Arabidopsis thaliana Flavoprotein AtHAL3a Catalyzes the Decarboxylation of 4'-Phosphopantothenoylcysteine to 4'-Phosphopantetheine, a Key Step in Coenzyme A Biosynthesis*

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The Arabidopsis thaliana flavoprotein AtHAL3a is related to plant growth and salt and osmotic tolerance. AtHAL3a shows sequence homology to the bacterial flavoproteins EpiD and Dfp. EpiD, Dfp, and AtHAL3a are members of the homo-oligomeric flavin-containing Cys decarboxylase (HFCD) protein family. We demonstrate that AtHAL3a catalyzes the decarboxylation of (R)-4'-phospho-N-pantothenoylcysteine to 4'-phosphopantetheine. This key step in coenzyme A biosynthesis is catalyzed in bacteria by the Dfp proteins. Exchange of His-90 of AtHAL3a for Asn led to complete inactivation of the enzyme. Dfp and AtHAL3a are characterized by a shortened substrate binding clamp compared with EpiD. Exchange of the cysteine residue of the conserved ACGD motif of this binding clamp resulted in loss of (R)-4'-phospho-N-pantothenoylcysteine decarboxylase activity. Based on the crystal structures of EpiD H67N with bound substrate peptide and of AtHAL3a, we present a model for the binding of (R)-4'-phospho-N-pantothenoylcysteine to AtHAL3a.

Coenzyme A is the principal acyl carrier group in all living cells and is required for many synthetic and degradative reactions in intermediary metabolism (1). In bacteria, coenzyme A is synthesized in five enzymatic steps from pantothenate (2). In the first step, pantothenate is phosphorylated to 4'-phosphopantothenate by pantothenate kinase. Then (R)-4'-phospho-N-pantothenoylcysteine (PPC)1 is synthesized by the addition of cysteine to 4'-phosphopantothenate. In the next step, PPC is decarboxylated to 4'-phosphopantetheine (PP). 4'-Phosphopantetheine is converted to coenzyme A by the enzymes phosphopantetheine adenylyltransferase and dephospho-CoA kinase.

The coenzyme A biosynthetic pathway is not understood in plants. The pantothenate kinase has been partially purified and characterized from spinach (3), but all the other enzymes involved in coenzyme A biosynthesis in plants are not characterized.

Recently, the flavoprotein AtHAL3a from Arabidopsis thaliana has been characterized as a protein that is related to plant growth, salt, and osmotic tolerance (4). AtHAL3a is similar to the flavoprotein EpiD from Staphylococcus epidermidis, the N-terminal domain of the Dfp flavoproteins from bacteria (Fig. 1) and to one of the domains of the SIS2 (HAL3) protein from Saccharomyces cerevisiae (4–6). EpiA catalyzes the oxidative decarboxylation of peptidylcysteines to peptidylaminoen-thiols and is involved in biosynthesis of the peptide antibiotic epidermin (7–11), which belongs to the lantibiotics (12). Dfp was originally described as a flavoprotein involved in DNA and pantothenate metabolism (13, 14). Recently, it was shown that Dfp catalyzes the decarboxylation of PPC to PP (6), a reaction that had been attributed before to a pyruvoyl-containing enzyme (15). The SIS2 protein influences ion homeostasis and cell cycle control of S. cerevisiae via the Ppz1p Ser/Thr protein phosphatase (16–19). Expression of the AtHAL3a gene in yeast hal3 mutants partially complements their LiCl sensitivity, and its overexpression in transgenic Arabidopsis plants improves growth rates and salt and drought tolerance (4). Environmental salt and drought stress response in plants include a myriad of cellular and physiological adaptations ranging from stress signaling mitogen-activated protein kinase (MAP kinase, MAPK) cascades to Ca2+-mediated salt transport and compartmentation by kinase-activated plasma membrane entry channels and Na+/H+ extrusion and vacuolar antiports (update reviews are found in Refs. 20–22). The protective effects observed in AtHAL3a flavoprotein-engineered plants further confirm the complexity of salt and drought stress tolerance.

