**4’-Phosphopantetheine Biosynthesis in Archaea**

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Coenzyme A as the principal acyl carrier is required for many synthetic and degradative reactions in intermediary metabolism. It is synthesized in five steps from pantothenate, and recently the CoaA biosynthetic genes of eubacteria, plants, and human were all identified and cloned. In most bacteria, the so-called Dfp proteins catalyze the synthesis of the coenzyme A precursor 4’-phosphopantetheine. Dfp proteins are bifunctional enzymes catalyzing the synthesis of 4’-phosphopantetheine (CoaB activity) and its decarboxylation to 4’-phosphopantetheine (CoaC activity). Here, we demonstrate the functional characterization of the CoaB and CoaC domains of an archaebacterial Dfp protein. Both domains of the *Methanocaldococcus januschii* Dfp protein were purified as His-tag proteins, and their enzymatic activities were then identified and characterized by site-directed mutagenesis. Although the nucleotide binding motif II of the CoaB domain resembles that of eukaryotic enoyl-CoA hydratase/isomerases, the 4’-phosphopantothenoylaminoenethiol intermediate is not present. Moreover, the conserved Asn residue otherwise is involved in the reduction of an aminoenethiol reaction intermediate, is not present. The CoaB activity differs significantly from those of other characterized CoaB proteins. In particular, the active site cysteine residue, which is synthesized (2–4). Crystal structure analysis of *E. coli* CoaB (5) revealed the 4’-phosphopantothenoate and CTP binding motifs of CoaB. The nucleobase binding motif II of eubacterial CTP-binding CoaBs deviates from that of eukaryotic ATP-binding PPC synthetases. In *E. coli* Dfp the sequence reads 307-PNP-DIV, whereas the sequence is 210-VPK-PLL in the human enzyme (residues in boldface are those conserved in eubacterial/eukaryotic enzymes). In the next step, PPC is oxidatively decarboxylated to 4’-phosphopantothenolamine by the NH₂-terminal FMN-binding CoaC domain of Dfp (6–9). Subsequent reduction of 4’-phosphopantothenolamine by 4’-phosphopantetheine to CoaC activity by the reduced cofactor FMNH₂ depends on the conserved cysteine residue of the 16-amino acid 4’-phosphopantothenoylcysteine binding clamp, 131-PDS-GSQAC-GDIPGRGM (6, 8, 9). Binding of 4’-phosphopantothenoylcysteine also involves the Asn residue of the 5′-coaC part and the 3′-coaA part of the archaebacterial dfp gene. Both the expressed His protein and the purified His tag proteins His-CoaB and His-CoaA were characterized functionally. Although several active site residues of eubacterial and eukaryotic CoaC activities are not conserved in *Methanocaldococcus Dfp* (12), we were able to demonstrate its PPC decarboxylase activity. Furthermore, it is shown that His-CoaC (and thus Dfp) forms homododecamers like the *E. coli* Dfp protein and the related peptidyl-cysteine decarboxylases (10, 12, 13). We show that *M. januschii* CoaB is a CTP-dependent enzyme, although the nucleotide binding motif II contains a Lys residue, which is characteristic for the ATP-dependent PPC synthetase activity.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction**

*Materials—*All PCR amplifications were performed with Vent-DNA polymerase (New England Biolabs). For amplification of parts of the dfp gene, purified chromosomal DNA of *M. januschii* DSM 2661 was used.

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²The abbreviations used are: PP, 4’-phosphopantetheine; HFDCL, homo-oligomeric flavin-dependent (3)-desaturase; MBP, maltose-binding protein from *Escherichia coli*; R-N-NTA, nickel-nitritriacetic acid; PPC, (R)-4’-phospho-N-pantetheine; RPC, reversed phase chromatography; Tricine, (N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; wt, wild type.
Cloning of coaB from M. jannaschii—The 3′/H11032 part of the dfp gene encoding the carboxyl-terminal CoaB domain Asn 190–Ser403 of M. jannaschii Dfp was amplified by PCR and cloned into the single BamHI site of the expression vector pQE8 (Qiagen). For PCR amplification, the primers (i) forward, 5′-GTTATTGAAAAATCAAATTCACTCCTAGCCTAACTTTAAGAG-3′, and (ii) reverse, 5′-CCCAGCACTTGTTGACAATCTTATTTCCTTCTT-3′, were used (nucleotides shown in bold letters were exchanged compared with the sequence of the chromosomal DNA to introduce the BglII sites (underlined)). The amplified coaB gene was digested with BglII and cloned into pQE8 BamHI. The pQE8-derived plasmids were transformed into the expression strain E. coli M15 (pREP4) (Qiagen) by electroporation. The expression plasmid pQE8 coaB encodes an NH2-terminal His tag fusion protein of the M. jannaschii CoaB protein (His-CoaB: MRGSHHHHHHGS-Dfp-Asn190-Ser403).

