Plant Phenylacetaldehyde Synthase Is a Bifunctional Homotetrameric Enzyme That Catalyzes Phenylalanine Decarboxylation and Oxidation

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We have isolated and characterized Petunia hybrida cv. Mitchell phenylacetaldehyde synthase (PAAS), which catalyzes the formation of phenylacetaldehyde, a constituent of floral scent. PAAS is a cytosolic homotetrameric enzyme that belongs to group II pyridoxal 5′-phosphate-dependent amino-acid decarboxylases and shares extensive amino acid identity (∼65%) with plant L-tyrosine/3,4-dihydroxy-L-phenylalanine and L-tryptophan decarboxylases. It displays a strict specificity for phenylalanine with an apparent Kₘ of 1.2 mM. PAAS is a bifunctional enzyme that catalyzes the unprecedented efficient coupling of phenylalanine decarboxylation to oxidation, generating phenylacetaldehyde, CO₂, ammonia, and hydrogen peroxide in stoichiometric amounts.

Aldehydes are intermediates in a variety of biochemical pathways, including those involved in the metabolism of carbohydrates, vitamins, steroids, amino acids, benzylisoquinoline alkaloids, hormones, and lipids (1, 2). In plants, they are also synthesized in response to environmental stresses such as salinity, cold, and heat shock (3, 4) or as flavors and aromas in fruits and flowers (5, 6). For example, phenylacetaldehyde (PHA), 2-phenylethanol, and its acetate ester are important scent compounds in numerous flowers (5), including petunias (7, 8) and roses (9). They also contribute to the aromas of tomato (6), grape (10), and tamarind (11) fruits and to the flavor of tea (12). PHA has been identified in some animals and fungi as well (13, 14).

PHA, 2-phenylethanol, and phenethyl acetate each contain a benzene ring with a 2-carbon side chain and are therefore referred to as C-6–C-2 compounds. The synthesis of PHA in yeast proceeds from L-phenylalanine to phenylpyruvate via transamination followed by decarboxylation (14, 15). Hazen et al. (16) postulated that oxidative decarboxylation of Phe by the myeloperoxidase-hydrogen peroxide-chlorine system can also lead to PHA. However, little is known about the biosynthesis of volatile C-6–C-2 compounds in plants.

Feeding of petunia petals with deuterium (2H₅)-labeled Phe results in a PHA labeling pattern consistent with synthesis from Phe via negligible pools of intermediates (8). Moreover, 2-aminoindane phosphate, a specific inhibitor of L-phenylalanine ammonia-lyase (EC 4.3.1.5), does not inhibit PHA synthesis but instead increases it by ∼2-fold, suggesting that the formation of PHA from Phe does not occur via trans-cinnamic acid and is in competition with trans-cinnamic acid synthesis for Phe utilization (8). Similar labeling experiments were carried out with [2H₈]Phe to determine the route to PHA and subsequent phenylethanol biosynthesis in rose petals (17, 18). A mixture of PHA isomers differing in hydrogen labeling ([2H₄] or [1H]) at C-1 was obtained. The occurrence of two competing pathways to PHA synthesis, one via the decarboxylation of Phe to 2-phenylethylamine followed by amine oxidation and the second via deamination of Phe to phenylpyruvate followed by its decarboxylation, as occurs in yeast, was suggested (17, 18). These two postulated pathways are shown in Fig. 1. However, to date, no enzyme activities have been demonstrated for either of the postulated pathways in plants. Here, we describe the isolation of a cDNA encoding phenylacetaldehyde synthase (PAAS) and the biochemical characterization of the recombinant protein, which catalyzes an unusual, combined decarboxylation-amine oxidation reaction, leading to the formation of PHA from Phe.
Plant Phenylacetaldehyde Synthase

FIGURE 1. Predicted labeling of PHA synthesized from L-^{2H}Phe in rose petals via two hypothetical routes. The scheme is based on the suggestions of Watanabe et al. (17, 18). The asterisks denote the positions of ^2H atoms.

EXPERIMENTAL PROCEDURES

Plant Material—Petunia hybrida cv. Mitchell plants (Ball Seed, West Chicago, IL) were grown under standard greenhouse conditions (8). Labeling experiments were performed as described previously using corolla limbs of 2-day-old petunia flowers (8), and emitted volatiles were collected by a closed-loop stripping method over 4 h during the day (19).

Radiolabeled- and Stable Isotope-labeled Compounds—Deuterium-labeled L-phenylalanine (C$_6$D$_5$CD$_2$CD(NH$_2$)COOH; 1-^{2H}Phe) was purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA), and L-[U-^{14}C]Tyr (434 mCi/mmol) from American Radiolabeled Chemicals, Inc. (St. Louis, MO).

Other Reagents—All other reagents were purchased from Sigma or Aldrich.