Molecular characterization of EpiD and the determined enzymatic function of the Dfp protein indicated that all proteins having a EpiD homologous domain catalyze the decarboxylation of cysteine residues (6). This idea was further confirmed by crystal structure analysis of the active-site mutant EpiD H67N with bound substrate peptide DSYTC. The substrate of EpiD is embraced by a substrate recognition clamp comprising residues Pro-143 to Met-162. Substrate binding also involves an N-terminal substrate binding helix (Fig. 1). The binding clamp and the substrate peptide form a three-stranded β-sheet (5). Residues such as Asn-117 of the so-called PXMXXXMW motif of EpiD, which are important for binding of the cysteine residue of the substrate peptide, are conserved in Dfp and AtHAL3a.

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1 The abbreviations used are: PPC, (R)-4'-phospho-N-pantothenoylcysteine; PP, 4'-phosphopantetheine; HFCD, homooligomeric flavin-containing Cys decarboxylases; ESI-FT-ICR-MS, electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry; IMAC, immobilized metal affinity chromatography; RPC, reversed phase chromatography; Ni-NTA, nickel nitrilotriacetic acid; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.
EpiD and Dfp are homododecameric proteins (6), whereas AtHAL3a forms trimers (23). We therefore proposed that enzymes having a EpiD homologous domain are homo-oligomeric flavin-containing Cys decarboxylases and suggested the name HFC proteins for this protein family (5, 6).

Here, we ascribe an enzymatic function to the flavoprotein AtHAL3a, demonstrating that AtHAL3a but not the active-site mutant AtHAL3a H90N catalyzes the decarboxylation of (R)-4'-phospho-N-pantothenoylsteine to 4'-phosphopantetheine. The PPC decarboxylases Dfp and AtHAL3a are distinguished from EpiD by a shortened substrate recognition clamp containing the sequence motif ACGD (5). In this study we analyze the importance of the conserved cysteine residue of the ACGD motif for substrate binding of the PPC decarboxylases and present a theoretical model for the binding of PPC to AtHAL3a.

The data show that 4'-phosphopantetheine and/or coenzyme A biosynthesis is linked to salt tolerance indicating that AtHAL3a is not necessarily involved in signal transduction as has been proposed earlier.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction**

In General—Polymerase chain reaction amplifications were performed with Vent DNA polymerase (New England Biolabs) or PfuTurbo DNA polymerase (Stratagene). The entire sequences of the dfp and AtHAL3a-coding regions of the constructed plasmids were verified. The used oligonucleotides were purchased from MWG Biotech and Sigma, respectively.

pET28α (+) AtHAL3a H90N—The mutant AtHAL3a gene was constructed by QuikChange site-directed mutagenesis (Stratagene) using pET28α (+) AtHAL3a (4) as a template. The oligonucleotides (i) forward, 5'-GGTATCGCTGTCTTAAACATGCTAGGTTAAGCT; (ii) reverse, 5’-CAACGTTAATGAACGCGGCTAATAGCAGCAGGTACAC-3’, exchanging the codon CAC (codon 90 of the AtHAL3a-coding sequence for AAC) were used as mutagemesis primers. The pET28α (+) derived plasmid was transformed into the expression strain E. coli BL21 (DE3) by electroporation. The expression plasmids pET28α (+) AtHAL3a and pET28α (+) AtHAL3a H90N encode N-terminal His tag fusion proteins of the AtHAL3a protein (His-AtHAL3aHis-AtHAL3a H90N). MGSSSHHHHHHHSSGLYPQRSMACTGCGQQMQGRS-AtHAL3a/AtHAL3a H90N).