Cloning of coaC from M. jannaschii—The 5′ part of the dfp gene encoding the amino-terminal CoaC domain Met1–Arg197 of M. jannaschii Dfp was amplified by PCR and cloned into the single BamHI site of the expression vector pQE8 (Qiagen). For PCR amplification, the following primers, (i) forward, 5′-GGTTTAAATGACATGATAGTGGATCTAGGTTCTTCTTCT-3′, and (ii) reverse, 5′-CTCCGTTTAATATTAAA-GATCTATTTTCTCCT-3′, were used. The amplified coaC gene was digested with BglII and cloned into pQE8 BamHI as described above. The expression plasmid pQE8 coaC encodes an NH2-terminal His tag fusion protein of the M. jannaschii CoaC protein (His-CoaC: MRGSHHHHHHGS-Dfp-Met1-Arg197-Ser) (the additional serine residue at the COOH terminus was introduced because of the cloning procedure used).

(DSMZ, Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany). Oligonucleotides were purchased from MWG Biotech.

**Cloning of coaB from M. jannaschii**—The 3′ part of the dfp gene encoding the carboxyl-terminal CoaB domain Asn190–Ser403 of M. jannaschii Dfp was amplified by PCR and cloned into the single BamHI site of the expression vector pQE8 (Qiagen). For PCR amplification, the primers (i) forward, 5′-GTTATTGAAAAATCAAATTCACTCCTAGCCTAACTTTAAGAG-3′, and (ii) reverse, 5′-CCCAGCACTTGTTGACAATCTTATTTCCTTCTT-3′, were used (nucleotides shown in bold letters were exchanged compared with the sequence of the chromosomal DNA to introduce the BglII sites (underlined)). The amplified coaB gene was digested with BglII and cloned into pQE8 BamHI. The pQE8-derived plasmids were transformed into the expression strain E. coli M15 (pREP4) (Qiagen) by electroporation. The expression plasmid pQE8 coaB encodes an NH2-terminal His tag fusion protein of the M. jannaschii CoaB protein (His-CoaB: MRGSHHHHHHGS-Dfp-Asn190-Ser403).

Cloning of coaC from M. jannaschii—The 5′ part of the dfp gene encoding the amino-terminal CoaC domain Met1–Arg197, of M. jannaschii Dfp was amplified by PCR and cloned into the single BamHI site of the expression vector pQE8 (Qiagen). For PCR amplification, the following primers, (i) forward, 5′-GGTTTAAATGACATGATAGTGGATCTAGGTTCTTCTTCT-3′, and (ii) reverse, 5′-CTCCGTTTAATATTAAA-GATCTATTTTCTCCT-3′, were used. The amplified coaC gene was digested with BglII and cloned into pQE8 BamHI as described above. The expression plasmid pQE8 coaC encodes an NH2-terminal His tag fusion protein of the M. jannaschii CoaC protein (His-CoaC: MRGSHHHHHHGS-Dfp-Met1-Arg197-Ser) (the additional serine residue at the COOH terminus was introduced because of the cloning procedure used).