Expression of PAAS in Escherichia coli and Purification of Recombinant Protein—The coding region of petunia PAAS was amplified by PCR using forward and reverse primers (5'-ATTCATATGGGATCCTTCTACGCATTCAGCATCATAG-3' and 5'-GATCTCTGCTAGATGTAATCATATGGATACTATCAAAATCAACCC-3', respectively) and subcloned into the expression vector pET-28a containing an N-terminal hexahistidine tag (Novagen, Madison, WI). The forward and reverse primers for rose PAAS were 5'-GCCCATGGGTAGCTTCCCATTCAACCC-3' and 5'-GGGATCCTTCTACGCATTAGCATTAG-3', respectively. Sequencing revealed no errors introduced during PCR amplifications. For functional expression, E. coli BL21 Rosetta competent cells were transformed with the resulting recombinant plasmid and the pET vector without an insert as a control and grown in LB medium containing 50 g/ml kanamycin at 37 °C. Induction, harvesting, and induction of the hexahistidine tag.

PAAS Assay and Product Verification—The standard reaction mixture (50 µl) contained purified PAAS protein (3.5–15 µg) and 2 mM L-[^14]C]-Phe (662 mCi/µmol) in assay buffer containing 50 mM Tris-HCl (pH 8.5), 0.2 mM PLP, and 0.1 mM EDTA. Fractions with the highest PAAS activity were examined by SDS-PAGE, followed by Coomassie Brilliant Blue staining of the gel. The purity of the isolated protein was examined using gel electrophoresis and Coomassie Brilliant Blue staining of the gel. The purity of the enzyme was taken into account for calculations of the stoichiometry of PLP binding and for determination of $k_{cat}$ values.

Product verification was performed by radio TLC and gas chromatography-mass spectrometry (GC-MS). For radio TLC analysis, 50 µl of the reaction product was spotted onto a Silica Gel 60 F$_{254}$-precoated TLC plate (EM Chemicals Inc., Gibbstown, NJ) and co-chromatographed with authentic compounds as standards using ethyl acetate/methanol (4:1, v/v) as a solvent. For GC-MS analysis, the enzyme reaction was scaled to 1 ml and contained 5 mM unlabeled L-Phe or L-[^{2H}Phe].

Determination of H$_2$O$_2$, NH$_4^+$, and CO$_2$—H$_2$O$_2$, and NH$_4^+$ were measured in 96-well plates at room temperature using a plate reader and a fluorimeter (BioTek Instruments Inc., Winoski, VT) for data analysis. For H$_2$O$_2$ determinations, the reaction mixture contained 100 µl of assay buffer (100 mM Tris-HCl (pH 8.5), 0.4 mM PLP, 0.2 mM Na$_2$EDTA, and 4 mM Phe), 8 µl of purified PAAS (8.2 µg of protein), and 100 µl of coupling reagent (4 mM phenol, 6 mM 4-aminoantipyrine, and 0.2 units of horseradish peroxidase) (22). The absorbance increase at 505 nm was monitored at 10-min intervals, and the amount of H$_2$O$_2$ formed was determined by reference to standards (0–300 µM) in the same buffer.

The production of NH$_4^+$ was determined using glutamate dehydrogenase. The standard reaction mixture consisted of 200 µl of assay buffer containing 50 mM Tris-HCl (pH 8.5), 0.2 mM nitrotriloltriacetic acid-agarose (0.5-ml bed volume). The nickel-nitrotriloltriacetic acid-agarose was washed with wash buffer containing 0.02 M sodium phosphate (pH 7.4), 0.5 M NaCl, and 20 mM imidazole until the $A_{280}$ of the material eluting from the column was near that of the wash buffer. The His$_6$-tagged protein was eluted from the column with 15 ml of the same buffer containing 500 mM imidazole. The eluate fractions (1 ml each) with the highest PAAS activity were desalted on NAP$^{TM}$-5 columns (Amersham Biosciences AB, Uppsala, Sweden) into assay buffer (50 mM Tris-HCl, pH 8.5, 0.2 mM PLP, and 0.1 mM EDTA). Fractions with the highest PAAS activity were examined using SDS-PAGE, followed by Coomassie Brilliant Blue staining of the gel. The purity of the isolated protein was between 75 and 85%, varying among different purifications (supplemental Fig. S1). The purity of the enzyme was taken into account for calculations of the stoichiometry of PLP binding and for determination of $k_{cat}$ values.

PAAS Assay and Product Verification—The standard reaction mixture (50 µl) contained purified PAAS protein (3.5–15 µg) and 2 mM L-[^{14}C]-Phe (662 mCi/µmol) in assay buffer containing 50 mM Tris-HCl (pH 8.5), 0.2 mM PLP, and 0.1 mM EDTA. After incubation for 30 min at room temperature, the reaction was stopped with 5 µl of 10 M NaOH. The product was extracted with 250 µl of ethyl acetate, and 200 µl of the organic phase was counted in a liquid scintillation counter. The raw data were converted to nanokatals based on the specific activity of the substrate and efficiency of counting. Protein concentrations were determined by the method of Bradford (21) using the Bio-Rad protein reagent and bovine serum albumin as a standard.

Product verification was performed by radio TLC and gas chromatography-mass spectrometry (GC-MS). For radio TLC analysis, 50 µl of the reaction product was spotted onto a Silica Gel 60 F$_{254}$-precoated TLC plate (EM Chemicals Inc., Gibbstown, NJ) and co-chromatographed with authentic compounds as standards using ethyl acetate/methanol (4:1, v/v) as a solvent. For GC-MS analysis, the enzyme reaction was scaled to 1 ml and contained 5 mM unlabeled L-Phe or L-[^{2H}Phe].

