Role for an Essential Tyrosine in Peptide Amidation*

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The catalytic core of the peptidyl-α-hydroxyglycine α-amidating lyase (PAL) domain of peptidylglycine α-amidating monooxygenase was investigated with respect to its ability to function as a ureidoglycolate lyase and the identity and role of its bound metal ions. The purified PAL catalytic core (PALcc) contains molar equivalents of calcium and zinc along with stoichiometric amounts of iron and functions as a ureidoglycolate lyase. Limiting iron availability in the cells synthesizing PALcc reduces the specific activity of the enzyme produced. Concentrated samples of native PALcc have an absorption maximum at 560 nm, suggestive of a phenolate-Fe(III) charge transfer complex. An essential role for a Tyr residue was confirmed by elimination of PAL activity following site-directed mutagenesis. Purified PALcc in which the only conserved Tyr residue (Tyr654) was mutated to Phe was secreted normally, but was catalytically inactive and lacked bound iron and bound zinc. Our data demonstrate an essential role for Tyr654 and suggest that it serves as an Fe(III) ligand in an essential iron-zinc bimetallic site.

C-terminal amidation, a required post-translational modification for the bioactivation of many neuropeptides, is catalyzed by a bifunctional enzyme, peptidylglycine α-amidating monooxygenase (PAM4; EC 1.14.17.3), in a two-step reaction (see Fig. 1) (1–5). The first step, hydroxylation at the α-carbon of the terminal glycine, is catalyzed by peptidylglycine α-hydroxylating monooxygenase (PHM), a copper-, ascorbate-, and molecular oxygen-dependent enzyme, and yields a peptidyl-α-hydroxyglycine intermediate. The second step, dealkylation of this intermediate, is catalyzed by peptidyl-α-hydroxyglycine α-amidating lyase (PAL; EC 4.3.2.5), leaving the carboxamide moiety at the C terminus of the bioactive peptide and generating glyoxylic acid (1, 6, 7). PAL is also referred to as peptidylamidoglycolate lyase and α-hydroxyglycine amidating dealkylase (6).

In many species (e.g. human, mouse, rat, and Xenopus), PHM and PAL are encoded by the same transcript, yielding a bifunctional protein, PAM, with separate catalytic domains (8). In other species (e.g. Drosophila, Cnidaria, and Planaria), PHM and PAL are encoded by separate genes (9–11). The Drosophila genome encodes two active PAL proteins (12); whereas Drosophila PAL2 is a soluble protein localized to secretory granules, Drosophila PAL1 is an integral membrane protein localized to the endoplasmic reticulum. Although yeast and humans use similar subtilisin-like endopeptidases to produce bioactive peptides from inactive precursors (13, 14), yeast cells do not produce amidated peptides, and the yeast genome does not contain sequences homologous to PHM or PAL.

PHM belongs to a small family of monooxygenases that use molecular oxygen and ascorbate to catalyze the hydroxylation of their substrates (EC 1.14.17.x) (8). Dopamine β-monooxygenase (also known as dopamine β-hydroxylase; EC 1.14.17.1) (15) converts dopamine into norepinephrine during catecholamine biosynthesis. Substrates have not been identified for the remaining family members: monooxygenase X (16) and dopamine β-hydroxylase-like protein (17). Searches for homologous sequences consistently fail to identify proteins related to PAL.

The reaction catalyzed by PAL closely resembles the reaction catalyzed by ureidoglycolate lyase (UGL; EC 4.3.2.3), conversion of ureidoglycolate into urea and glyoxylate (Fig. 1) (18). UGL was first described in Streptococcus allantiosicus and Pseudomonas (19, 20). UGL activity has been studied in bacteria, yeast, fish, and rat, and UGL has been purified from fish liver (21), chick pea (22), and Burkholderia cepacia (23). The only natural sequence data available are for B. cepacia UGL and place it in the fumarylacetoacetate hydrolase family (23). Fumarylacetoacetate hydrolase (FAH; EC 3.7.1.2) catalyzes the hydrolytic cleavage of a carbon–carbon bond in fumarylacetoacetate, yielding fumarate and acetoacetate in the final step of Phe and Tyr degradation (24). In humans, loss of FAH activity causes tyrosinemia type I, a fatal metabolic disease (25). The sequence of B. cepacia UGL shows no homology to PAL (23).