**Purification of Proteins**

**Growth of Strains**—The E. coli strains used were grown to an OD600 = 0.4 in 0.5 liters of B-broth (10 g of casein hydrolysate 140 (Life Technologies, Inc.), 5 g of yeast extract (Difco), 5 g of NaCl, 1 g of glucose, and 1 g of K2HPO4 liter, pH 7.0) in 2-liter shaker flasks, induced with 1 ml of isopropyl-1-thio-β-D-galactopyranoside, and harvested 2 h after induction. E. coli BL21 (DE3) pET28α (+) AtHAL3a cells were grown in the presence of 100 µg/ml kanamycin, and E. coli M15 (pREP4) pQE12/dfp cells were grown in the presence of 100 µg/ml ampicillin and 25 µg/ml kanamycin. The growth temperature was 37 °C.

**Expression and purification**—AtHAL3a and AtHAL3a H90N—500 ml of isopropyl-1-thio-β-D-galactopyranoside-induced E. coli BL21 (DE3) pET28α (+) AtHAL3a and AtHAL3a H90N cells were harvested and disrupted by sonication in 10 ml of 20 mM Tris-HE, pH 8.0. 5 ml of the cleared lysate obtained by two centrifugation steps (each 20 min at 30,000 x g at 4 °C) was diluted with 5 ml of column buffer (20 mM Tris-HE, pH 8.0, 10 mM imidazole, 300 mM NaCl) and applied to an equilibrated Ni-NTA column containing 1 ml of Ni-NTA-agarose (Qiagen). The column was then washed with 10 ml of column buffer. His-AtHAL3a and mutant His-AtHAL3a H90N proteins, respectively, were eluted with column buffer containing 250 mM instead of 10 mM imidazole, and the yellow peak fractions (~80 µl) were collected. 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 eluted to a Superdex 200 PC 3.2/30 column equilibrated in running buffer (20 mM Tris-HE, 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. The Ni-NTA and gel filtration eluates were used for activity assays.

**Dfp and Dfp C158A—**500 ml of isopropyl-1-thio-β-D-galactopyranoside-induced E. coli M15 (pREP4) pQE12/dfp/pQE-12/dfp C158A cells were harvested and disrupted by sonication in 10 ml of column buffer (20 mM Tris-HE, pH 8.0). 5 ml of the cleared lysate obtained by two centrifugation steps (each 20 min at 30,000 x g at 4 °C) was diluted with 5 ml of column buffer and loaded on a 1 ml HiTrapQ column (Amersham Pharmacia Biotech) 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 was eluted with column buffer containing 0.25 M NaCl, and the yellow peak fraction (~400 µl) was collected. A 25-µl aliquot of this HiTrapQ eluate was then immediately subjected to Superdex 200 PC 3.2/30 gel filtration as described above for AtHAL3a. The fraction containing the maximum amount of Dfp/Dfp C158A (elution volume 1.02–1.10 ml; Ref. (6)) was used for the activity assay.

**SDS-PAGE and Immunoblotting—**Proteins were separated using Tricine-sodium dodeyl sulfate-polyacrylamide (10%) gel electrophoresis (24) under reducing conditions. After SDS-polyacrylamide gel electrophoresis, proteins were electrophoretically transferred to polyvinylidene difluoride membranes (25). AtHAL3a was detected by polyclonal anti-AtHAL3a antiserum. Immuno-reactive proteins were visualized by enhanced chemiluminescence.
Activity Assays and Modeling

*AtHAL3a and Dfp Assays*—Approximately 50–100 μg of PPC as Ca²⁺ salt (6) were incubated with 1–5 μg of Dfp or AtHAL3a 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 AtHAL3a H90N and Dfp C158A, which were supposed to be inactive, were used at approximately double concentration relative to the bound FMN. The reaction mixture was then separated by RPC. The obtained fractions were analyzed by electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry (ESI-FT-ICR-MS). ESI-FT-ICR-MS was performed using a 4.7-tesla APEXIII-ESI-FT-ICR mass spectrometer from Bruker.

Modeling—Modeling was done interactively using the program MAIN (26). The coordinates of the PPC molecule were generated with the software SYBYL molecular modeling software (Version 6.4, Tripos, Inc., St. Louis, MO), which was also used to optimize its geometry and to minimize the energy of the resulting model.

