Molecular Characterization of the Arabidopsis thaliana Flavoprotein AtHAL3a Reveals the General Reaction Mechanism of 4′-Phosphopantothenoylcysteine Decarboxylases*

Pilar Hernández-Acosta‡, Dietmar G. Schmid§, Günther Jung‡, Francisco A. Culiáñez-Macià‡, and Thomas Kupke¶

From the ²Instituto de Biología Molecular y Celular de Plantas, Universidad Politécnica de Valencia-CSIC, Camino de Vera s/n, 46022 Valencia, Spain, §Institut für Organische Chemie, Universität Tübingen, Auf der Morgenstelle 18, 72076 Tübingen, and ¶Lehrstuhl für Mikrobielle Genetik, Universität Tübingen, Auf der Morgenstelle 15, Verfüngsgebäude, 72076 Tübingen, Germany

The Arabidopsis thaliana flavoprotein AtHAL3a, which is linked to plant growth and salt and osmotic tolerance, catalyzes the decarboxylation of 4′-phosphopantothenoylcysteine to 4′-phosphopantetheine, a key step in coenzyme A biosynthesis. AtHAL3a is similar in sequence and structure to the LanD enzymes EpiD and MrsD, which catalyze the oxidative decarboxylation of peptidylcysteines. Therefore, we hypothesized that the decarboxylation of 4′-phosphopantothenoylcysteine also occurs via an oxidatively decarboxylating intermediate containing an aminoenithiol group. A set of AtHAL3a mutants were analyzed to detect such an intermediate. By exchanging Lys175 in AtHAL3a mutants, we found that AtHAL3a is not only able to decarboxylate 4′-phosphopantothenoylcysteine but also pantothenoylcysteine to pantetheinylcysteamine. Exchanging residues within the substrate binding clamp of AtHAL3a (for example of Gly179) enabled the detection of the proposed aminoenithiol intermediate when pantethynoylcysteine was used as substrate. This intermediate was characterized by its high absorbance at 260 and 280 nm, and the removal of two hydrogen atoms and one mole of CO2 was confirmed by ultrahigh resolution mass spectrometry. Using the mutant AtHAL3a C175S enzyme, the product pantetheinylcysteamine was not detectable; however, oxidatively decarboxylated pantetheinoylcysteine could be identified. This result indicates that reduction of the aminoenithiol intermediate depends on a redox-active cysteine residue in AtHAL3a.

The biosynthesis of coenzyme A from pantothenate includes the decarboxylation of (R)-4′-phospho-N-pantothenoylcysteine (PC)1 to 4′-phosphopantetheine (PP), a reaction that introduces the reactive cysteamine residue of coenzyme A. In eubacteria, the decarboxylation of PPC is catalyzed by the NH2-terminal CoaC domain of the Dfp proteins (1, 2). Dfp is a bifunctional enzyme, and the COOH-terminal CoaB domain catalyzes the synthesis of PPC from 4′-phosphopantetheine and i-cysteine using cytidine 5′-triphosphate as the activating nucleotide (3). The coenzyme A biosynthetic pathway in plants is not fully understood. However, it was recently shown that the Arabidopsis thaliana trimeric flavoprotein AtHAL3a, which is linked to plant growth and salt and osmotic tolerance (4), catalyzes the same reaction as the CoaC domain of Dfp, the decarboxylation of PPC (5).