A previous study defined the PAL catalytic core (PALcc; Asp948–Val1230), assigned its two disulphide bonds, and eliminated an essential role for N-glycosylation (26). In defining the PALcc, it was clear that many truncation mutants were misfolded. Thermal stability and protease resistance studies revealed a role for divalent metals in maintaining the stability of PALcc (26). Mutagenesis studies led to the postulate that PALcc contains both a structural and a catalytic metal-binding site (26). Consistent with our hypothesis of multiple metal-binding sites, we demonstrate here that purified PALcc con-
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A. PHM:

\[
\begin{align*}
\text{Peptide} & \text{C} - \text{COO}^- \\
\text{Peptidylglycine} & \begin{array}{c}
\text{2 Ascorbate} \\
\text{2 Semidehydroascorbate}
\end{array}
\rightarrow \\
\text{Peptide} & \text{N} - \text{C} - \text{COO}^- \\
\text{Peptidyl-α-hydroxyglycine} & \begin{array}{c}
\text{2Cu}
\rightarrow
\text{Peptide-} \text{NH}_2 + \text{HC-} \text{COO}^- \\
\text{Peptide-amide + Glyoxylate}
\end{array}
\end{align*}
\]

B. PAL:

\[
\begin{align*}
\text{Peptide} & \text{C} - \text{COO}^- \\
\text{Peptidyl-α-hydroxyglycine} & \begin{array}{c}
\text{PAL} \\
\text{Peptide-NH}_2 + \text{HC-} \text{COO}^- \\
\text{Peptide-amide + Glyoxylate}
\end{array}
\end{align*}
\]

C. UGL:

\[
\begin{align*}
\text{Ureidoglycolate} & \text{H}_2\text{N-C} - \text{NH}_2 \\
\text{Urea} & \text{Glyoxylate}
\end{align*}
\]

FIGURE 1. Reactions catalyzed by PHM, PAL, and UGL. A, copper is the only metal that supports PHM activity. B, purified PALcc, which contains equimolar amounts of zinc, is known to be inhibited by chelators of divalent metal ions. C, UGL is also inhibited by chelators of divalent metal ions (48). Both UGL and PAL react preferentially with substrates with the 2- enantiomer (23).

contains molar amounts of both zinc and calcium. Our observation that concentrated solutions of PAL had a lavender color led to the identification of bound iron and to the identification of an essential Fe(III)-tyrosinate charge transfer complex. Mutation of the only conserved Tyr residue (Tyr\(^{654}\)) eliminated catalytic activity; the lavender color; and the binding of zinc, calcium, and iron to PAL.

EXPERIMENTAL PROCEDURES

Cell Culture and Purification of PALcc—PALcc was purified from the spent medium of stably transfected Chinese hamster ovary (CHO) cells as described (26). Cells maintained in roller bottles were fed daily with complete serum-free medium (CSFM), and assays were carried out at pH 5.5 in 100–150 mM NaTES containing 0.05% Thesit (Roche Applied Science) and 1.0 mM CdCl\(_2\) as described (26). Based on scans of Coomassie Brilliant Blue R-250-stained membranes, 93 ± 2% of the protein in all preparations analyzed was PALcc. The concentration of PALcc was determined by measuring the absorbance at 280 nm and using the measured extinction coefficient (1 mg/ml = 1.42; molecular mass of 38.3 kDa) (26).

Iron Loading of PAL-producing CHO Cells—Stably transfected CHO cells expressing PALcc were rinsed for 4 h in CSFM or in CSFM containing the iron-nitritoacetate complex (200 \(\mu\)M) (27), holotransferrin (iron-loaded, 10 or 100 \(\mu\)M), or apotransferrin (iron-free, 10 or 100 \(\mu\)M). Cells were then incubated for two sequential 24-h periods with fresh medium of the same composition. Spent media were centrifuged to remove non-adherent cells; after the second 24-h incubation, cells were extracted into 20 mM NaTES, 10 mM mannitol, and 1% Triton X-100 (pH 7.4) (26). Cell lysates and media were assayed for PAL activity as described (26). Aliquots of cell extract and medium were fractionated on 4–15% Criterion gels (Bio-Rad), transferred to Immobilon membranes, and visualized using rabbit polyclonal antibody to bovine PAM-(561–579) (JH256) (28). The amount of PAL protein in each sample was quantified using GeneSnap (Syngene, Frederick, MD). The specific activity of the PAL contained in the cell extract or secreted into the medium was calculated by dividing the activity measured by the amount of PAL protein present. The experiment was replicated three times.

