Molecular Characterization of the 4′-Phosphopantothenoylcysteine Decarboxylase Domain of Bacterial Dfp Flavoproteins*

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The NH2-terminal domain of the bacterial flavoprotein Dfp catalyzes the decarboxylation of (R)-4′-phospho-N-pantothenoylcysteine to 4′-phosphopantetheine, a key step in coenzyme A biosynthesis. Dfp proteins, LanD proteins (for example EpiD, which is involved in epidermin biosynthesis), and the salt tolerance protein AtHAL3a from Arabidopsis thaliana are homooligomeric flavin-containing Cys decarboxylases (HFCF protein family). The crystal structure of the peptidyl-cysteine decarboxylase EpiD complexed with a pentapeptide substrate has recently been determined. The peptide is bound by an NH2-terminal substrate binding helix, residue Asn117, which contacts the cysteine residue of the substrate, and a COOH-terminal substrate recognition clamp. The conserved motif G-G/S-I-A-Y-K of the Dfp proteins aligns partly with the substrate binding helix of EpiD. Point mutations within this motif resulted in loss of coenzyme binding (G14S) or in significant decrease of Dfp activity (G15A, I16L, A17D, K20N, K20Q). Exchange of Asn257 of Dfp, which corresponds to Asn117 of EpiD, and exchange of Cys258, which is within the proposed substrate recognition clamp of Dfp, led to inactivity of the enzyme. Molecular analysis of the conditional lethality of the Escherichia coli dfp-707 mutant revealed that the single point mutation G11D of Dfp is related to decreased amounts of soluble Dfp protein at 37°C.

Coenzyme A and 4′-phosphopantetheine are essential cofactors for many enzymatic reactions and acyl-CoA derivatives are key intermediates in energy metabolism. In bacteria, coenzyme A is synthesized in five enzymatic steps from pantothenate (Fig. 1) (1, 2). In the first step, pantothenate is phosphorylated to 4′-phosphopantethenate by pantohenate kinase, which is encoded by the coaA gene (3). Then, (R)-4′-phospho-N-pantothenoylcysteine (PPC)1 is synthesized by the addition of cysteine to 4′-phosphopantethenate and in the next step, PPC is decarboxylated to 4′-phosphopantetheine (PP). 4′-Phosphopantetheine is converted to coenzyme A by the enzymes 4′-phosphopantetheine adenylyltransferase and dephospho-CoA kinase (Fig. 1). The conversion of 4′-phosphopantethenate to coenzyme A has only recently been attributed to purified enzymes and cloned genes, respectively. In 1999, Geerlof et al. (4) showed that the kdtB gene encodes the 4′-phosphopantetheine adenylyltransferase and renamed the gene to coaD. The molecular characterization of the lantibiotic-synthesizing enzyme EpiD, a peptidyl-cysteine decarboxylase (5–7), was the basis for studying the enzymatic activity of the Dfp protein from Escherichia coli. It was demonstrated that the NH2-terminal domain of the Dfp protein, which shows sequence homology to EpiD, catalyzes the decarboxylation of (R)-4′-phospho-N-pantothenoylcysteine to 4′-phosphopantetheine (8). Recently, it was shown that the Arabidopsis thaliana flavoprotein AtHAL3a, which is related to plant growth and salt and osmotic tolerance (9), is catalyzing the same reaction as the amino-terminal domain of Dfp (10). This year the identification of enzymes/genes involved in coenzyme A biosynthesis in E. coli was completed. Strauss et al. (11) showed that the Dfp protein is a bifunctional enzyme and catalyzes not only the decarboxylation of PPC but also its synthesis from 4′-phosphopantethenate and L-cysteine using cytidine 5′-triphosphate as the activating nucleotide (Fig. 1). As a consequence, Strauss et al. (11) renamed the dfp gene to coaBC. Mishra et al. (12) showed that the dephospho-coenzyme A kinase is a 22.6-kDa protein encoded by the yacE (coaE) gene.

EpiD, Dfp, and AtHAL3a belong to a new superfamily of flavoproteins, which was named HFCF ( homo-oligomeric flavin containing Cys decarboxylases). They share a new flavin-binding motif, conserved active-site residues, are trimeric or dodecameric enzymes, and decarboxylate cysteine residues (8, 10, 13, 14). The crystal structure of EpiD complexed with a pentapeptide substrate revealed the active-site architecture and gave first insights into the mechanism of the oxidative decarboxylation of peptides catalyzed by EpiD. It was proposed that decarboxylation occurs via a thioaldehyde intermediate (14). In case of the PPC decarboxylases Dfp and AtHAL3a, decarboxylation of the substrate may also involve the formation of a thioaldehyde/ethenol intermediate (8, 10).