RESULTS AND DISCUSSION

**Purification of His-AtHAL3a H90N—**AtHAL3a and AtHAL3a H90N were expressed as His-tag fusion proteins and purified from the corresponding E. coli clones by immobilized metal affinity chromatography (IMAC) and additionally by gel filtration (Figs. 2 and 3). Purification of His-AtHAL3a has already been described (4), but there are only incomplete data for the molecular weight determination by gel filtration (23). The mutant H90N has the same elution volume as wild-type His-AtHAL3a, and a comparison with standard proteins revealed an apparent molecular mass of 110 kDa (Fig. 2). The calculated molecular mass of trimeric His-AtHAL3a-FMN is 82 kDa. The ratio of the absorbance values at 280 and 450 nm was not significantly altered by the H90N mutation. These data show that the introduced mutation did not affect the trimeric structure and coenzyme binding of AtHAL3a H90N, indicating that the overall three-dimensional structure is not significantly altered.

**AtHAL3a Catalyzes the Decarboxylation of (R)-4′-Phospho-N-Pantothenoylcysteine**—Crystal structure analysis of flavoprotein EpiD H67N with bound peptide DSYTC showed that substrate binding of EpiD involves the N-terminal helix H1 of EpiD (substrate binding helix), the His motif, the PXM-NXXMW motif, and the residues Pro-143 to Met-162 (substrate recognition clamp) (Fig. 1; Ref. 5). We assume that sequences that are homologous to the substrate binding helix and the substrate recognition clamp of EpiD are also involved in substrate binding of AtHAL3a and Dfp, respectively. Interestingly, substrate binding helix and substrate recognition clamp of AtHAL3a are more similar to the corresponding sequences of Dfp than that of EpiD. Especially, the substrate recognition clamp of AtHAL3a and Dfp is shortened by four residues compared with EpiD and contains the conserved ACGD motif (5). This led to the assumption that AtHAL3 might be able to decarboxylate (R)-4′-phospho-N-pantothenoylcysteine as had already been shown for Dfp, although it had been proposed that AtHAL3a regulates, via a Ppz1-like protein phosphatase, the expression of genes related to cell cycle and ion homeostasis (4, 23). To examine this idea, we incubated His-AtHAL3a purified by IMAC and gel filtration with PPC. By using RPC separation of the reaction mixture, it was shown that PPC was converted to 4′-phosphopantetheine (Figs. 4A and 5). The synthesized PPC contained minor amounts of d-pantothenoylcysteine; however, ESI-MS data indicated that pantothenoylcysteine was not decarboxylated by His-AtHAL3a as has also been shown for Dfp (6).

At the moment, it is not obvious how the observed activity is correlated with salt and osmotic tolerance mediated by AtHAL3a, since the cofactors 4′-phosphopantetheine and coenzyme A are involved in a lot of different biochemical reactions, and plants use different principles to resist high salt concentrations (reviewed in Refs. 20–22). There is also the possibility...
that in plants 4'-phosphopantetheine is not only an intermediate in coenzyme A biosynthesis but also in taurine biosynthesis. For bacteria, it is known that taurine has an osmo-protective effect (27). In principle, it is possible that taurine synthesis from PP occurs via cysteamine that is released from PP by a 4'-phosphopantetheinylase activity (compare Ref. 28). Plants that overexpressed the AtHAL3a gene showed a faster growth rate than the wild type (4). This effect can be explained by a higher coenzyme A content of AtHAL3a-overexpressing plants.

His90 Is an Active-site Residue of AtHAL3a—The histidine residue His-90 of AtHAL3a is conserved in EpiD, Dfp, and all other HFCD proteins. Recently, it has been shown by site-directed mutagenesis and crystal structure analysis that this histidine residue is an active-site residue of EpiD and Dfp (5, 6). For AtHAL3a, a cysteine residue has been modeled into the active site, and it has been proposed that oxidation of this cysteine residue occurs via concerted $\alpha,\beta$-dehydrogenation and depends on the basic His-90–Glu-77 diad (23).