Determination of H$_2$O$_2$, NH$_4^+$, and CO$_2$—H$_2$O$_2$, and NH$_4^+$ were measured in 96-well plates at room temperature using a plate reader and KC4 software (BioTek Instruments Inc., Winoski, VT) for data analysis. For H$_2$O$_2$ determinations, the reaction mixture contained 100 µl of assay buffer (100 mM Tris-HCl (pH 8.5), 0.4 mM PLP, 0.2 mM Na$_2$EDTA, and 4 mM Phe), 8 µl of purified PAAS (8.2 µg of protein), and 100 µl of coupling reagent (4 mM phenol, 6 mM 4-aminoantipyrine, and 0.2 units of horseradish peroxidase) (22). The absorbance increase at 505 nm was monitored at 10-min intervals, and the amount of H$_2$O$_2$ formed was determined by reference to standards (0–300 µM) in the same buffer.

The production of NH$_4^+$ was determined using glutamate dehydrogenase. The standard reaction mixture consisted of 200 µl of assay buffer containing 50 mM Tris-HCl (pH 8.5), 0.2 mM
PLP, 0.1 mM Na2EDTA, 2 mM Phe, 0.25 mM NADH, 2.5 mM α-ketoglutarate, and 4 units of glutamate dehydrogenase (Calzyme Laboratories, Inc., San Luis Obispo, CA) and 8 μl of purified PAAS (8.2 μg of protein). The decrease in absorbance at 340 nm was recorded at 10-min intervals and subtracted from a blank lacking Phe. The amount of product formed was determined using the molar absorptivity of NADH (6.22 ml/mmol).

The production of CO2 was determined by trapping released CO2 (23). Reactions at low O2 concentration were performed in a Type B anaerobic chamber with 40 ppm O2 and 0.1% (v/v) H2 (Coy Laboratory Products Inc., Grass Lake, MI).

**Electrochemical Detection of H2O2 Formation and O2 Consumption**—For electrochemical detection, enzyme assays were performed in a microscale assay chamber. To detect H2O2, glass-encased platinum electrodes were modified by coating them with Nafion, an anionic polymer that repels negatively charged interferents such as ascorbate from the positively charged analytical surface of the electrode, which was polarized to 0.65 V versus an Ag/AgCl reference. To detect O2, similarly constructed glass-encased platinum electrodes were modified by application of a nitrocellulose membrane and operated at −0.75 V versus a separate Ag/AgCl reference electrode (Whalen configuration). The reference electrode was fabricated from a 100-μm diameter silver wire. The electrodes were calibrated before and after the experiment, and any drift was compensated by linear drift analysis. The rate of oxygen consumption was determined by measuring the decrease in dissolved O2 in the reaction mixture covered with mineral oil.

**PLP Determination**—PLP was measured according to the method of Wada and Snell (24).

**RNA Isolation and RNA Gel Blot Analysis**—Total RNA was isolated from petunia floral tissues, petals at different stages of flower development, and at 10 time points during a daily light/dark cycle and analyzed as described previously (8). A 1.5-kb fragment containing the coding region of the PAAS gene was used as a probe.

**Generation and Analysis of Transgenic PAAS RNA Interference Petunia Plants**—A 464-bp fragment of the PAAS gene obtained by PCR with primers F100 (5′-ATCTCGAGCAACCCAGAATTTGATGGTCA-3′) and R564 (5′-AGAATTFIGURE 2. Characterization of petunia PAAS gene expression. A, tissue specificity of PAAS mRNA. Shown is an RNA gel blot of total RNA isolated from the young leaves, sepals, corolla tubes and limbs, pistols, stamens, and ovaries of 2-day-old petunia flowers. The RNA was hybridized with the PAAS gene as a probe (upper panel). The blot shown here, as well as in B and C, was rehybridized with an 18 S rDNA probe (lower panel) to standardize samples. Each gel contained 5 μg of total RNA/lane, and autoradiography was performed overnight. B, RNA gel blot (upper panel) showing developmental changes in steady-state PAAS mRNA levels in petunia corolla limbs. Total RNA was isolated at different stages of flower development from mature buds to day 7 post-anthesis. The RNA gel blots shown here, as well as in C, were scanned with a Storm 860 PhosphorImager, and values were corrected by standardizing for the amounts of 18 S rRNA measured in the same run. The maximum transcript level was taken as 1. Each point is an average of four experiments. S.E. values are indicated by error bars. C, RNA gel blot analysis of steady-state PAAS mRNA levels in petunia flowers during a normal light/dark cycle. Total RNA was isolated from the limbs of 2–4-day-old flowers. D, GC-MS headspace analysis of volatile compounds emitted from flowers of P. hybrida PAAS transgenic and control non-transgenic plants. Volatiles were collected from detached flowers for 24 h, and representative gas chromatograms are shown. Peak 1, benzaldehyde; peak 2, benzyl alcohol; peak 3, PHA; peak 4, methyl benzoate; peak 5, 2-phenylethanol. I.S., internal standard (isobutylbenzene).
CACACCACCACCACCACCA-3’ was cloned into pRNA69 (25) 3’ to the cauliflower mosaic virus 35S promoter in the sense and antisense orientations separated by an intron. The construct was mobilized in the binary vector pART27 by NotI digestion. Transformation and verification of stable transformants were performed as described (26). Floral volatiles were collected from detached petunia flowers and analyzed by GC-MS as described previously (27).