Measurement of UGL Activity—UGL activity was determined by measuring glyoxylate formed through its reaction with phenylnitrile (29). The reaction mixture (100 \(\mu\)l/well in a 96-well plate) contained 100 mM NaTES (pH 7.8), 2.0 mM ureidoglycolate (freshly prepared and kept on ice), 3.0 mM phenylhydrazine, and enzyme. After incubation for 30 min at 37 °C, the absorbance was measured at 360 nm using a Wallac VICTOR\(^\text{TM}\) plate reader (PerkinElmer Life Sciences). Controls lacking enzyme were assayed to determine nonenzymatic production of glyoxylate. A standard curve generated using 0.156–5.0 mM glyoxylate was used to quantify glyoxylate produced from ureidoglycolate by PAL; samples were assayed in duplicate (30).

Effect of Metals, Oxidizing Agents, and Reducing Agents on the Activity of PALcc—Purified PALcc was exposed to the indicated concentrations of trivalent metal (e.g. GaCl\(_3\), Fe(NO\(_3\))\(_3\), FeCl\(_3\)) or oxidizing/reducing agent (K\(_3\)Fe(CN)\(_6\), K\(_4\)Fe(CN)\(_6\), ascorbate, freshly dissolved dithionite) for 30 min on ice; diluted 10\(^{10}\)-fold into 20 mM NaTES, 10 mM mannitol, 1% Triton X-100, and 1 mg/ml bovine serum albumin (pH 7.4); and assayed under standard assay conditions (26). Each modifying reagent and salt were individually assayed for the ability to support nonenzymatic conversion of substrate into product under standard reaction conditions; no significant amount of product was generated.

To determine the effect of K\(_3\)Fe(CN)\(_6\) treatment on the chromophore, purified PALcc (0.59 mg/ml, 15.5 \(\mu\)M) was treated with 10 mM K\(_3\)Fe(CN)\(_6\) in 25 mM NaTES and 100 mM NaCl (pH 7.4) for 30 min on ice. The reaction mixture was passed through a NICK\(^\text{TM}\) column equilibrated with NH\(_4\)HCO\(_3\) (pH 8) to remove low molecular mass products contributing to the absorption spectrum. PALcc recovered in the void volume was lyophilized and redissolved; based on As\(_{280}\), the protein concentration was 8 \(\mu\)M. The absorption spectrum was recorded from 200 to 800 nm.

Modification of Tyrosine Residues in PALcc—Purified PALcc was exposed to the indicated molar excess of tetraniomethane (TNM) or N-acetylimidazole on ice for 30 min. After incubation, the reaction was diluted 10\(^{10}\)-fold into 20 mM NaTES, 10 mM mannitol, 1% Triton X-100, and 1 mg/ml bovine serum albumin (pH 7.4) for assessment of enzyme activity (26). The ability of substrate to protect PAL from modification by TNM was assessed by preincubating a concentrated sample of purified PALcc (0.65 mg/ml) with ureidoglycolate or \(\alpha\)-hydroxyhippuric acid before exposure to TNM or ice for 1 h. After incubation with TNM, purified PALcc was diluted and assayed as described (26). PALcc subjected to the same manipulations but not exposed to the modifying agent served as the control.

To determine the effect of TNM treatment on the chromophore, purified PALcc (100 \(\mu\)l, 0.55 mg/ml or 14.4 \(\mu\)M) was treated with a 20-fold molar excess of TNM for 1 h at room temperature in 25 mM NaTES and 100 mM NaCl (pH 7.4). The reaction mixture was passed through a NICK column equili-
brated with NH₄HCO₃ (pH 8) to remove nitroformate, which has a strong 350 nm absorption peak. Fractions containing the void volume were lyophilized and dissolved in 100 μl of 20 mM Tris-HCl (pH 9.0); based on A₂₈₀, the protein concentration was 8.3 mg/ml. The absorption spectrum was recorded from 200 to 800 nm; 20 mM Tris-HCl (pH 9.0) was used as a blank. TNM-treated PALcc digested with endoproteinase Lys-C or endoproteinase Glu-C was subjected to liquid chromatography-tandem mass spectrometry analysis in the University of Connecticut Facility; in both digests, the only nitro-Tyr residue identified was Tyr582, and modification was partial.