**FIGURE 1. The 4′-phosphopantetheine and coenzyme A biosynthetic pathway in bacteria.** Coenzyme A is synthesized from pantothenate in five steps. The enzymes CoaA, CoaB, CoaC, CoaD, and CoaE catalyze the phosphorylation of pantothenate, the CTP-dependent ligation of cysteine and 4′-phosphopantothenate to 4′-phosphopantetheinoylcysteine, the decarboxylation of PPC to 4′-phosphopantetheine, the formation of dephospho-coenzyme A, and the phosphorylation of the 3′-hydroxyl group of dephospho-coenzyme A. In eu- and archaebacteria, the CoaB and CoaC activities are domains of the bifunctional Dfp proteins. The CoaB-catalyzed synthesis of PPC occurs in two half-reactions starting with the formation of 4′-phosphopantetheinoyl-CMP and the subsequent formation of the amide bond of PPC (shaded in gray) by reaction of this activated intermediate with cysteine.

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Pantothenic acid

CoaA: Pantothenate kinase

4′-Phosphopantothenoylcysteine decarboxylase

CoaB

4′-Phosphopantothenoylcysteine synthetase

PPi + CMP + CO2 + 4′-Phosphopantetheine

CoaD: 4′-Phosphopantetheine adenyllyltransferase

Dephospho Coenzyme A

CoaE: Dephospho-Coenzyme A Kinase

Coenzyme A
Site-directed Mutagenesis of coaB and coaC—All point mutations were introduced by using sequential PCR and appropriate mutagenesis primers as described recently (14). For the PCR mutagenesis of coaB, pQE8 coaB was used as template, and the oligonucleotides (i) forward, 5'-CAATTGTGAGGGGATAAACATATTTCAC-3', and (ii) reverse, 5'-CAGCTAATAGCCTAGTATTTC-3', were used as terminal primers (the introduced BglII site underlined). For the PCR mutagenesis of coaC, pQE8 coaC was used as template and the oligonucleotides (i) forward, 5'-CAATTGTGAGGGGATAAACATATTTCAC-3', and (ii) reverse, 5'-AATCCAGATGGAGAGCTCATTTAC-3', were used as terminal primers (introduced BglII site is underlined). The amplified coaB and coaC genes, respectively, were digested with EcoRI/BglII and cloned into pQE12 EcoRI/BglII. The pQE12-derived plasmids were transformed into the expression strain E. coli M15 (pREP4) (Qiagen) by electroporation. The entire sequences of the codon regions of the constructed pQ12 plasmids were verified. Because of the cloning procedure, the pQ12 plasmids encode the same His tag proteins as the pQE8 plasmids except for the introduced point mutation.

Purification of Proteins

Growth of Strains—The E. coli strains used were grown to A600 = 0.4 in 0.5 liters of B-broth (10 g casein hydrolysate 140 (Invitrogen), 5 g of yeast extract (Difco), 5 g of NaCl, 1 g of glucose, and 1 g of K2HPO4/liter, pH 7.3) in 2-liter shaker flasks, induced with 1 mM isopropyl-

Amersham Biosciences. The elution volumes of the standard proteins used for calibration were obtained from a SMART system (Amersham Biosciences). Compounds were eluted with a linear gradient of 0–50% acetonitrile-0.1% trifluoroacetic acid in 5.8 ml with a flow rate of 200 μl/min. The absorbance was measured simultaneously at 214, 260, and 280 nm to enable identification of reaction products.

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SITE-DIRECTED MUTAGENESIS—Because of the importance of 4'-phosphopANTetheinE adenylyltransferase activity and/or substrate or cofactor binding in eubacterial and eukaryotic enzymes are conserved in M. jannaschii (2, 3, 5, 12). From sequence comparisons, we propose that the 16-alpha amino acid peptide PKFTEEGKAVNEDIV is specific for 4'-phosphopantothenoyl-CoA (15). However, the active site cysteine residue 158 is not conserved in the M. jannaschii protein. Mutation of Cys158 to Ala or Ser led to loss of PPC decarboxylase activity in E. coli Dfp (12). Crystal structure analysis of the plant PPC decarboxylase AtHAL3a complexed with the oxidatively decarboxylated reaction intermediate suggested that reduction of the C=C bond of this intermediate can be performed by direct hydride transfer from N-5 of FMNH2 to C9 supported by protonation of C8 by the conserved cysteine residue (8). The function of the conserved cysteine residue as active site acid in the protonation on the eneithiolate intermediate is further supported by mechanistic studies on the human PPC decarboxylase (9). It is not clear which residue of the M. jannaschii PPC synthetase (15).