RESULTS

Isolation and Heterologous Expression of Petunia and Rose PAASs—Petal-specific expressed sequence tag data bases for P. hybrida cv. Mitchell (8) and rose (28) were searched for sequences potentially involved in PHA biosynthesis, including putative aminotransferases, decarboxylases, and amine and monoamine oxidases. This analysis yielded two putative aminotransferases, one amine oxidase, one monoamine oxidase, and one putative L-tyrosine/L-Dopa decarboxylase (TYDC). RNA gel blot analysis for these genes revealed that the petunia (clone 1–2-D07) and rose (clone fc0444) cDNAs designated PAAS (see below), both homologs to TYDC, had expression patterns positively correlated both temporally and spatially with the production of PHA in petunia and 2-phenylethanol in rose flowers (Fig. 2, A–C; data for rose not shown) (8, 28). Flowers of five independent transgenic petunia lines with suppressed PAAS gene expression showed no PHA emission compared with controls (Fig. 2D). These plants also produced no 2-phenylethanol (Fig. 2D), supporting our previous conclusion that PHA is a precursor of 2-phenylethanol (8). Petunia PAAS encodes a protein of 506 amino acids with a calculated molecular mass of 57,074 kDa and pI of 5.46 (GenBank™ accession number DQ243784) (Fig. 3A). This protein belongs to group II PLP-dependent amino-acid decarboxylases, including histidine, glutamate, serine, and aromatic L-amino-acid decarboxylases.

FIGURE 3. Comparison of the predicted amino acid sequences of petunia and rose PAASs with three plant TYDCs and a phylogenetic tree of these and additional related decarboxylases with different substrate specificities. P. hybrida (PhPAAS) and Rosa hybrida (RhPAAS) PAAS sequences were aligned with Thalictrum flavum (Thl) TYDC1 (NCBI accession number AAG60665), Papaver somniferum (Ps) TYDC6 (accession number AAC61844), and Petroselinum crispum (Pc) TYD2 (accession number Q06086) using ClustalW. Alignment was shaded using the BoxShade Version 3.21 software program (Human Genome Sequencing Center, Houston, TX). Residues shaded in black indicate conserved amino acids identical in at least three sequences shown, and residues shaded in gray represent similar matches. The asterisk indicates the PLP-binding lysine residue (Ref. 35 and references therein), and the arabic numbers indicates the position corresponding to Tyr152 in pig kidney DDC. The distance bar is shown under the tree, and bootstrap values (1000 replicates) are given for the nodes. AtGAD1 and PhGAD, Arabidopsis thaliana (accession number AAA93132) and P. hybrida (accession number AAA33709) glutamate decarboxylases, respectively; AtSDE and BnSDE, A. thaliana (accession number AAK77493) and Brassica napus (accession number BAA78331) serine decarboxylases, respectively; CaTDC2 and CrTDC, C. acuminata (accession number AAB39709) and C. roseus (accession number CAA47898) L-tryptophan decarboxylases, respectively; LeHDC, Lycopersicon esculentum histidine decarboxylase (accession number P54772); PsTYDC1, P. somniferum tyrosine/Dopa decarboxylase (accession number U08597). The unrooted neighbor joining tree was created using ClustalX and TreeView for visualization.
boxylases (29). It shares 63–67% amino acid identity (79–81% similarity) with TYDCs (tyrosine/L-Dopa decarboxylase, EC 4.1.1.28) from opium poppy (30), meadow rue (NCBI accession number DQ192639) encodes a protein of 509 residues and is 64% identical to petunia PAAS. Neither petunia nor rose PAAS contains signal peptides at its N termini (Fig. 3), suggesting cytosolic localization.

Characterization of Recombinant Petunia PAAS—Purified petunia and rose PAAS proteins produced in E. coli were tested with several amino acids, including L-Phe, L-Tyr, L-Tryp, L-Dopa, L-Met, L-Gln, and L-His as substrates. Both enzymes displayed strict substrate specificity for Phe, and the product of this reaction detected by GC-MS was PHA (Fig. 4, A and B; shown for petunia PAAS). Kinetic characterization of purified recombinant petunia PAAS revealed an apparent $K_m$ for Phe of 1.18 ± 0.04 mM (mean ± S.E., $n = 3$), which is very similar to the $K_m$ values of opium poppy TYDC for Tyr and Dopa, both equal to 1 mM (34). In 2-day-old petunia petals, the level of Phe at night is 445 ± 1 nmol/g (fresh weight) (8), which corresponds to a concentration of 5.5 mM, a value that is severalfold higher than the PAAS $K_m$ for Phe of 1.2 mM. The PAAS $k_{cat}$ per homotetramer was 0.80 ± 0.06 s$^{-1}$ (mean ± S.E., $n = 3$). Enzyme activity was maximum at pH 8.5. The native molecular mass of petunia PAAS determined by gel filtration chromatography was ~250 kDa, suggesting that the native enzyme is a tetramer containing identical 57-kDa subunits. Whereas L-tryptophan decarboxylase and TYDC were found to be homodimeric enzymes (35), a tetrameric structure was reported for L-serine decarboxylase from Arabidopsis (36) and histidine decarboxylase from Morganella (37).