In this study, the molecular characterization of the NH2-terminal CoaC domain of Dfp, catalyzing the decarboxylation of PPC, is presented. I show that the CoaC domain is responsible for dodecamer formation of Dfp. Conserved sequence motifs of the CoaC domain are investigated by site-directed mutagenesis continuing the molecular characterization of MBP-Dfp fusion proteins (8). These studies lead to the definition of a PPC decarboxylase signature sequence enabling the identification of further PPC decarboxylases. Furthermore, the molecular characterization of the conditional lethal E. coli dfp-707 mutant (15, 16) is reported.

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1 The abbreviations used are: PPC, (R)-4′-phospho-N-pantothenoylcysteine; PP, 4′-phosphopantetheine; HFCF, homooligomeric flavin-containing Cys decarboxylases; RPC, reversed phase chromatography; MBP, maltose-binding protein; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis.
FIG. 1. The role of Dfp in coenzyme A biosynthesis. A, coenzyme A is synthesized in five steps from pantothenate. The bifunctional enzyme Dfp catalyzes the two-step conversion of 4′-phosphopantetheinyl acid to 4′-phosphopantetheine. B, domain organization of Dfp. The NH2-terminal domain of Dfp (Ser5′ to Asn5′) catalyzes the synthesis of PPC and the enzymatic activity of the CoaC domain and the structures of PPC and PP.

Experimental Procedures

Plasmid Construction

In General—PCR amplifications were performed with Vent-DNA-polymerase (New England BioLabs). The entire sequences of the dfp coding regions of the constructed plasmids were verified. The used oligonucleotides were purchased from MWG Biotech.

Cloning of the 5′-Port of the dfp Gene—The 5′-part of the dfp gene encoding the NH2-terminal domain Met1–Asn190 of Dfp (numbering of the Dfp amino acid residues is based on the experimentally determined start codon of the dfp gene (8) and differs from the previously used nomenclature) was amplified by PCR and cloned into the single EcoRI and BglII sites of the expression vector pQE12 (Qiagen) in such a way that no fusion with the His tag codons occurred. For PCR amplification, the template plasmid pQE12 dfp and the primers (i) forward, 5′-GATAACAATTTCACACAGAATTCTGGAAAG-3′ and (ii) reverse, 5′-GGTCATTACTGGATCTATCAACAGG-3′ were used. The restriction sites EcoRI and BglII used for cloning are underlined, and the introduced stop codon is in bold letters.

Site-directed Mutagenesis of dfp—The mutant dfp genes were constructed by sequential PCR using appropriate mutagenesis primers and pQE12 dfp (8) as a template. The mutant dfp genes were then cloned into the single EcoRI and BglII sites of the pQE12 expression vector (Qiagen) in such a way that no fusion with the His tag codons occurred. The primers (i) forward, 5′-GATAACAATTTCACACAGAATTCTGGAAAGCGC-3′ and (ii) reverse, 5′-CCGGCCGGGCGTGTAAGATCTTTAAGTTCAGATGGTTTCAG-3′ were used. The restriction sites EcoRI and BglII used for cloning are underlined, and the introduced stop codon is in bold letters.

Cloning of the 5′-Port of the dfp Gene—The 5′-part of the dfp gene encoding the NH2-terminal domain Met1–Asn190 of Dfp (numbering of the Dfp amino acid residues is based on the experimentally determined start codon of the dfp gene (8) and differs from the previously used nomenclature) was amplified by PCR and cloned into the single EcoRI and BglII sites of the expression vector pQE12 (Qiagen) in such a way that no fusion with the His tag codons occurred. The primers (i) forward, 5′-GATAACAATTTCACACAGAATTCTGGAAAGCGC-3′ and (ii) reverse, 5′-CCGGCCGGGCGTGTAAGATCTTTAAGTTCAGATGGTTTCAG-3′ were used. The restriction sites EcoRI and BglII used for cloning are underlined, and the introduced stop codon is in bold letters.

