mM $MgCl_2$. The proteins were separated on a 10% native polyacrylamide gel. Lanes 1 and 4, assay without dephospho CoA; lanes 2 and 5, with dephospho CoA; lanes 3 and 6, with dephospho CoA and apo ACP (0.35 nmol).

acyl carrier proteins (26% identity), this result is not surprising. CitX like MdcG, however, forms a tight complex with triphosphoribosyl-dephospho-CoA, which in analogy to $MdcG_i$ was designated $CitX_i$. This is indicated by the band shift of purified CitX observed in native PAGE after incubation with precursor synthase, ATP and dephospho-CoA (Figure 6, lane 2). We neither observed in our in vitro assay the transfer of the prosthetic group from $CitX_i$ to the apo ACP of malonate decarboxylase nor the formation of a complex between CitX and ACP. Nevertheless, the formation of the $CitX_i$ complex strongly indicates that this protein is a holo ACP synthase as well and that the mechanism of holo ACP biosynthesis in citrate lyase is the same as described for malonate decarboxylase (*4*).

DISCUSSION

Enzymes of the holo ACP synthase family contain a conserved motif with two aspartic acid residues essential for the transfer of 2′-(5″-phosphoribosyl)-3′-dephospho-CoA to a specific serine of the ACP. An analogous motif is found within other enzymes with nucleotidyltransferase activity, such as the uridylyltransferase/uridylyl-removing enzyme GlnD, adenylyltransferase GlnE, and aminoglycoside nucleotidyltransferases. On the basis of sequence and structure comparison, it has been postulated that the respective enzymes are all members of an ancient superfamily of nucleotidyltransferases (*22*, *23*). Other members of this family are DNA polymerase β (*24*) from the eukaryotic DNA polymerase X family that is involved in base excision repair and gap filling, poly(A) polymerase (*25*) that creates the 3′-polyadenylyl tail of mRNA, terminal deoxynucleotidyltransferase (*26*) that catalyzes the random addition of dNTPs at the 3′-end of DNA, and tRNA adenylyltransferase (CCA-adding enzyme; *27*) that is responsible for the posttranscriptional 3′-addition of the CCA terminus of tRNA. All these enzymes have a conserved amino acid signature in common starting with GG or GS, followed by a variable stretch of 7−13 amino acids before the catalytic important aspartic acid residues. Mutation of these invariant carboxylic amino acids results in a dramatic loss of activity in various members of the nucleotidyltransferase family (*28*, *29*). The same signature is also present in holo ACP synthases encoded by malonate decarboxylase operons in *K. pneumoniae* and *M. rubra*, except that the variable stretch is 16 amino acids long, therefore escaping the attention of previous databank researches (*22*, *23*). However, the presence of this specific motif in *K. pneumoniae* holo ACP synthase together with the facts that the enzyme has an unspecific nucleotidyltransferase activity and that the aspartic acid residues 134 and 136 are essential for the transferase activity strongly suggests that the holo ACP synthases form a new enzyme class in the large nucleotidyltransferase superfamily.

Nucleotidyltransferases have a rather broad substrate specificity. Next to the obvious ability of DNA polymerases to transfer more than one specific nucleotide, aminoglycoside nucleotidyltransferases have been shown to transfer in vitro a wide variety of nucleotides and deoxynucleotides (*30*, *31*), although under physiological conditions adenylylation is the predominant reaction. GlnD has been shown to transfer any nucleotide or deoxynucleotide when the activating molecule ATP was present in the assay (*32*). Broad substrate specificity is also shown by *K. pneumoniae* holo ACP synthase that may transfer all NTPs and dATP to the apo ACP.

One remarkable feature of this enzyme is its affinity for the prosthetic group of malonate decarboxylase. Complex formation is observed either with the precursor of the prosthetic group or with holo ACP but not with dephospho-CoA, NTP, or apo ACP alone. Hence, the tight binding of the prosthetic group to MdcG should involve both the dephospho-CoA moiety and the phosphoribosyl moiety.