The absence of PLP during both purification and assay resulted in a 98.4% loss of enzyme activity. However, the addition of PLP in the initial extraction buffer followed by purification and assay in the absence of PLP resulted in a 90% loss of PAAS activity (Fig. 5A). The addition of PLP restored the activity in a saturable fashion (Fig. 5A), indicating that PAAS is indeed a PLP-dependent enzyme. The dissociation constant ($K_d$) for PLP was 11.3 ± 1.8 μM (mean ± S.E., $n = 3$). The PLP antagonists hydroxylamine, phenylhydrazine, and semicarbazide (at 2 mM) abolished PAAS activity (hydroxylamine and phenylhydrazine) or decreased it by ~70% (semicarbazide). The purified PAAS protein exhibited an absorption maximum at 424 nm, consistent with the presence of a PLP cofactor. The PLP content in PAAS was 4.08 ± 0.07 mol/mol of PAAS subunit as determined by the phenylhydrazine method (24). A value of 1.2 mol of PLP/mol of subunit was reported for tetrameric Arabidopsis L-serine decarboxylase (36).

TYDCs from a variety of plants are sensitive to L-phenylalanine ammonia-lyase inhibitors (35). 2-Aminooxyacetate (100 μM), a PLP antagonist with modest inhibitory action on L-phenylalanine ammonia-lyase (38), reduced PAAS activity by 38%, whereas 2-aminoindane phosphonate (100 μM), a L-phenylalanine ammonia-lyase inhibitor (39), inhibited PAAS activity by ~13%. The latter result was consistent with our previous observation showing that 2-aminoindane phosphonate treatment of excised petunia petals does not inhibit PHA emission (8).
requires an \( \alpha \)-keto acid acceptor, followed by decarboxylation, resulting in the formation of PHA, \( \text{CO}_2 \), and the corresponding amino acid, with phenylpyruvate as an intermediate; 2) decarboxylation followed by oxidative deamination, which results in the formation of PHA, \( \text{CO}_2 \), ammonia, and possibly \( \text{H}_2\text{O}_2 \) (depending on the nature of the deamination reaction), with 2-phenylethylamine as an intermediate; 3) oxidative deamination of Phe followed by decarboxylation, leading to the formation of the same products as in mechanism 2 but with phenylpyruvate as an intermediate; and 4 and 5) decarboxylation-dependent transamination (a “full” or “half”-transamination, respectively), which converts Phe into PHA with simultaneous conversion of enzyme-bound PLP into pyridoxamine 5’-phosphate (PMP). The full transamination requires an \( \alpha \)-keto acid to convert PMP back to PLP (mechanism 4), whereas the half-transamination requires PMP oxidase activity (mechanism 5).

Analysis of the reaction products revealed that \( \text{CO}_2 \), \( \text{NH}_4^+ \), and \( \text{H}_2\text{O}_2 \) were produced in stoichiometric amounts in addition to PHA (Fig. 5B). Over a 30-min reaction, the rates of \( \text{CO}_2 \), \( \text{NH}_4^+ \), \( \text{H}_2\text{O}_2 \), and PHA formation were \( 2.44 \pm 0.21 \), \( 2.79 \pm 0.03 \), \( 2.28 \pm 0.22 \), and \( 2.27 \pm 0.07 \) nanokatals/mg of protein (mean \( \pm \) S.E., \( n = 3 \)), respectively. Consumption of \( \text{O}_2 \) (Fig. 5C) confirmed the \( \text{O}_2 \) dependence of the PAAS-catalyzed reaction. Unlike in the experiment shown Fig. 5B, the level of \( \text{O}_2 \) consumed in the experiment shown in Fig. 5C was determined under restricted \( \text{O}_2 \) conditions in which \( \text{O}_2 \) penetration into the reaction mixture from the surrounding air was eliminated. Under these conditions, \( \text{O}_2 \) and \( \text{H}_2\text{O}_2 \) formation was almost equal (0.11 and 0.12 nmol/mg of protein/s, respectively). Enzyme-generated \( \text{H}_2\text{O}_2 \) inhibited PAAS beginning 40 min after initiation of the reaction. In the presence of bovine liver catalase, however, the formation of PHA was linear for at least 4 h (Fig. 5B). The amount of PHA produced in 4 h (69 nmol, 1.38 mmol in the 0.05-ml reaction mixture) under aerobic conditions greatly exceeded the initial PLP content (10 nmol, 0.2 mM in the reaction mixture).

When the PAAS reaction was performed at a very low \( \text{O}_2 \) concentration (40 ppm) in a closed chamber, neither ammonia nor \( \text{H}_2\text{O}_2 \) could be detected, whereas the rate of PHA production (at 0.2 mmol PLP) was \( \sim 35 \% \) of the rate under aerobic conditions, suggesting that the level of ambient \( \text{O}_2 \) can play a role in the type of reaction catalyzed by PAAS. Similarly, under anaerobic conditions, the rate of Dopa decarboxylation catalyzed by pig kidney 3,4-dihydroxyphenylalanine decarboxylase (DDC) is about half of that measured in the presence of \( \text{O}_2 \) (40). The mechanism of this reaction and its physiological significance remain to be determined.