Dfp and AtHAL3a belong to a new family of flavoproteins that was named HFCD (homo-oligomeric flavin containing Cys decarboxylases (2, 6)). Other members of this flavoprotein family include the LanD flavoenzymes EpiD and MrsD, which catalyze the oxidative decarboxylation of peptidylcysteines to peptidyl-β-aminothenethiols (7–11), a reaction involved in the biosynthesis of lantibiotics containing unsaturated thioether bridges such as epidermin and mersacidin. Lantibiotics are a group of ribosomally synthesized and posttranslationally modified antibiotic peptides containing the thioether amino acid lanthionine and other unusual amino acid residues (12, 13). The structure of the EpiD peptidyl-β-aminothenethiol reaction products has been elucidated by NMR spectroscopy and mass spectrometry (9, 14). EpiD, AtHAL3a, and Dfp all bind the cofactor FMN (4, 10, 15), whereas MrsD is a FAD-dependent enzyme (11). The HFCD proteins share the flavin binding motif and conserved active-site residues and are trimeric or dodecameric enzymes catalyzing the decarboxylation of cysteine residues (2, 6).

The crystal structure of an active-site mutant of EpiD with a bound substrate peptide gave the first insight into the decarboxylation mechanism used by the HFCD proteins (6). Surprisingly, it looks like Cα-Cα dehydrogenation is not the initial reaction but rather the oxidation of the thiol group by the flavin cofactor. The spontaneous decarboxylation of the thialdehyde group-containing intermediate then leads to the peptidylaminothenethiolate reaction product (6).

The existence of oxidatively decarboxylated reaction products in the case of the LanD enzymes is a direct hint that the homologous PPC decarboxylases also use oxidation of the thiol group to enable decarboxylation of cysteine residues. Therefore, it was concluded that a “peptidyl”-aminoethiol is an intermediate of the PPC decarboxylases (2, 5) and that this compound is finally reduced to 4′-phosphopantetheine (see Fig.

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† To whom correspondence and reprint requests should be addressed.

‡ The abbreviations used are: PPC, (R)-4′-phospho-N-pantothenoylcysteine; PP, 4′-phosphopantetheine; HFCD, homo-oligomeric flavin-containing Cys decarboxylases; PC, p-pantothenoylcysteine; Ni-NTA, nickel nitritoletricarboxylic acid; His-AtHAL3a, MGSSHHHHHHSS-GLVPRGSHMAMTGTKGGGQMGGS-HTHAL3a; DTT, dithiothreitol; RPC, reversed phase chromatography; ESI-FTICR-MS, electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry; IMAC, immobilized metal affinity chromatography; wt, wild type.
Mechanistic studies on the Dfp enzyme such as detection of substrate/product-induced charge-transfer complexes also favor a mechanism via oxidation of the thiol group and the presence of a thioualdehyde group-containing intermediate (16). However, the existence of this oxidatively decarboxylated intermediate has not been proven, and up to now, the intermediate itself has not been purified or characterized.

The four characterized HFCD proteins EpiD, MrsD, Dfp (CoaC) and AthAL3a are similar in sequence and the reaction they catalyzed, but they use different substrates, with peptidylestines on one hand and 4-phosphopantothenoylcysteine on the other. Crystal structure analysis of EpiD with bound substrate peptide and a theoretical model for the binding of PPC to AthAL3a showed that substrate binding involves an N-terminal substrate binding helix and a COOH-terminal substrate binding clamp (5, 6). The substrate binding clamp of EpiD forms an antiparallel β-sheet with the residues SSG (Ser-152 to Gly-154) in the turn region. The binding clamp and substrate peptide together form a three-stranded β-sheet (6). The substrate binding clamp of the PPC decarboxylases AthAL3a and Dfp is four residues shorter than that of EpiD and contains a conserved ACGD motif. The conserved Cys residue of this motif (Dfp, Cys158; AthAL3a, Cys175) aligns with Ser153 of EpiD, and modeling of the AthAL3a-PPC complex suggests that the conserved Cys residue is in the vicinity of the substrate cystein moiety and might participate in catalysis (5).

For Dfp the substrate binding structural elements have been partially characterized and a PPC decarboxylase signature defined. The conserved lysine residue of the N-terminal G(G/S)IAXKY motif of the Dfp proteins is probably important for the binding of the phosphate group of PPC, whereas the exchange of the residue Cys158 led to loss of PPC decarboxylase activity (1).