From two members of the nucleotidyltransferase superfamily, i.e., DNA polymerase β (*33*−*35*) and kanamycin nucleotidyltransferase (*36*), the crystal structures have been solved and allow insights into the catalytic mechanism of these enzymes. In the structure of human DNA polymerase β complexed with gapped DNA and ddCTP (2.2-Å resolution), the two conserved aspartic acid residues together with a third invariant aspartate coordinate two Mg^{2+} ions. The first (nucleotide-binding) Mg^{2+} ion coordinates the α-, β-, and γ-phosphates of ddCTP, whereas the second (catalytic) Mg^{2+} ion coordinates the 3′-OH moiety of the DNA primer and the α-phosphate moiety of ddCTP. This in-line geometry of the 3′-OH group, the α-phosphorus and the oxygen of the $\alpha-\beta$-phosphodiester bond allows a direct nucleophilic attack of the deprotonated 3′-terminus of the primer DNA on the α-phosphate group of ddCTP with $\beta-\gamma$-pyrophosphate as a leaving group and under reversion of conformation at the α-phosphate. Both Mg^{2+} ions together are supposed to stabilize the pentacovalent transition state of the α-phosphate of the nucleosidetriphosphate. This unified two metal-ion catalysis has first been proposed for the 3′-5′ exonuclease domain of the Klenow fragment from DNA polymerase I of *E. coli* and has been adopted for the polymerase β family (*37*). A similar catalytic mechanism has been proposed for the kanamycin nucleotidyltransferase, although only one of the two Mg^{2+} ions has been found in the crystal structure.

It is very likely that the mechanism of the phosphoribosyl-dephospho-CoA transfer reaction catalyzed by the newly described holo ACP synthase family obeys the two metal ion catalysis. A model for the active site of *K. pneumoniae* holo ACP synthase, adopted from the crystal structure of human DNA polymerase β (*35*) is depicted in Figure 7. The two catalytic important aspartic residues 134 and 136, together with a third conserved carboxylic amino acid, coordinate two magnesium ions that facilitate the nucleophilic attack of ACP-serine 25 on the α-phosphate of the

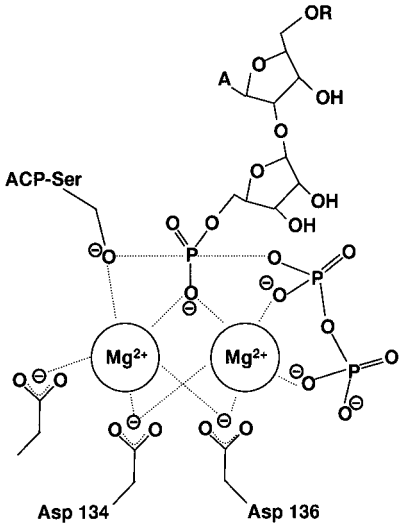

FIGURE 7: Schematic of proposed phosphoribosyl-dephospho-CoA transfer by holo ACP synthase showing the pentacovalent transition state according to the two metal ion catalysis mechanism. The deprotonated hydroxyl group of serine 25 from the acyl carrier protein (ACP) makes an in-line nucleophilic attack on the α-phosphate of the prosthetic group precursor under reversion of the conformation at the α-phosphate. The transition state is stabilized by two magnesium ions, which are coordinated by three invariant aspartates. A, adenine; R, 4′-diphosphopantetheine moiety of the prosthetic group precursor.

prosthetic group precursor. The third invariant carboxylate, whose position is more variable in the secondary structures of different members of the nucleotidyltransferase superfamily is in proximity to the other carboxylates in the three-dimensional structure of DNA polymerase β and kanamycin nucleotidyltransferase. Indeed, two carboxylic acids, aspartic acid 165 and glutamic acid 179, are conserved among holo ACP synthases. They could have the function of the third conserved carboxylic acid involved in Mg^{2+} coordination.

The nucleotidyltransferase superfamily is a good example for divergent evolution. In archaea, and to a lesser extent in bacteria, genes have been identified that code for small proteins (approximately 90 amino acids) that contain the nucleotidyltransferase motif. The function of these "minimal" nucleotidyltransferases is unknown, but they are supposed to recognize their targets with the help of other proteins (*23*). During evolution, the nucleotidyltransferase domain may have been fused to other domains responsible for target recognition or substrate specificity. So far, a wide spectrum of targets for nucleotidyltransferases are known, ranging from RNA and DNA to proteins and antibiotics. However, the substrate to be transferred always remained a nucleoside- or deoxynucleosidetriphosphate. In this context, the holo ACP synthase family expands the variety of substrates to other triphosphates, i.e., 2′-(5″-triphosphoribosyl)-3′-dephospho-CoA, the prosthetic group precursor of malonate decarboxylase.

Another enzyme carrying phosphoribosyl-dephospho-CoA as a prosthetic group is citrate lyase. In *E. coli*, the two enzymatic activities for prosthetic group biosynthesis are encoded by *citG* and *citX* (*14*). Interestingly, the relationship of the citrate lyase holo ACP synthase CitX to the nucleotidyltransferase superfamily is less obvious than that of MdcG and MadK. Only the two aspartates of the nucleotidyltransferase consensus sequence are conserved. An even further step of divergent evolution has been reached in citrate lyase of *Leuconostoc mesenteroides* or *Weissella paramesenteroides*. In these organisms, the *citX* and *citG* genes are fused, giving rise to a bifunctional enzyme (*38*, *39*).

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