Formation of \( \text{NH}_4^+ \) and \( \text{H}_2\text{O}_2 \) under aerobic conditions excluded the possibility of transamination activity in the PAAS reaction (mechanisms 1 and 4). This was confirmed by the addition of various \( \alpha \)-keto acids (pyruvate, \( \alpha \)-ketoglutarate, oxalacetate, and glyoxylate) as amino group acceptors. The rate of PHA formation was not increased by the addition of these \( \alpha \)-keto acids.

Because the formation of PHA from Phe by PAAS involves the removal of both the carboxyl and amino groups, the sequence of these removals (mechanism 2 versus 3) was determined by experiments with \( 1\text{-}^\text{H}_3 \text{Phe. Conversion of} \)

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**Proposed Oxidative Decarboxylation Mechanism**—Synthesis of PHA from Phe could theoretically proceed via one of at least five possible reaction mechanisms: 1) transamination, which

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**FIGURE 5. Biochemical characterization of the PAAS-catalyzed reaction.** A, PLP dependence of the PAAS reaction. The reactions at different PLP concentrations were each performed for 30 min. Standard reaction mixtures containing 8.2 \( \mu \)g of PAAS and the indicated concentrations of PLP were incubated for 20 min, followed by quantitation of PHA. Note that PHA formation is stoichiometric with the concentration of PLP in the initial reaction mixture. *nkat*, nanokatals. *B*, product formation of the PAAS reaction. *Circles*, \( \text{NH}_4^+ \); *diamonds*, \( \text{H}_2\text{O}_2 \); *squares*, PHA. The inset shows the effect of catalase on PHA formation. *Triangles and squares*, amount of PHA formed in the presence and absence of catalase, respectively. C, \( \text{O}_2 \) consumption during the PAAS reaction. To measure \( \text{O}_2 \) consumption, the vessel had to be closed off, which limited the amount of \( \text{O}_2 \) available for the reaction, whereas in B, \( \text{O}_2 \) was not limited because the reaction mixture was open to the ambient air. The trace was obtained from a standard reaction mixture containing 3.4 \( \mu \)g of PAAS protein.
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L-[^2H_8]Phe to phenylpyruvate, which then serves as the precursor of PHA, requires that all of the label in the α-position of L-[^2H_8]Phe be replaced by 1H at the aldehyde position of the product, resulting in PHA molecules only +7 atomic mass units larger (Fig. 1). However, PHA was labeled by +7 and +8 atomic mass units at almost a 1:1 intensity (m/z 127:m/z 128) (Fig. 4C), suggesting that decarboxylation precedes nitrogen removal (Fig. 1 and mechanism 2). A similar PHA labeling pattern was obtained in vivo when petunia petals were fed L-[^2H_8]Phe, and floral scent was collected over a 4-h period (Fig. 4D). Moreover, the formation of phenylacetone from L-α-methyl-Phe (Fig. 4E) also ruled out mechanism 3 and confirmed that phenylpyruvate cannot be an intermediate because a deamination reaction would require the presence of an α-C–H bond, which α-methyl-Phe lacks.

Synthesis of PHA via mechanism 2 or 5 assumes that phenylethylamine or PMP is the intermediate, respectively. However, no phenylethylamine (mechanism 2) was detected during the PAAS reaction. Moreover, when PHA was incubated with 2-phenylethylamine as a substrate, no PHA, H_2O_2, or NH_4+ was produced, suggesting that PAAS cannot use 2-phenylethylamine directly in the amine oxidase reaction or that free 2-phenylethylamine is not a true intermediate in this reaction.

Oxidative deamination of 2-phenylethylamine to PHA, NH_4+, and H_2O_2 requires amine oxidase activity. When several potent amine oxidase inhibitors (2 mM) were used, benzaldoxime inhibited the PAAS reaction by 80%, whereas ironizond and pyridoxine had a slight (8%) or no effect on PAAS activity, respectively. Amine oxidases generally contain flavin (flavin-containing amine oxidase, EC 1.4.3.4) or topaquinone (copper-containing amine oxidase, EC 1.4.3.6) as a prosthetic group (41, 42). The addition of FAD or FMN at 0.2–4 mM, as well as the presence of ascorbic acid (10 mM) or Cu^2+ -chelating agents such as sodium azide and sodium diethyldithiocarbamate (at 1 mM), did not affect the rate of PHA formation. Moreover, preincubation of PHA for 48 h at 4 °C in 10 mM EDTA had no effect on its activity, whereas the addition of Cu^2+ (0.1 mM) inhibited it by >30%. An inductively coupled plasma mass spectrometric analysis also did not show the presence of any metals. These findings suggest that the PLP cofactor itself might participate in redox chemistry in the active site of PAAS.

Another possible source of NH_4+ and H_2O_2 in the PAAS-catalyzed reaction could be PMP oxidase activity, which recovers PLP from PMP (mechanism 5). When high levels of PMP (2 mM) were supplied to PAAS protein, no H_2O_2 or NH_4+ formation was detected, excluding contamination of purified PAAS protein with E. coli PMP oxidase or the ability of PAAS to catalyze a PMP deaminase reaction.