To elucidate the reaction mechanism of PPC decarboxylases, two central questions have to be answered. First of all, it has to be proven that decarboxylation of PPC involves an oxidatively decarboxylated substrate. Second, elucidation of the mechanism by which this compound is reduced by FMNH2 to complete the reaction cycle of the flavoprotein must occur. To address these questions, we chose the following approach. It should be possible to trap the reaction at the oxidatively decarboxylated intermediate and to prevent complete reduction by changing the geometry between the enzyme-bound intermediate and reduced flavin coenzyme. This could be achieved by changing residues involved in the binding of PPC, such as residues of the N-terminal binding helix or the substrate recognition clamp and/or by changing the substrate. Using site-directed mutagenesis, it should also be possible to identify amino acid residues involved in reoxidation of FMNH2 and to prevent complete reduction by changing residues involved in reoxidation of the oxidized intermediate.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction**

*In General*—PCR amplifications were performed with Vent-DNA polymerase (New England BioLabs). The entire sequences of the AthAL3a-coding regions of the constructed plasmids were verified. The oligonucleotides used were purchased from MWG Biotech.

**Site-directed Mutagenesis of AthAL3a**—AthAL3a mutants were constructed by sequential PCR steps (47) using appropriate mutagenesis primers and pET28a(+)/AthAL3a (4) as template. The primers (i) forward, 5'-GGTTGTGGTTAGCAGCCGGATCTCAGTG-3', and (ii) reverse, 5'-GGTGCCGCGCGGCAGCCATATGGCTAG-3' were used as 5' and 3' terminals for the sequential PCR and bind upstream and downstream of the BamHI and HindIII sites of pET28a(+)/AthAL3a, respectively. The mutant AthAL3a genes were then cloned into the single BamHI and HindIII sites of the pET28a(+) vector (Novagen) so that a fusion with the His-tag codons occurred. The pET28a(+) derived plasmids were transformed into the expression strain Escherichia coli BL21 (DE3) by electroporation. The pET28a(+)/AthAL3a expression plasmids encode NH2-terminal His-tag fusion proteins of the mutant AthAL3a proteins (His-AthAL3a, MGSSHHHHHHSSGLVPRGSHM ASMTGGQMMRGVS-AthAL3a).

**Purification and Characterization of His-AthAL3a Proteins**

**Growth of Strains**—The *E. coli* BL21 (DE3) pET28a(+) AthAL3a strains were grown at 37 °C in the presence of 100 μg/ml kanamycin to A578 = 0.4 in 0.5 liters of B-broth (10 g of casein hydrolysate 140 (GibcoBRL (Life Technologies)), 5 g of yeast extract (GibcoBRL (Life Technologies)), 5 g of NaCl, 1 g of glucose, and 1 g of K2HPO4/liter, pH 7.3) in 2 liters shaker flasks induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside and harvested 2 h after induction.

**Purification of His-AthAL3a Proteins**—500 ml of isopropyl-1-thio-β-D-galactopyranoside-induced *E. coli* BL21 (DE3) pET28a(+) AthAL3a cells were harvested and disrupted by sonication in 10 ml of 20 mM Tris-HCl, pH 8.0. 3 ml of the cleared lysate, obtained by two centrifugation steps (each 20 min at 30,000 × g at 4 °C), was diluted with 3 ml of column buffer (20 mM Tris-HCl, pH 8.0, 10 mM imidazole, 300 mM NaCl) and applied to an equilibrated Ni-NTA column containing ~0.5 ml of Ni-NTA-agarose (Qiagen). The column was then washed with 10 ml of column buffer. His-AthAL3a and mutant His-AthAL3a proteins were eluted with column buffer containing 250 mM instead of 10 mM imidazole, and the yellow peak fractions (~400 μl) were collected. If not otherwise stated, immediately after elution from the column, DTT was added to a final concentration of 5 mM. For gel filtration, a 25-μl aliquot of the Ni-NTA eluate was subjected to a Superdex 200 PC 2.3/30 column equilibrated in running buffer (20 mM Tris/HCl, pH 8.0, 200 mM NaCl) at a flow rate 40 μl/min. The Superdex 200 PC 3.2/30 column and the standard proteins used for calibration were obtained from Amersham Pharmacia Biotech. For activity assays (see below), the Ni-NTA eluates were used.