Purification and Characterization of Dfp Proteins

Growth of Strains—E. coli M15 (pREP4, pQE12) cells were grown in the presence of 100 μg/ml ampicillin and 25 μg/ml kanamycin in 0.5 liter of B-broth in 2-liter shaker flasks. At A600 = 0.4, the cells were induced with 1 mM isopropyl-β-D-thiogalactopyranoside and harvested 2 h after induction. Growth temperature was 37 °C if not otherwise stated.

Purification of Mutant Dfp Proteins and of the NH2-Terminal Domain of Dfp—500 ml of isopropyl-β-D-thiogalactopyranoside-induced E. coli M15 (pREP4, pQE12 dfp) cells were harvested and disrupted by sonication in 10 ml of column buffer (20 mM Tris-HCl, pH 8.0). 5 ml of the cleared lysate obtained by two centrifugation steps (each 25 min at 30,000 × g at 4 °C) was diluted with 5 ml of column buffer and loaded on a 1-ml HiTrapQ column equilibrated with column buffer. The column was then washed with 5 ml of column buffer and 5 ml of column buffer containing 0.1 M NaCl. Dfp proteins and Dfp S2-N190, respectively, were eluted with column buffer containing 0.25 mM NaCl, and the yellow peak fractions (~400 μl) were collected. A 25-μl aliquot of this HiTrapQ eluate was then immediately subjected to a Superdex 200 PC 3.2/30 gel filtration column (Amersham Pharmacia Biotech) equilibrated in running buffer (20 mM Tris-HCl, pH 8.0, 200 mM NaCl) at a flow rate of 40 μl/min. The elution was followed by absorbance at 280, 378, and 450 nm. Molecular weight information was obtained as described previously (8), determining the elution volumes of standard proteins and the void volume of the column.

Dfp Assays—Approximately 50–100 μg of PPC as calcium salt were incubated with 1–5 μg of Dfp for 15–30 min at 37 °C in a total volume of 0.75–1 ml of 50 mM Tris-HCl, pH 8.0, 3 mM dithiothreitol. The mutant proteins Dfp N125D, Dfp N125Q, and Dfp C158S, which were supposed to be inactive, were used at approximately double concentration compared with the bound FNM. The reaction mixture was then subjected to reversed phase chromatography (RPC) to separate PPC and the reaction product PP. RPC was carried out with a µRPC C8/C18 SC 2.1/10 column on a SMART system (Amersham Pharmacia Biotech) as described recently (8).
Recently, the NH$_2$-terminal CoaC domain of Dfp proteins was shown to catalyze the decarboxylation of \((\text{R})-4'\)-phospho-N-pantothenoylcysteine to \(4'\)-phosphopantetheine (Fig. 1; Ref. 8), a key step in coenzyme A biosynthesis. EpID and \(E.\ coli\) Dfp S2-R406 (CoaBC; the NH$_2$-terminal Met residue is cleaved off by methionine aminopeptidase activity in \(E.\ coli\)) form homododecamers, whereas the plant PPC decarboxylase AtHAL3a (CoaC) forms homotrimers (8, 13, 14). To verify that the CoaC domain of the Dfp protein is responsible for dodecamer formation, the 5'-part of the \(E.\ coli\) dfp gene was cloned and overexpressed. Gel filtration experiments revealed a molecular mass of about 270 kDa for the CoaC domain (Fig. 2), whereas the calculated molecular mass of the Dfp S2-N190-FMN monomer is 20.1 kDa. The experimentally determined value of 270 kDa is in accordance with homododecamer formation allowing for the inaccuracy of molecular weight determination by gel filtration.

To purify the NH$_2$-terminal domain to homogeneity, the ribosomal binding site of the dfp gene was optimized. Expression was increased drastically, but did not yield native protein. Most of the expressed protein formed high molecular weight aggregates, as shown by gel filtration chromatography of the soluble protein fraction. Moreover, the ratio of the absorbance values at 280 and 450 nm of Dfp S2-N190 eluted from the gel filtration column indicated that the flavin coenzyme FMN is not bound in 1:1 proportion (12 FMN molecules should be bound to one native dodecameric protein). Therefore, molecular characterization of the CoaC domain was carried out using the complete Dfp protein, which can easily be purified without affinity tag in a two-step procedure if cloned and expressed as described previously (8).