RESULTS AND DISCUSSION

Cloning and Sequence Comparison of M. jannaschii 4'-Phosphopantetheine Biosynthetic Genes coaB and coaC—The complete genome sequence of M. jannaschii was published as early as 1996 (17), and the dfp gene is annotated in the data banks. Several but not all residues shown to be important for PPC synthetase and decarboxylase activity and/or substrate or cofactor binding in eubacterial and eukaryotic enzymes are conserved in M. jannaschii (2, 3, 5, 12). From sequence comparisons, we propose that the 16-alpha amino acid peptide PKFTEEGKAVNEDIV corresponds to the substrate recognition clamp 152DSGSGACGCDIGPRGM166 of the E. coli Dfp protein (10); however, the active site cysteine residue 158 is not conserved in the M. jannaschii protein. Mutation of Cys158 to Ala or Ser led to loss of PPC decarboxylase activity in E. coli Dfp (12). Crystal structure analysis of the plant PPC decarboxylase AtHAL3a complexed with the oxidatively decarboxylated reaction intermediate suggested that reduction of the C=C bond of this intermediate can be performed by direct hydride transfer from N-5 of FMNH2 to C9 supported by protonation of C8 by the conserved cysteine residue (8). The function of the conserved cysteine residue as active site acid in the protonation on the eneithiolate intermediate is further supported by mechanistic studies on the human PPC decarboxylase (9). It is not clear which residue of the M. jannaschii PPC synthetase (15).
PC decarboxylase functionally aligns with this active site cysteine residue. A second residue that is important for the decarboxylase activity is also not conserved in *M. jannaschii* Dfp. In peptidyl-cysteine decarboxylases (LanD enzymes) and most eubacterial and eukaryotic PPC decarboxylases an Asn residue is conserved in the so-called PXM-NXXMW motif and proposed to be involved in binding of the COOH-
group of the substrates (8, 10, 12); in the *M. jannaschii* enzyme this Asn residue is exchanged for His (His139).

To characterize the PPC synthetase and decarboxylase activities of the *M. jannaschii* enzyme, the 5′ part (encoding the CoaC domain Met1–Arg197) and the 3′ part (encoding the CoaB domain Asn190–Ser403) of the *dfp* gene were cloned in the pQE8 expression vector. The coding regions of both plasmids were verified by DNA sequencing.

**Expression, Purification, and Characterization of 4′-Phosphopantetheine Biosynthetic Enzymes**—The *M. jannaschii* His-CoaB and His-CoaC proteins were purified from overexpressing *E. coli* clones in a single immobilized metal affinity chromatography purification step (purity of the enzymes was verified by SDS-PAGE; data not shown). To obtain information about the cofactor dependence of His-CoaB, we analyzed the mutant enzyme His-CoaB N217H. Recently, it has been shown that residues Arg206, Asn210, and Ala276 of *E. coli* CoaB are involved in the second half-reaction of the PPC synthesis and that the 4′-phosphopantetheynoyl-CMP intermediate is copurified with mutant *E. coli* His-CoaB N210K protein but not with *E. coli* His-CoaB wt (2, 3). This residue, Asn210, corresponds to residue Asn217 in *M. jannaschii* CoaB.

Treatment of *M. jannaschii* His-CoaB N217H with trifluoroacetic acid and RPC separation of the copurified substances revealed that also in this case 4′-phosphopantetheynoyl-CMP is bound (Fig. 3A), indicating that the archaeobacterial PPC synthetase is CTP- and not ATP-dependent. The ATP dependence of the *M. jannaschii* PPC synthetase was suggested recently because the nucleotide binding motif II contains a Lys residue (Lys310; Fig. 2) that is conserved in eukaryotic ATP-dependent enzymes but not in eu- or catabacterial CTP-dependent ones (5).