**SDS-PAGE**—Proteins were separated using Tricine-sodium dodecyl
sulfate-polyacrylamide (10%) gel electrophoresis (18) under reducing conditions.

**AtHAL3a Assays** — Approximately 50–100 μg of PPC as a calcium salt (2) were incubated with 0.5–1.0 μg of His-AtHAL3a for 20 min at 37 °C in a total volume of 0.70 ml of 50 mM Tris/HCl, pH 8.0, 5 mM DTT. The decarboxylation of pantothenoylcysteine used as barium salt (2) was assayed in the same way; however, for each assay 475 μg of substrate were used in a total volume of 1 ml. The purity of both substrates was in the range of 60–80%, and it was verified by mass spectrometry that no 4'-phosphopantetheine or pantothenoylcysteamine was present in the synthesized substrates. However, PPC contained minor amounts of the unphosphorylated pantothenoylcysteine. The His-AtHAL3a mutants were adjusted to the same absorbance at 455 nm to enable a direct comparison of their activities. The reaction mixtures were kept at −80 °C and then were successively separated by reversed phase chromatography (RPC) with a μRPC C18 SC 2.1/10 column on
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 oxidatively decarboxylated intermediates (7, 8). The fractions obtained were analyzed by electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry (ESI-FTICR-MS) as described below.

High Resolution Mass Spectrometry—ESI-FTICR-MS (19) measurements were carried out with a passively shielded 4.7-Tesla APEX™H-ESI/matrix-assisted laser desorption ionization-FTICR mass spectrometer (Bruker Daltonik). The mass spectrometry software XMass version 5.0.10 (Bruker Daltonik) was used for mass calculation, data acquisition, and processing. Mass calculation was performed with the standard elemental mass compilation of Audi and Wapstra (20). The measured mass range was m/z 200–2000, and broadband excitation took place from m/z 100 to 2000. In general, 524,288 data points were acquired. For positive ion investigations, 50 μl of methanol and 1% acetic acid were added to the sample solutions (~40–70-μl fractions from RPC). Electrospray ionization was performed in the positive mode with a grounded capillary sprayer needle mounted 60° off-axis (Analytica of Branford). No nebulizer gas was necessary to support the spray process. In general, the spectrum of a blank run (solvent and 1% acetic acid) showed no activity.

FIG. 4. PPC decarboxylase activity of mutant AtHAL3a proteins. PPC was incubated with purified wild-type and mutant His-AtHAL3a proteins. The reaction mixtures were separated by RPC, and the elution was followed by absorbance at 214 nm. The reaction product PP eluted before PPC, as has been shown by electrospray ionization mass spectrometry (2). The mutant His-AtHAL3a proteins were analyzed under comparable conditions together with wild-type His-AtHAL3a in two sets of experiments (A, control, AtHAL3a wt, AtHAL3a V30I, AtHAL3a I33L, AtHAL3a I33V, and AtHAL3a K34N; B, AtHAL3a wt, AtHAL3a K34Q, AtHAL3a K34R, AtHAL3a R95Q, AtHAL3a N142D, AtHAL3a M145L, AtHAL3a A174S, AtHAL3a A174V, AtHAL3a C175S, AtHAL3a D177N, AtHAL3a G179A, and AtHAL3a G181A). The mutant His-AtHAL3a proteins N142D, M145L, and C175S showed no activity (only with high enzyme concentrations very low residual activities might be detectable), whereas AtHAL3a D177N and G179A very significantly reduced PPC decarboxylase activity. The mutant proteins A174S and G181A showed significantly reduced activity. An additional reaction product (labeled with an asterisk) was clearly detectable for the Lys34 mutants K34N and K34Q. However, minor amounts of this reaction product were also detectable for all active AtHAL3a mutants (including the R95Q mutant, data not shown) and wild-type enzyme. Further experiments showed that the substance labeled with an asterisk is pantothenoylcysteamine, which is derived from pantothenoylcysteine by decarboxylation (compare Fig. 5 and Fig. 7).
FIG. 5. PC decarboxylase activity of mutant AtHAL3a proteins and detection of the oxidatively decarboxylated intermediate.