To verify that His-90 of AtHAL3a is an active-site residue, we investigated the activity of His-AtHAL3a H90N (Fig. 4B). His-AtHAL3a H90N was inactive in decarboxylation of PPC, verifying that the observed activity of AtHAL3a is not due to contaminating Dfp protein of E. coli. A side product of the PPC synthesis was also decarboxylated by AtHAL3a (Fig. 4B) but not by Dfp (not shown), indicating a slightly different substrate specificity of AtHAL3a. This difference in substrate specificity can be related to the different size of the sequence insertion in front of the His motif (see below and Fig. 1).

The Conserved Cys Residue of the Substrate Binding Clamp of PPC Decarboxylases—The binding clamp of the peptidylcysteine decarboxylase EpiD forms a twisted antiparallel $\beta$-sheet with residues Ser-152, Ser-153, and Gly-154 in the turn region. Ser-153 of EpiD aligns with the conserved cysteine residue of the ACGD motif of the PPC decarboxylases (Cys-158 of Dfp and Cys-175 of AtHAL3a). To investigate the importance of this residue for the activity/substrate binding of the PPC decarboxylases, we characterized the Dfp mutant Dfp C158A. Dfp C158A was purified by anionic exchange chromatography and gel filtration as described for Dfp (6). Dfp C158A eluted exactly at the same volume as Dfp from the Superdex 200 PC 3.2/30 column. The ratio of the absorbance values at 280 and 450 nm was not significantly altered by the mutation (not shown). These data show that the introduced mutation did not affect the dodecameric structure and coenzyme binding of Dfp C158A, indicating that the overall three-dimensional structure is not significantly altered. The mutant protein Dfp C158A was inactive in decarboxylation of PPC (Fig. 6). Modeling of the enzyme-substrate complex suggests that Cys-175 of AtHAL3a and Cys-158 of Dfp will be in direct vicinity of the substrate cysteinyl moiety and might participate in catalysis (see below and Fig. 7).

Model for Binding of PPC to AtHAL3a and Reaction Mechanism—The theoretical model for the binding mode of PPC to AtHAL3a is based on the crystal structure of EpiD with bound peptide DSYTEC (Protein Data Bank accession code 1G5Q; Ref. 5) and the known crystal structure of AtHAL3a (Protein Data Bank accession code 1E20 (23)). For modeling, the position of the cysteinyl moiety common to both substrates was taken from the EpiD crystal structure, and an elongated conformation of the PPC molecule was chosen to match the location of the peptide backbone of DSYTEC. Both substrates are quite similar in length.

In the resulting model (Fig. 7), the cysteinyl moiety of PPC is tightly fixed by hydrophobic interactions of its methylene group to Val-88 and Ile-91. The carboxylic group could form H-bonds to the backbone NH of Val-88, to the guanidinium group of Arg-95, and to Asn-142, which is highly conserved within the HFCD family. The presence of Val-88 constrains the binding site near the FMN considerably compared with the active site of EpiD and thereby restricts the rotation of the methylene-thiol moiety. This might be important to avoid oxidation of the substrate and restrict the overall reaction to a decarboxylation.

The crystal structure of EpiD H67N with bound substrate peptide DSYTEC suggested an FMN-dependent oxidation of $\mathrm{S}_{\text{Y}}$ of the cysteine residue, yielding a thioaldehyde intermediate. It has been assumed that this thioaldehyde intermediate decarboxylates spontaneously, forming the enethiolate group of the reaction product (5, 29) and that the decarboxylation of PPC by AtHAL3a and Dfp follows a similar mechanism. Since we did not find any oxidative decarboxylated product but only PP, we concluded that the enethiol group is reduced immediately by $\mathrm{FMNH}_2$ of the PPC decarboxylase, completing the reaction cycle (6).