Our results indicate that PAAS is a PLP-containing enzyme that catalyzes both a decarboxylase reaction and an oxidase reaction at the same active site. The ability of PAAS to catalyze both decarboxylation and O_2-consumed oxidation is not unique. At least three other PLP-containing decarboxylases (E. coli glutamate decarboxylase (43), pig kidney DDC (44–48), and Hafnia alvei ornithine decarboxylase (49)) have been reported to catalyze O_2-consuming side reactions following decarboxylation of an amino acid or α-methylaminoo acid substrate. However, the oxidative decarboxylation reactions catalyzed by these enzymes are quite slow relative to the non-oxidative decarboxylation reactions, indicating that the structures of these enzymes have evolved to maximize decarboxylation and at the same time to minimize the oxidase side reaction. In contrast, PAAS appears to have evolved to fully (stoichiometrically) couple the oxidase reaction with the decarboxylation reaction, which appears to be unprecedented for a native PLP enzyme (Fig. 5B). Efficient coupling of these two reactions occurs presumably as a result of easy access of O_2 to the active site, stabilization of the peroxide anion through protonation (43), and enhanced radical character of the reaction intermediates (50).

We suggest that, under aerobic conditions, PAAS catalyzes oxidative decarboxylation by a radical mechanism (see “Discussion”) similar to that suggested previously for oxidative decarboxylation reactions catalyzed by bacterial glutamate decarboxylase and other decarboxylases (50, 51). Such a mechanism would readily explain why both PLP- and thiamine diphosphate-dependent decarboxylases commonly have oxygen-consuming side reactions (50).

**DISCUSSION**

Occurrence of Oxidative Decarboxylation Catalyzed by PLP-dependent Decarboxylases—Native pig kidney DDC (44, 47), E. coli glutamate decarboxylase (43, 52), and Lactobacillus 30a ornithine decarboxylase (52) can catalyze the oxidative decarboxylation of α-methyl-Dopa, α-methylglutamate, and α-methylornithine, respectively. Therefore, there is ample precedent for the present finding that a PLP-dependent decarboxylase such as PAAS can catalyze the oxidative decarboxylation of its α-methylamino acid analog. DDC has also been shown to oxidize D-tryptophan methyl ester to its corresponding α-keto acid methyl ester analog (48). However, the present finding that native PAAS can stoichiometrically catalyze the oxidative decarboxylation of its unsubstituted “natural” L-amino acid substrate is unprecedented.

Bertoldi et al. (46) showed that mutation of Tyr^{332} to Phe converts pig kidney DDC into a decarboxylation-dependent deaminase, in which the decarboxylation of amino acid substrate (L-Dopa) is stoichiometric with oxidation. Unlike in the reaction catalyzed by the native enzyme, the amine product cannot be detected in the reaction catalyzed by the mutant enzyme (46). Thus, mutant pig kidney DDC has catalytic properties very similar to those of PAAS. Native DDC cannot support the oxidative decarboxylation of Dopa, although it can oxidize dopamine at a relatively low rate. Evidently, only a slight perturbation of the topography of the active site is required to convert pig kidney DDC from an enzyme that catalyzes exclusively the non-oxidative decarboxylation of its L-amino acid substrate into an enzyme that catalyzes exclusively the oxidative decarboxylation of its L-amino acid substrate. Interestingly, Tyr^{332} in pig kidney DDC is replaced by phenylalanine and valine in rose and petunia PAASs, respectively (Fig. 3).

Comments on the Proposed Mechanism for PAAS-catalyzed Formation of PHA under Aerobic Conditions—We have presented strong evidence against the possibility of a flavin cofactor or PQQ in the active site of PAAS. Other cofactors involved in oxidation reactions, including tryptophan tryptophylquinone, trihydroxyphenylalanine quinone (topaquinone), and
lysine tyrosylquinone, and the galactose oxidase cofactor (53) also seem unlikely to be present in PAAS. Enzymes that make use of these cofactors also require Cu²⁺. PAAS activity is not affected by a copper chelator, and added Cu²⁺ is inhibitory. It is possible that PAAS contains a “new” type of unidentified cofactor. However, this possibility also seems unlikely, as PAAS is closely related to DDCs (Fig. 3), and only a simple mutation is required to convert pig kidney DDC from a non-oxidative decarboxylase to a fully oxidative decarboxylase (46). The only cofactor present in the active site of pig kidney DDC is PLP. PLP is most likely the only cofactor present in the active site of PAAS.

Vitamin B₆ in its active form (usually PLP, but occasionally PMP) is a remarkably versatile biological catalyst (54). Most reactions catalyzed by vitamin B₆-containing enzymes are not thought to involve free radical mechanisms. Rather, most proposed mechanisms involving PLP-dependent enzyme catalysis are based on stabilization of high energy anionic intermediates in the reaction pathways by the pyridinium moiety of PLP/PMP (55). Thus, PLP-dependent decarboxylation pathways are usually shown to proceed via a non-radical quinonoid intermediate (see, for example, Ref. 54 and references cited therein). One possible mechanism for the oxidative decarboxylation of Phe catalyzed by PAAS involves a “conventional” decarboxylation occurring through a quinonoid intermediate, followed by reaction of this intermediate with molecular O₂. Indeed, Bertoldi et al. (48) have suggested that the oxidation of D-tryptophan methyl ester catalyzed by DDC proceeds through a quinonoid intermediate, which then reacts with molecular O₂. However, a reaction mechanism that proceeds initially through a quinonoid intermediate should result in retention in the product aldehyde of all the deuterium originating from the α-position of deuterated Phe. The fact that only about half of the deuterium label was present in the aldehyde moiety of PHA (Fig. 4, C and D) suggests that a conventional quinonoid mechanism is not adequate to explain the observed isotopic labeling.