D-Pantothenoylcysteine was incubated with IMAC-purified wild-type and mutant His-AtHAL3a proteins (AtHAL3a K34Q, AtHAL3a K34R, AtHAL3a R95Q, AtHAL3a N142D, AtHAL3a M145L, AtHAL3a A174S, AtHAL3a A174V, AtHAL3a C175S, AtHAL3a D177N, AtHAL3a G179A, and AtHAL3a G181A). The reaction mixtures were separated by RPC, and the elution was followed by absorbance at 214 nm (A), 260 nm (B), and 280 nm (data not shown). From the column used, PC eluted as a broad peak between 20 and 25 min. In the presence of active His-AtHAL3a enzymes, PC was converted to a compound (pantothenoylcysteamine, see Fig. 6) that eluted at about 24 min (A), 260 nm (B), and 280 nm (data not shown). From the column used, PC eluted as a broad peak between 20 and 25 min. In the presence of active His-AtHAL3a enzymes, PC was converted to a compound (pantothenoylcysteamine, see Fig. 6) that eluted at about 24 min (corresponding to 13% acetonitrile; labeled with an asterisk). This compound is not present in the control reaction (PC incubated without any enzyme; not shown) and was not detected when the mutant His-AtHAL3a proteins N142D, M145L, and C175S were used. His-AtHAL3a A174S and G179A showed very low activity, whereas for D177N, the detection of the reaction product was only possible when higher enzyme concentrations were used. Interestingly, in the case of some of the mutants an additional compound eluted with a retention time of about 26–26.5 min. This compound (pantothenoylamino-ethanolthiol, see Fig. 6) showed an increased absorbance at 260 nm (B) and at 280 nm (not shown) and is labeled with an open circle in the figure (PC does not absorb at either 260 or 280 nm). Larger amounts of this compound were observed with the mutants R95Q, A174S, A174V, C175S, and G179A.
Mutations within the NH2-terminal Binding Helix of AtHAL3a—Recent molecular characterization of CoaC activity showed that mutations within the NH2-terminal G(G/S)IAXYK motif of the Dfp proteins, especially the exchange of the residue Lys for Gln or Asn, decreased PPC decarboxylase activity (1). It was suggested that the conserved Lys residue is involved in binding of the phosphate group of PPC. This assumption is supported by the model for binding of PPC to AtHAL3a (5).

Therefore, we were interested in characterizing the Lys residue within the motif GSVAAIK in the plant AtHAL3a protein. For the K34Q mutant, we could show a small decrease of PPC decarboxylase activity (Fig. 4). However, we also observed that exchange of the residue Lys for Asn or Gln led to an additional reaction product. This compound is only present in very low amounts if wild-type enzyme is used and, therefore, has not been detected before. Small amounts of this additional compound are also observed when the mutants I33V and K34R are used. Because we were aware that the synthetic PPC used is contaminated with pantothenoyleysteine, we decided to investigate if, in contrast to Dfp, AtHAL3a and mutant AtHAL3a proteins are able to decarboxylate PC (see below). We have previously shown that AtHAL3a is not as specific as Dfp (5).