Alignment of the CoaC Domains of Sequenced Dfp Proteins—EpID, AtHAL3a, and Dfp are HFCD proteins (8, 14). First site-directed mutagenesis studies on EpID, Dfp, and AtHAL3a and the crystal structure elucidation of EpID H67N with bound substrate peptide led to identification of a new flavin binding motif and revealed the active-site architecture and substrate binding mode of EpID (8, 10, 14). The motifs PASANT and PXXNXMXM are common to all HFCD proteins. They are involved in coenzyme binding and contribute contacts with the pyrimidine and phosphoribityl moiety of FMN. The PXXNXMXM motif is not only important for coenzyme but also for substrate binding. For EpID, it was shown that the conserved Asn residue of this motif (Asn$^{117}$) is in contact with the C$_b$ hydrogen atoms and the carboxylate group of the substrate cysteinyl moiety, whereas the conserved Met residue (Met$^{120}$) is located above the pyrimidine ring of the FMN coenzyme. In all HFCD proteins, a histidine residue (for example His$^{87}$ of EpID, His$^{75}$ of Dfp, and His$^{90}$ of AtHAL3a) is strictly conserved, and its mutation for Asn led to inactivation of the enzymes (8, 10). Crystal structure analysis of EpID and AtHAL3a showed that the conserved His residue is within the active site of the HFCD proteins and is likely to be involved in the reaction mechanism. Binding of the substrate peptide to EpID involves the amino-terminal helix of EpID and a substrate recognition clamp comprising residues Pro$^{143}$ to Met$^{162}$ of EpID (Fig. 3; Ref. 14).

In the published sequence alignments, the comparison of the CoaC domain of Dfp proteins from different bacteria was not addressed. To identify residues that are specific for PPC decarboxylases, a detailed comparison of the CoaC domains with each other, with the eukaryotic PPC decarboxylase AtHAL3a, and with the bacterial peptidyl-cysteine decarboxylase EpID was carried out (Fig. 3). This sequence comparison revealed that the conserved NH$_2$-terminal G-G/S-I-A-X-Y-K motif, the P-A-M-N-X-X-M-X (corresponding to the P-N-M-N-I-R-M-W sequence of EpID) motif, and the proposed substrate recognition clamp P-X-X-X-X-C-X-X-X-G-X-G are characteristic for bacterial PPC decarboxylases. This sequence comparison is the basis for the site-directed mutagenesis studies of the CoaC domain presented below.

**RESULTS AND DISCUSSION**

**The NH$_2$-terminal Domain of Dfp Forms Homododecamers—**

Recently, the NH$_2$-terminal CoaC domain of Dfp was shown to catalyze the decarboxylation of \((\text{R})-4'\)-phospho-N-pantothenoylcysteine to \(4'\)-phosphopantetheine (Fig. 1; Ref. 8), a key step in coenzyme A biosynthesis. EpID and \(E.\ coli\) Dfp S2-R406 (CoaBC; the NH$_2$-terminal Met residue is cleaved off by methionine aminopeptidase activity in \(E.\ coli\)) form homododecamers, whereas the plant PPC decarboxylase AtHAL3a (CoaC) forms homotrimers (8, 13, 14). To verify that the CoaC domain...
The Conserved Asn125 Residue of the PXMINXXMW Motif—Preliminary studies on the importance of the conserved Asn residue for the PPC decarboxylase activity have already been published (8). However, for these studies a fusion protein between the MBP from *E. coli* and the N-terminal domain of Dfp assuming the originally proposed GTG start codon was used. Therefore, the experiment was extended using purified mutant proteins Dfp N125Q and Dfp N125D (Figs. 4–6). In gel filtration experiments, both mutant proteins had the same elution volume as wild-type Dfp, and the ratio of the absorbance values at 280 and 27600 nm was imposable anyhow. The Conserved Asn125 Residue of the PXMINXXMW Motif—The Conserved Asn residue for the PPC decarboxylase activity have already been published (8). However, for these studies a fusion protein between the MBP from *E. coli* and the N-terminal domain of Dfp assuming the originally proposed GTG start codon was used. Therefore, the experiment was extended using purified mutant proteins Dfp N125Q and Dfp N125D (Figs. 4–6). In gel filtration experiments, both mutant proteins had the same elution volume as wild-type Dfp, and the ratio of the absorbance values at 280 and 450 nm was not significantly altered by the mutations. These data show that the introduced mutations neither affect dodecameric structure nor coenzyme binding, indicating that the overall three-dimensional structure is not significantly altered. Both mutant proteins were completely inactive even when used at