Purified *M. jannaschii* His-CoaC protein was green, and UV-visible spectroscopy showed absorbance maxima characteristic for flavoproteins and, additionally, a stable long-wavelength absorption with a maximum of about 706 nm (Fig. 3B), indicating the formation of a charge-transfer complex between wild-type enzyme and either the substrate PPC or the reaction product PP or with the oxidatively decarboxylated enethiolate intermediate (9). The flavin cofactor was identified by RPC as flavin mononucleotide (data not shown). Exchanging of the conserved His87 residue to Ala led to reduced flavin binding capacity (data not shown), indicating that in this case greater structural changes were introduced. His-CoaC H87N was also purified as a green enzyme; how-
ever, the long-wavelength absorption band is not stable and is no longer visible after a few hours (Fig. 3B). The absorbance maximum of the flavin cofactor is at 448 nm for the wt enzyme and at 453 nm for the mutant enzyme H87N. From the published crystal structures of PPC decarboxylases and LanD enzymes (8, 10, 18, 19), we know that the conserved His residue of HFCD (homo-oligomeric flavin-dependent Cys decarboxylases) enzymes is in direct neighborhood of the flavin cofactor. Therefore, it is not surprising that exchanging His87 led to changes in the UV-visible spectrum of the bound cofactor. Because His87 is part of the active site and proposed to be involved in substrate binding, it is also not surprising that the charge-transfer band is not as stable as in the wt enzyme.

Enzymes known to catalyze the decarboxylation of cysteine residues are trimeric (for example the PPC decarboxylase AtHAL3a (8, 18)) or dodecameric enzymes (built up from four trimers; for example the LanD enzymes EpiD and MrsD and the eubacterial Dfp proteins (10, 12, 13, 19)) and are named HFCD proteins (10). *M. jannaschii* His-CoaC eluted at an apparent molecular mass of ~242 kDa from the gel filtration column (Fig. 3D). The calculated molecular mass of His-CoaC-FMN is 24.06 kDa. Although the observed molecular mass deviates by 46.8 kDa from the theoretical value for a homododecamer, we assume (taking the inaccuracy of molecular weight determinations by gel filtration into account) that His-CoaC builds up homododecamers. In conclusion, the *M. jannaschii* Dfp protein is also a homododecameric enzyme, with the CoaC domain forming a dodecameric core structure as has recently been modeled for the *E. coli* enzyme (20).

**Identification and Characterization of the PPC Synthetase Activity of *M. jannaschii***—To analyze the biochemistry of the 4′-phosphopantetheine synthesizing activity of *M. jannaschii* Dfp, it is useful to separate PPC synthetase and PPC decarboxylase activities by separating the

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**FIGURE 4. The enzymatic activity of *M. jannaschii* bifunctional Dfp protein.** The activity of His-CoaB wt, His-CoaB N217H, His-CoaC wt, and His-CoaC H87N was determined at 60 °C by adding combinations of the enzymes (A, His-CoaB wt; B, His-CoaB N217H; C His-CoaB wt and His-CoaC wt; D, His-CoaB wt and His-CoaC H87N) to a reaction mixture (preincubated at 37 °C) containing pantothenate, ATP, CTP, Mg2+, cysteine, dithiothreitol, and His-CoaA (*E. coli*), separating the reaction products by RPC, and monitoring the absorbance at 214 nm, 260 nm (not shown), and 280 nm (not shown). The reaction products, PPC and PP, were identified by their known retention times and absorbance properties.
bifunctional Dfp protein into CoaB and CoaC. Using the published high pressure liquid chromatography method for the detection of PPC (13), we were able to show that His-CoaB synthesizes PPC from 4'-phosphopantothenate and cysteine in the presence of CTP (Fig. 4); 4'-phosphopantothenate was enzymatically synthesized in situ using E. coli CoaA. We could not observe any synthetase activity if CTP was omitted in the assay, indicating that ATP (which is always present as cofactor of the pantothenate kinase activity CoaA) is not the cofactor of the Methanocaldococcus enzyme, thus confirming the results described above. The PPC synthetase activity of His-CoaB was further confirmed in a coupled enzyme assay in which synthesized PPC was first decarboxylated to PP and then ligated with AMP to dephospho-CoA (Figs. 5 and 6). The mutant enzyme His-CoaB N217H had only residual PPC synthetase activity, and His-CoaB K310Q had very low activity (Fig. 5). From the crystal structure of E. coli His-CoaB we know that these residues are active site residues (compare also Fig. 2) (5).