Pantothenoyleysteine Is Decarboxylated by AtHAL3a—To analyze if AtHAL3a not only decarboxylates PPC but also PC, synthetic PC was incubated with wild-type and mutant His-AtHAL3a proteins. The reaction mixtures were then separated by RPC, and the masses of the eluted compounds were determined. AtHAL3a enzymes, which were active in decarboxylation of PPC, were also active in the decarboxylation of PC (Fig. 5). The mass difference between PC and the observed reaction product was determined to be 43.990 Da (not shown but see Fig. 6), which is in excellent agreement with the monoisotopic mass of CO2 (the calculated monoisotopic mass is 43.9898 Da). The reaction product pantothenoyleysteine eluted with a retention time of about 24.0–24.2 min. This retention time is in agreement with that of the observed additional reaction product of the AtHAL3a K34N/K34Q reaction with synthetic PPC (see above; minor shifts in the overall retention times are explained by changes in the column material with increasing numbers of applications). We conclude that, in a mixture of PPC and PC, wt AtHAL3a preferentially decarboxylates PPC and that this discrimination between both substrates depends on Lys. This is a direct hint that Lys of AtHAL3a binds the phosphate group of PPC. If no PPC is present, there should be no difference between AtHAL3a and AtHAL3a K34N/K34Q in the reaction with PC, because this substrate lacks the phosphate group (the data presented in Fig. 5 support this view). The elucidation of the kinetic parameters for AtHAL3a and AtHAL3a K34N/K34Q for both substrates is impeded by the lack of a suitable enzyme assay. Moreover, the determination of decarboxylase activities for different ratios of PPC and PC is only possible by separating the reaction products (as it is shown in Fig. 4).

The Conserved Asn and Met Residues of the PXNXXMW Motif—The PXNXXMW motif is conserved within the HFCD proteins, and crystal structure analysis of EpiD revealed the importance of this motif (residues 114–121 of EpiD) for binding of the substrate and the flavin cofactor (6). The conserved residue Asn of EpiD contacts the Cα hydrogen atoms and the carboxylate group of the substrate cysteinoyl moiety. It appears that this Asn residue (EpiD, Asn117; MrsD, Asn125; Dfp, Asn125; AtHAL3a, Asn142) and the conserved His residue of the HFCD proteins (EpiD, His67; MrsD, His75; Dfp, His78; AtHAL3a, His85) are essential for the decarboxylase reaction. Mut126 of EpiD is located above the pyrimidine system of the FMN cofactor. For the eubacterial enzyme Dfp, the
The importance of the conserved Asn and Met residues for the PPC decarboxylase activity has already been published (1, 2). To verify these data and the proposed model of PPC binding to AtHAL3a, the mutant proteins His-AtHAL3a N142D and His-AtHAL3a M145L were purified and characterized. As expected, both enzymes were inactive in decarboxylating PPC and PC (Figs. 4 and 5).

Mutations within the Substrate Recognition Clamp of AtHAL3a—Recently, crystal structure analysis of EpiD H67N with bound peptide DSYTC revealed that the pentapeptide is embraced by a 20-amino acid substrate recognition clamp comprising residues Pro143 to Met162. Residues of the NH2-terminal binding helix, the residue Asn 117, and the NH motif (containing the above mentioned conserved His residue) are also important for substrate binding (6). We were interested in changing the way that PC and PPC bind to AtHAL3a to detect the oxidatively decarboxylated intermediate. Therefore, we not only exchanged Asn142 and residues of the NH2-terminal binding helix (see above) but also residues within the proposed PPC binding clamp of AtHAL3a. Characterization of the substrate recognition clamp of HFCD proteins by site-directed mutagenesis has not been published apart from the preliminary characterization of the conserved Cys residue of the PPC decarboxylases (1, 5). We mutated residues Ala174, Cys175 (see below), Gly179, and Gly181 of the AtHAL3a substrate recognition clamp Pro143-Ile-Lys-Lys-Ala174-Cys175-Gly-Asp-Ile-Gly179-Pro-Gly181-Arg-Met183 and elucidated the activity of the altered enzymes with both PPC and PC (Figs. 4 and 5). All of these residues are conserved in the eubacterial Dfp proteins (1). The mutant protein AtHAL3a D177N was included in the studies, because both AtHAL3a and E. coli Dfp have the ACGD motif, which is not present in the LanD enzymes EpiD and MrsD. Activity of AtHAL3a A174S, G179A, and G181A was significantly reduced with both substrates compared with wild-type AtHAL3a. D177N showed very low activity. However, activity of AtHAL3a A174V was comparable with wild-type activity, indicating that a hydrophobic side chain in position 174 is important for hydrophobic interactions with the substrate (compare the published model of PPC binding to AtHAL3a (5)). As described below, further characterization of the AtHAL3a G179A reaction with pantothenoylcysteine then led to the identification of the proposed intermediate.