Molecular Characterization of the CoaC Activity

PCC decarboxylase signature: G-G-I-A-X-Y-K-X_{20-22}^\pm-H^+X_{22-24}-P_{24-27}^\pm-X_{27-30}^\pm-M_{30-33}^\pm-X_{33-36}^\pm-P_{36-39}^\pm-C-X_{40-43}^\pm-G-G-S\_L\_A\_I\_S\_A\_H\_T\_G\_A

FIG. 3. Sequence alignment of the CoaC domains of eubacterial Dfp proteins and the PPC decarboxylase signature pattern. The NH2-terminal CoaC domain of the Dfp protein from *E. coli* (framed) was compared with eubacterial Dfp proteins (listed in alphabetical order), the bacterial peptide-cysteine decarboxylase EpiD, and the eukaryotic PPC decarboxylase AtHAL3a. Only a part of the comparison emphasizing the motifs proposed to be involved in substrate binding is shown. Residues of EpiD that contact the substrate peptide (14) are in green. The G-G/S-I-A-X-Y-K motif of the PPC decarboxylases aligns with residues Ala14 and Ser15 of EpiD, which are involved in coenzyme binding (14) and partly with the amino-terminal substrate binding helix of EpiD (boxed in green). The PXMINXXMW motif contributes the conserved Asn residue (in dark blue; Asn125 of EpiD (in green), Asn125 of AtHAL3a, and Asn125 of the Dfp protein from *E. coli*) that was shown to contact the carboxylate group of the cysteinyI moiety of the peptide-cysteine substrate of EpiD. The substrate binding clamp of EpiD forms an antiparallel b-sheet with the residues -S-S-G- (Ser152 to Gly154) in the turn region (14). Ser153 of EpiD aligns with Lys20 of the Dfp protein from *E. coli*, this Gly residue is exchanged for Ala). The PPC decarboxylase signature includes residues that all HFCD proteins have in common. Besides residues from the PXMINXXMW motif and the substrate recognition clamp, these residues are (labeled with asterisks): the active-site His residue (dark red), the Pro residue (light blue) of the PASANT motif that is involved in coenzyme binding, and a conserved Gly residue (light blue; in the case of the Dfp protein from Helicobacter pylori, this Gly residue is exchanged for Ala).

EpiD (Fig. 3). This indicates that the G-G/S-I-A-X-Y-K motif is also involved in coenzyme and substrate binding. Moreover, the theoretical model for the binding mode of PPC to the eukaryotic PPC decarboxylase AtHAL3a showed that the phosphate group of PPC could be anchored by Lys24 (10). Lys24 of AtHAL3a aligns with Lys20 of the Dfp protein from *E. coli* (Fig. 3). To elucidate the function of the G-G/S-I-A-X-Y-K motif, the following 12-mutant Dfp proteins were purified and analyzed for coenzyme binding and activity: Dfp G14S, Dfp G15A, Dfp I16L, Dfp I16V, Dfp A17D, Dfp A17G, Dfp A17S, Dfp Y19F, Dfp Y19L, Dfp K20N, Dfp K20Q, and Dfp K20R (Figs. 4–6). All mutant proteins showed exactly the same elution volume as Dfp in the gel filtration experiments, indicating that the mutant proteins are completely inactive when used at

Acinetobacter baylyi, Dfp I16L is significantly less active than Dfp I16V, indicating that the methyl groups bound to the b-C atom of Ile16 are important for substrate binding. This is in excellent agreement with the theoretical model for the binding of PPC to AtHAL3a proposing hydrophobic interactions between the side chain of Val10 with the two methylene groups of the b-alanine part of PPC (compare Fig. 1C; Ref. 10). The mutant protein Dfp Y19F was as active as wild-type Dfp, whereas Dfp Y19L was less active (not shown). At the moment, an accurate determination of the kinetic parameters of the mutants is not possible. This determination is hampered by the factors described by Strauss et al. (11) and by the lack of a good working enzyme assay. The most important mutants presented in this paper are inactive or slightly active, and therefore, determination of the K_m values is impossible anyhow.