Favorable hydrophobic interactions between the two methylene groups of the $\beta$-alanine part of PPC with Val-30 next to FMN and Leu-173 from the binding clamp and of the dimethylmethylene group with Ile-33 and Ile-84 represent a nice match in surface properties. The phosphate group could be anchored by Lys-34 at the bottom of the binding site and Lys-171 from the binding clamp. This could explain how AtHAL3a discriminates between PPC and pantothenoylecysteine. It is very likely that AtHAL3a does not recognize peptidylcysteines because the
**FIG. 4.** *AtHAL3a* catalyzes the decarboxylation of PPC. Decarboxylation of PPC was analyzed by RPC following the elution of the compounds by absorbance at 214 nm. A, PPC was incubated with equal volumes of the His-AtHAL3a-containing fractions of the gel filtration experiment (compare Fig. 3). PPC eluted at 21.51 min, corresponding to 7.8% acetonitrile. In presence of AtHAL3a, PPC was converted to a compound that eluted at 20.95 min (corresponding to 6.8% acetonitrile). Retention time and absorbance properties of this compound are indistinguishable from the reaction product of Dfp (compare Fig. 7), and by ESI-FT-ICR-MS analysis it was verified that this compound is PP. The observed activity is proportional to the AtHAL3a content of the gel filtration fractions. B, activity of AtHAL3a and AtHAL3a H90N was investigated using Ni-NTA-purified proteins. No activity was observed for AtHAL3a H90N. RPC separation of synthetic PPC and ESI-MS analysis revealed that minor amounts of PPC are also eluted at 29.13 min together with very small amounts of an unknown compound $x$ (side product of PPC synthesis) that has a mass of 916.3003 Da. This unknown compound is converted by AtHAL3a to a compound $y$ that has a mass of 828.3197 Da and eluted at 29.43 min. The mass difference between the compounds is 87.9806 Da, indicating the loss of two molecules CO$_2$. The structure of compound $x$ is not known.
spacing between the peptide bonds will be different and, therefore, hydrophobic parts in PPC do not mimic amino acid side chains. The binding clamp crosses the PPC substrate at the NH-CO-CHOH part of the molecule, with potential H-bonding contacts to the protein backbone at Leu-173 and Ala-174. The shorter binding clamp of AtHAL3a places the connecting loop at Ala-174 to Gly-176 (ACG). The latter residue appears to be highly conserved within the HFCD family as the corresponding Gly-154 (SSG) in EpiD also marks the turn connecting both β-strands of the clamp. The position of Cys-175 suggests contacts to the backbone of the insertion segment, anchoring its position, but after a simple side chain rotation it could also make a direct contact to a PPC carboxamide group.

Dfp and AtHAL3a perform the same reaction and, interestingly, show identical lengths of the substrate binding clamp. The sequence motif ACGD that presumably forms the turn region within the clamp is present in both proteins. The difference between AtHAL3a and Dfp is the length of the insertion in front of the His motif (Fig. 1). Residues Asp-76 to Leu-89 of AtHAL3a, which connect β-strand S3 and α-helix H4, deviate in the three-dimensional structure from EpiD. An insertion of eight residues is included and substitutes for the contacts between trimers present in the dodecameric EpiD (5, 23). Val-88 appears to have the additional function of constricting the active site of AtHAL3a. Dfp forms dodecamers and not trimers (6), but an insertion of five residues is present in Dfp, which might constrict the active site.

Conclusion—In this paper we have ascribed the PPC decarboxylase activity to the plant flavoprotein AtHAL3a. This result is of great importance for the elucidation of the in vivo role of AtHAL3a in salt and osmotic tolerance. On the other hand, the presented data are the starting point for a detailed investigation of coenzyme A biosynthesis in plants. The results confirm that proteins with an EpiD homologous domain are homo-oligomeric flavin containing Cys decarboxylases (HFCD proteins). The elucidation of the crystal structure of Dfp/AtHAL3a with bound PPC will give more detailed insights into
the substrate binding and the reaction mechanism of PPC decarboxylases.

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