Purification and Characterization of Oxidatively Decarboxylated Pantothenoylcysteine—From the characterization of the flavoenzyme EpiD it was known that oxidatively decarboxylated peptidylcysteines are characterized by their absorbance properties. The aminoenethiol group NH=CH=CH=SH is a strong chromophore, and the UV
spectra of the EpiD reaction products were pH-dependent, with an absorption maximum of 259 nm under acidic conditions (pH ≤ 4.2) for the enethiol form and 283 nm at pH 7.0 for the enethiolate form; the isosbestic point was determined to be 271 nm. It was shown that the molar extinction coefficient (ε) of SPNSYV—NH—CH=CH—SH at 259 nm is at least ε = 6,800 M⁻¹cm⁻¹ and that the pKₐ value of the enethiol group is about pH 6.0 (7). Therefore, the reversed phase separation of the AtHAL3a reaction mixtures was not only followed by absorbance at 214 nm but also by absorbance at 260 and 280 nm. Using PPC as substrate, we did not unambiguously detect compounds with an increased absorbance at 260 nm, even if mutant AtHAL3a proteins were used in the decarboxylation assay (data not shown). However, the combination of pantothenoylsteine as a substrate and mutant His-AtHAL3a enzymes such as R95Q, A174S, A174V, C175S, D177N, and G179A enables the identification of a compound that showed strong absorption at 260 nm (Fig. 5B). The residues Ala₁⁷⁴, Cys₁⁷⁵, Asp₁⁷⁷, and Gly₁⁷⁹ are within the proposed substrate recognition clamp of AtHAL3a and could directly contact the substrate. Gly₁⁷⁹ could also be important for the maintenance of the correct secondary/tertiary structure of the clamp. We believe that changing the structure of the recognition clamp results in a different active-site architecture and that the different geometry of the substrate/intermediate FMN/FMNH₂ pair prevents complete reduction of the oxidatively decarboxylated intermediate by FMNH₂. It is also possible that structural changes within the recognition clamp enable reoxidation of FMNH₂ by oxygen. Modeling of PPC binding to AtHAL3a shows that also residue Arg-95 could contact the substrate (5).

The putative oxidatively decarboxylated PC (pantothenoylaminoenethiol) was purified in larger amounts by incubation of PC with His-AtHAL3a C179A, because incubation with this enzyme led to the largest amounts of the intermediate. After separation of the reaction mixture by RPC, the reaction products were analyzed by ESI-FTICR-MS (Fig. 6). The MS data prove that PC is decarboxylated to pantothenoylcysteamine and that the compound with increased absorbance at 260 nm (and 280 nm) is pantothenoylaminoenethiol. Interestingly, a second compound with the mass of pantothenoylaminoenethiol could also be detected, and we propose that the two compounds are cis and trans isomers of the aminoenethiol intermediate.