From the investigated mutants, only Dfp G14S did not bind FMN anymore (and therefore was inactive in PPC decarboxylation). The mutant proteins Dfp G15A and Dfp A17D showed drastically reduced PPC decarboxylase activity (less than 5% of wild-type activity). Dfp K20N and Dfp K20Q showed decreased activity, whereas Dfp K20R showed activity comparable with wild-type Dfp. Supporting the model that the conserved positively charged Lys residue contacts the phosphate group of PPC. It is likely that this Lys residue causes discrimination of PPC and pantothenoylcysteine by Dfp (8). Dfp I16V and Dfp I16L both have reduced activity compared with wild-type Dfp. However, Dfp I16L is significantly less active than Dfp I16V, indicating that the methyl groups bound to the b-C atom of Ile16 are important for substrate binding. This is in excellent agreement with the theoretical model for the binding of PPC to AtHAL3a proposing hydrophobic interactions between the side chain of Val10 with the two methylene groups of the b-alanine part of PPC (compare Fig. 1C; Ref. 10). The mutant protein Dfp Y19F was as active as wild-type Dfp, whereas Dfp Y19L was less active (not shown).

Acinetobacter baylyi, Dfp I16L is significantly less active than Dfp I16V, indicating that the methyl groups bound to the b-C atom of Ile16 are important for substrate binding. This is in excellent agreement with the theoretical model for the binding of PPC to AtHAL3a proposing hydrophobic interactions between the side chain of Val10 with the two methylene groups of the b-alanine part of PPC (compare Fig. 1C; Ref. 10). The mutant protein Dfp Y19F was as active as wild-type Dfp, whereas Dfp Y19L was less active (not shown). At the moment, an accurate determination of the kinetic parameters of the mutants is not possible. This determination is hampered by the factors described by Strauss et al. (11) and by the lack of a good working enzyme assay. The most important mutants presented in this paper are inactive or slightly active, and therefore, determination of the K_m values is impossible anyhow.
higher concentrations than wild-type Dfp (showing that residual wild-type Dfp can only be present in very low amounts). This is in accordance with the observation that Asn₁₁⁷ of EpiD contacts the C₄b hydrogen atoms and the carboxylate group of the substrate cysteinyl moiety (14) and with the proposed model of PPC binding to AtHAL3a.

**FIG. 4.** Purification of mutant Dfp proteins by gel filtration. HiTrap Q-enriched Dfp proteins were separated by gel filtration on a Superdex 200 PC 3.2/30 column, and the elution was followed by absorbance at 280 nm (upper line), 378 nm (not shown), and absorbance at 450 nm (lower line). Wild-type and mutant Dfp proteins (labeled with asterisks) eluted at 1.04 ml corresponding to a molecular mass of about 600 kDa (8). Similar results were obtained for all mutants investigated in this study, but only the results for Dfp G14S, I16L, A17D, K20Q, H75N, N125Q, and C158S are shown. Overexpression of mutant dfp genes in an E. coli dfp wild-type strain is a suitable approach, since expression of the chromosomal dfp gene is very low (no Dfp is detected when a nonoverexpressing E. coli strain is used for the described two-step purification procedure). Moreover, a knockout of the dfp gene will be lethal, and expression in foreign host strains is not recommendable, since dfp is an ubiquitous gene.

**FIG. 5.** SDS-PAGE analysis of purified mutant Dfp proteins. Proteins eluted between 1.02 and 1.10 ml from the Superdex 200 PC 3.2/30 gel filtration column were analyzed by SDS-PAGE. M, molecular weight marker. In this figure only wild-type Dfp, Dfp G14S, G15A, I16L, A17D, A17S, Y19F, K20N, K20Q, K20R, H75N, N125D, N125Q, and C158S are shown. However, purity of all investigated Dfp mutants was comparable.
The Conserved Cys158 Residue of the Substrate Recognition Clamp—The proposed substrate recognition clamp of the PPC decarboxylase is shortened by four residues compared with EpiD and contains the conserved Cys residue that aligns with Ser153 of EpiD. Ser153 is within the turn region of the binding clamp of EpiD. Substrate peptide and the binding clamp of EpiD together form a three-stranded $\beta$-sheet (14). Recently, it was shown that Dfp C158A is completely inactive, and it was suggested that the Cys residue participate in the reaction mechanism (10). Interestingly, also the C158S mutation (simulating Ser153 in EpiD) led to complete inactivation of Dfp (Fig. 6). Therefore, it looks like the cysteine residue of the substrate recognition clamp is one of the residues characteristic for PPC decarboxylases. Interestingly, the conserved Cys residue is exchanged for Gly in the Dfp proteins from Mycobacterium tuberculosis and Streptomyces coelicolor. However, in these proteins the conserved Asn residue of the PXXNXXXMW motif is exchanged for His (Fig. 3), and it will be interesting to learn if this second exchange restores PPC decarboxylase activity.