Identification of the PPC Decarboxylase Activity of M. jannaschii—Crystal structure analysis of HFCD proteins and the known site-directed mutagenesis studies showed the importance of the conserved Cys residue within the substrate recognition clamp and of the conserved Asn residue within the PXMNXXMW motif for the decarboxylase activity (6, 8–10, 12, 19). Both residues are not present in M. jannaschii His-CoaC, and therefore we did not expect PPC decarboxylase activity. However, when His-CoaC was added to the PPC synthetase assay and the reaction mixture separated by RPC, we observed that synthesized PPC was partially converted to a substance that was identified by its retention time as 4'-phosphopantetheine (Fig. 4) (13). To verify these data in a second assay, synthesized 4'-phosphopantetheine was converted to dephospho-CoA by adding a 4'-phosphopantetheine adenylyltransferase to the assay (Figs. 5 and 6). These experiments clearly showed that His-CoaC is active in decarboxylating PPC.

Active Site Residues of the PPC Decarboxylase Domain of M. jannaschii Dfp—To characterize the PPC decarboxylase domain in more detail, the activity of several mutant enzymes was investigated. All mutants bind the flavin cofactor (however, for the H87A mutant see above) and showed charge-transfer bands indicating that no major structural changes were introduced by the amino acid exchanges. As expected, exchanging the active site residue His for Asn reduced the activity of M. jannaschii His-CoaC (Fig. 6). However, the PPC decarboxylases from E. coli and A. thaliana were completely inactivated by exchanging the active site His residue for Asn (13, 21). Exchanging His for Asp led to a significant change in the UV-visible spectrum of the CoaC enzyme and to significant decrease in enzymatic activity (Fig. 7). The absorbance maximum of the charge-
transfer band was shifted by about 100 nm to lower wavelengths (Fig. 3C), indicating a change in the mode of substrate binding. It is possible that to some extent an Asp residue in position 139 is also present in the H139N mutant, derived by deamidation from Asn at higher temperatures (explaining the low activity of the H139N mutant?). Exchange of Asn141 (which is not conserved in PPC decarboxylases) for Asp also led to a significant shift of the charge-transfer band (Fig. 3C) but no decrease in activity was observed (Fig. 7). Surprisingly, exchanging His139 for Ala had no significant impact on activity. By accident, a second mutation (E168K) occurred in one of the H139A clones, and this mutant enzyme, His-CoaC H139A/E168K, had drastically reduced PPC decarboxylase activity (Fig. 6); the residue Glu168 is within the proposed PPC recognition clamp of *M. jannaschii* His-CoaC. To investigate the role of the residue Glu168 in more detail, we investigated the PPC decarboxylase activities of the mutant proteins His-CoaC H168A, His-CoaC H168D, and His-CoaC H168K (Fig. 7). We observed only residual activity for the mutant E168K, drastically reduced activity for E168A, and a significantly decreased activity for E168D. Exchange of the neighboring Glu167 for Ala did not influence the PPC decarboxylase activity. Because the active site cysteine residue conserved in eubacterial and eukaryotic
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PPC decarboxylases is not present in the substrate binding clamp of the *M. jannaschii* CoaC domain, we analyzed whether one of the four cysteine residues of CoaC is essential for activity. However, the activity of the mutant enzymes His-CoaC C54A, His-CoaC C73A, His-CoaC C94A, and His-CoaC C96A was comparable with that of the wild-type enzyme (data not shown). At the moment it is not clear whether Glu^{168} of the substrate binding clamp takes over the role of the Cys residue as active site acid (in this case an increased apparent pK_a value has to be assumed for Glu^{168}).

**Conclusions**—The biosynthetic pathway of 4′-phosphopantetheine from 4′-phosphopantothenate has now been elucidated in eubacteria, Archaea, and eukaryotes. The active site architecture of the archaeabacterial CoaC domain differs significantly from that of eubacterial and eukaryotic enzymes. The conserved Asn residue of the PXMNXXMW motif is exchanged for His, and the active site Cys residue of the substrate recognition clamp is not present. Crystal structural analysis of *M. jannaschii* CoaC will be useful to elucidate the reaction mechanism of archaeabacterial PPC decarboxylases.

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