The Conserved Cys₁⁷⁵ Residue of the Substrate Binding Clamp—Recently, it has been shown that the conserved Cys residue of the substrate recognition clamp of Dfp proteins, which is missing in the LanD enzymes EpiD, MrsD, and MutD (6), is essential for activity (1, 5). We could prove this for AtHAL3a, because His-AtHAL3a C179A, because His-AtHAL3a C175S is inactive in decarboxylation of PC, because incubation of PC with His-AtHAL3a G179A, because incubation with this enzyme led to the largest amounts of the intermediate. After separation of the reaction mixture by RPC, the reaction products were analyzed by ESI-FTICR-MS (Fig. 6). The MS data prove that PC is decarboxylated to pantothenoylaminoenethiol and that the compound with increased absorbance at 260 nm (and 280 nm) is pantothenoylaminoenethiol. Interestingly, a second compound with the mass of pantothenoylaminoenethiol could also be detected, and we propose that the two compounds are cis and trans isomers of the aminoenethiol intermediate.

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REFERENCES
1. Kupke, T. (2001) J. Biol. Chem. 276, 27597–27604
2. Kupke, T., Uebele, M., Schmid, D., Jung, G., Blaesoe, M., and Steinbacher, S. (2000) J. Biol. Chem. 275, 31838–31846
3. Strauss, E., Kinsland, C., Ge, Y., McLafferty, F. W., and Begley, T. P. (2001) J. Biol. Chem. 276, 13513–13516
4. Espinosa-Ruiz, A., Bélès, J. M., Serrano, R., and Cúlliaíz-Macià, F. A. (1999) Plant J. 20, 529–539
5. Kupke, T., Hernández-Acosta, P., Steinbacher, S., and Cúlliaíz-Macià, F. A. (2001) J. Biol. Chem. 276, 19109–19106
6. Blaesoe, M., Kupke, T., Huber, R., and Steinbacher, S. (2000) EMBO J. 19, 6299–6310
7. Kupke, T., and Götz, F. (1997) J. Biol. Chem. 272, 4759–4762
8. Kupke, T., Kempter, C., Gnau, V., Jung, G., and Götz, F. (1994) J. Biol. Chem. 269, 5653–5659
9. Kupke, T., Kempter, C., Jung, G., and Götz, F. (1995) J. Biol. Chem. 270, 11282–11289
10. Kupke, T., Stevanovic, S., Sahl, H.-G., and Gotz, F. (1992) J. Bacteriol. 174, 5354–5361
11. Majer, F., Schmid, D. G., Altena, K., Bierbaum, G., and Kupke, T. (2002) J. Bacteriol. 184, 1234–1243
12. Sahl, H.-G., and Bierbaum, G. (1998) Annu. Rev. Microbiol. 52, 41–79
13. Schnell, N., Entian, K.-D., Schneider, U., Gotz, F., Zahner, H., Kellner, R., and Jung, G. (1988) Nature 333, 276–278
14. Kempter, C., Kupke, T., Kaiser, D., Metzger, J. W., and Jung, G. (1996) Angew. Chem. Int. Ed. Engl. 35, 2104–2107
15. Spitzer, E. D., and Weiss, B. (1985) J. Bacteriol. 164, 994–1003
16. Strauss, E., and Begley, T. P. (2001) J. Am. Chem. Soc. 123, 6449–6450
17. Cormack, B. (1991) in Current Protocols in Molecular Biology (Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K., eds) Vol. 1, pp. 8.5.1–8.5.9, John Wiley & Sons, Inc., New York
18. Schägger, H., and Jagow, G. (1987) Anal. Biochem. 166, 368–379
19. Marshall, A. G., Hendrickson, C. L., and Jackson, G. S. (1998) Mass Spectrom. Rev. 17, 1–35
20. Audi, G., and Wapstra, A. H. (1995) Nucl. Phys. A 595, 409–480
21. Albert, A., Martinez-Ripoll, M., Espinosa-Ruiz, A., Yenush, L., Culiánez-Maciá, F. A., and Serrano, R. (2008) Structure Fold. Des. 8, 961–969