The Conserved Active-site Base His75—His75 of Dfp had already been studied by characterization of MBP fusion proteins. These mutant MBP fusion proteins bound coenzyme but were nevertheless inactive (8). It looks like that coenzyme binding is impaired, since Dfp H75N did not show absorbance at 450 nm (Fig. 4). However, drastically overexpressing the mutant gene by optimizing the ribosomal binding site led to yellow Dfp H75N protein, which precipitated after HiTrapQ purification (data not shown).

Molecular Characterization of the Conditional Lethality of the E. coli dfp-707 Mutant—Molecular characterization of Dfp started about 15 years ago when Spitzer et al. (15, 16) showed that the conditional-lethal dfp mutant dfp-707 (originally designated dna-707) caused a slow cessation of DNA synthesis at 42 °C and required either pantothenate or $\beta$-alanine for growth on minimal medium at 30 °C, although the mutant had wild-type activity levels of aspartate-1-decarboxylase. Even when supplemented with 1 mM pantothenate, dfp-707 strains did not grow on rich media at 42 °C. Although complete molecular characterization of the dfp-707 mutant was not achieved, Spitzer et al. (15, 16) could already show that the mutation was within the NH$_2$-terminal half of the Dfp protein, which was later shown to be the CoaC domain (8). For further characterization of the dfp-707 mutation, the dfp-707 gene was cloned into pQE12, sequenced, and overexpressed. Sequence analysis revealed...
that dfp-707 has a point mutation in codon 11, substituting the wild-type GGC (Gly) with GAC (Asp) (Fig. 7), whereas in the parental E. coli strain the dfp sequence is not changed. This G-A transition concurs with the use of nitrosoguanidine as the mutagenizing agent (18). As expected, the amino acid exchange is within the CoaC domain of Dfp; however, Gly11 is not strictly conserved in the \( \text{P} \)PC decarboxylases (Fig. 3). To elucidate the reason for the temperature sensitivity of the E. coli dfp-707 strain, Dfp G11D was purified from an overexpressing strain grown at 30 °C and in a second experiment from cells grown at 37 °C. Interestingly, only very low amounts of Dfp G11D could be purified from cells grown at 37 °C (Fig. 7). Dfp G11D purified from cells grown at 30 °C was assayed for \( \text{P} \)PC decarboxylase activity. The enzyme was active at 30 and 37 °C even when preincubated at 42 °C (Fig. 7). These experiments indicate that the conditional lethality of E. coli dfp-707 can be attributed to a decrease in soluble Dfp protein. How the G11D mutation influences folding or oligomerization of Dfp at higher temperatures remains open for investigation.

The PPC Decarboxylase Signature—Sequence comparison of the \( \text{P} \)PC decarboxylases AtHAL3a and Dfp with the peptidyl-cysteine decarboxylase EpiD and the molecular analysis of the CoaC domain presented in this paper led to the definition of a sequence motif specific for \( \text{P} \)PC decarboxylases (Fig. 3). On one hand, this PPC decarboxylase signature includes residues that all HFCD proteins have in common. On the other hand, the G-G/S-I-A-\( \text{X} \)-Y-K motif together with the conserved Asn residue of the P-A-M-N-\( \text{X} \)-M motif and the motif P-X-\( \text{X} \)-C-X-G-X-G of the substrate recognition clamp very likely define the \( \text{P} \)PC binding site of the \( \text{P} \)PC decarboxylases. In Dfp enzymes from
archaeabacteria, the PPC decarboxylase signature is not completely conserved, and it has to be investigated if coenzyme A biosynthesis in archaeabacteria occurs similar to that of bacteria.

The PPC decarboxylase from horse liver has been described as a pyruvoyl-dependent enzyme (19). However, all known pyruvoyl-dependent enzymes catalyze their reactions via the formation of Schiff bases and require a free amino group in the substrate (20). With the exception of slight modifications of the G-G/S-I-A-X-Y-K motif, the sequence of the eukaryotic PPC decarboxylase AtHAL3a is in accordance with the bacterial PPC decarboxylase signature. It is likely that also other eukaryotic PPC decarboxylases are not pyruvoyl-dependent enzymes but HFCD proteins and share the bacterial signature sequence.

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