As noted above, a free radical mechanism has been proposed for the oxidative decarboxylation of glutamate catalyzed by E. coli and human glutamate decarboxylase (50, 51). If the quinonoid intermediate illustrated in Fig. 6 had substantial radical character (i.e., existed at least in part in a biradical form), then this is likely to be the form that reacts with molecular oxygen. The formation of a quinonoid biradical could account for the observed labeling pattern, in which about half of the deuterium label in the α-position of deuterated Phe is lost in the aldehyde moiety of the PHA product (Fig. 4C). Hydrogen radical migration from -CH or from the adjacent NH would result in scrambling of label at C-1 of PHA. Restriction of hydrogen migration between C-1 of the imine moiety of the quinonoid intermediate and the iminium hydrogen would provide the observed extent of isotopic dilution (reduction by 50%). Although oxygen is shown reacting with the pyridoxal ring in Fig. 6 and then migrating to C-1 of the imine moiety, this is only one of various possible reaction pathways. After decarboxylation, the potential for negative charge (carbonium, paired electrons) would be distributed from C-1 of the imine moiety to the pyridoxal ring. Similarly, the unpaired electrons in a radical mechanism would have a similar distribution. Oxygen could potentially react at any position where there is an unpaired electron density. By analogy between the quinonoid resonance form and the reduced

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**FIGURE 6. Proposed mechanism for the PAAS-catalyzed formation of PHA from Phe.** The mechanism shows how the deuterium label (D) at the α-carbon of phenylalanine is partially replaced in the product PHA (aldehyde) by hydrogen derived from solvent water. For further details, see “Discussion.”
isoalloxazine flavin ring, the first hydroperoxide formed in Fig. 6 would be equivalent to the 4a-hydroperoxide known to be formed in some flavin-catalyzed reactions (56). Although the reaction of reduced flavins with oxygen is also thought to be a radical process, both electrons are transferred nearly concurrently, with virtually none of the radical semiquinone form of flavin being present as an intermediate. Similar to the reaction of reduced flavins with molecular oxygen, the uncharged quinonoid intermediate (a carbanion resonance form) of PAAS is shown to react in Fig. 6 without indicating possible radical reaction intermediates. In addition to migrating to C-1 of the imine moiety, as illustrated in Fig. 6, the peroxide intermediate could independently release hydrogen peroxide and the other products.

A few examples in which PLP (or PMP) is known to participate in free radical reactions have been well documented. One such example is Clostridium subterminale lysine 2,3-aminomutase (57). This enzyme catalyzes the PLP-dependent interconversions of l-lysine and l-β-lysine. The reaction mechanism includes 1-electron chemistry and uses a [4Fe-4S] cluster and S-adenosylmethionine (57). Another example of a PLP enzyme that catalyzes a free radical mechanism is Clostridium sticklandii lysine 5,6-aminomutase. This enzyme catalyzes the vitamin B12-dependent interconversion of d-lysine with 2,5-diaminohexanoate and of l-β-lysine with 3,5-diaminohexanoate (58). The role of PLP in these two enzymes is not certain (57), but the cofactor may stabilize high energy radical intermediates by providing a conjugated π-electron system, over which the unpaired electron may delocalize (57, 59). A third vitamin B6-dependent enzyme that participates in a free radical mechanism is CDP-6-deoxy-l-threo-d-glycero-4-hexulose-3-dehydrase. This enzyme catalyzes the C-3 deoxygenation in the biosynthesis of 3,6-dideoxyhexoses in Yersinia pseudotuberculosis and is a PMP-dependent enzyme that also contains a [2Fe-2S] center but does not require S-adenosylmethionine or vitamin B12 (60).

In summary, PAAS is the first PLP enzyme to be described that, in its native state, catalyzes the stoichiometric oxidative decarboxylation of an L-amino acid substrate. Although PAAS and DDC exhibit many similarities, a major difference between these two enzymes is that, under aerobic conditions, decarboxylation is an oxidative process in the reaction catalyzed by PAAS and a non-oxidative process in the reaction catalyzed by DDC. Under aerobic conditions, PAAS catalyzes a strictly oxidative decarboxylation of L-amino acid substrate, whereas pig kidney DDC catalyzes a strictly non-oxidative decarboxylation of L-amino acid substrate. PAAS utilizes Phe as an oxidative substrate, but not phenylethylamine, whereas pig kidney DDC utilizes dopamine as an oxidative substrate, but not L-Dopa. Finally, O2 utilization by PAAS under aerobic conditions is associated with the formation of H2O2, whereas O2 utilization by pig kidney decarboxylase is not associated with the production of H2O2. Recently, Tieman et al. (61) identified in tomato another member of the PLP decarboxylase family that catalyzes the decarboxylation of Phe to phenylethylamine, which is released from the enzyme and can then be converted to phenylacetaldehyde through the action of a hypothesized amine oxidase. An understanding of the structural elements that result in subtle differences in the reactions catalyzed by these closely related enzymes must await more detailed studies.

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