Production and Purification of PALcc(Y654F)—CHO-DG44 cells transfected with the pCIS/PALcc(Y654F) vector were selected using α-minimal essential medium containing dialyzed fetal bovine serum (31). Positive subclones were selected by Western blot analysis of the spent medium. Cells from a single subclone were expanded into six 150-mm dishes and used to seed a CellMax® artificial capillary cartridge (outer surface area of 1.1 m², 95% molecular mass cutoff of 20 kDa; catalog number 360-400-003, Spectrum Laboratories, Inc., Rancho Dominguez, CA) precoated inside with NuSerum (BD Biosciences). The cartridge was maintained on the outside in medium containing Dulbecco’s modified Eagle’s medium/nutrient mixture F-12 supplemented with 10% fetal calf serum, penicillin/streptomycin, HEPES, and Fungizone for 7 days. Cells were then rinsed twice with CSFM; 50 ml of spent medium was collected and replaced daily for 8 weeks. Secreted proteins were concentrated by precipitation with 65% (NH₄)₂SO₄; pellets were resuspended in 20 mM NaTES and 1.5 M NaCl (pH 7.0) and applied to a hydrophobic interaction column (HiTrap™ phenyl) equilibrated with the same buffer and eluted with 20 mM NaTES (pH 7.0). Fractions containing PALcc(Y654F) were identified by Western blot analysis, pooled, and concentrated using an Amicon stirred flow cell with a YM-30 membrane. The concentrated protein was loaded onto a Superdex™ 200 gel filtration column (25 × 350 mm) equilibrated and eluted with 20 mM NaTES and 100 mM NaCl (pH 7.0). Fractions containing PALcc(Y654F) were identified by Western blotting and pooled, and the buffer was exchanged using an Amicon stirred flow cell and 20 mM Tris-HCl (pH 9.0). Following application to a SOURCE™ 15Q ion exchange column (10 × 30 mm; Amersham Biosciences), PALcc(Y654F) was eluted with a gradient to 20 mM Tris-HCl and 1 M NaCl (pH 9.0). Fractions containing PALcc(Y654F) were pooled, concentrated using an Amicon stirred flow cell, and subjected to gel filtration on a Superose 6 column (10 × 300 mm) eluted with 20 mM NaTES and 100 mM NaCl (pH 7.0). The purity of the fractions containing the N-glycosylation site at Asn⁷⁶⁵ by mutating Ser⁷⁶⁷ to Ala. Each cDNA construct was sequenced in its entirety.

Wild-type pCIS/pro-PALcc(Q551R) and the three mutant proteins were transiently expressed in PEAKrapid cells (Edge Biosystems) (26). Confluent 35-mm wells of cells were transfected with 2 μg of plasmid using Lipofectamine 2000 (Invitrogen). After 24 h, cells were fed with CSFM. After an additional 24-h incubation, the medium was collected, centrifuged to remove non-adherent cells, and stored with protease inhibitors. Cells were extracted and analyzed as described above.

Transient Expression of Mutant PALcc—The QuikChange site-directed mutagenesis kit (Stratagene) was used to replace three single Tyr residues in rat pro-PALcc with Phe: Tyr582, Tyr595, and Tyr654. The 21-mers used for mutagenesis (with the mutated nucleotides underlined) were as follows: Y582F, 5’-GGCGAGAACCTGTTTTTTTTAACACAGGCGC-3’ (forward) and 5’-GCGGTGGTGTGAAAAAAGGGTTTGGCC-3’ (reverse); Y595F, 5’-GATACAGATGGAAATTTTTGGGTC-3’ (forward) and 5’-CATCTGTGACCCAAAAATTCTCCCATGTGATC-3’ (reverse); and Y654F, 5’-CGTGTCCAGAGCGTTTTGTTAAGCTCGG-3’ (forward) and 5’-CCGACGTGTTACAGAAACCCTCAGACCG-3’ (reverse). The pCIS vector (26) used to test the effect of these single mutations encoded rat pro-PALcc containing an inconsequential mutation (Q551R) (26). Rat PALcc was engineered to lack the N-glycosylation site at Asn⁷⁶⁵ by mutating Ser⁷⁶⁷ to Ala. Each cDNA construct was sequenced in its entirety.

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Promoter and Purification of PALcc(Y654F)—CHO-DG44 cells transfected with the pCIS/PALcc(Y654F) vector were selected using α-minimal essential medium containing dialyzed fetal bovine serum (31). Positive subclones were selected by Western blot analysis of the spent medium. Cells from a single subclone were expanded into six 150-mm dishes and used to seed a CellMax® artificial capillary cartridge (outer surface area of 1.1 m², 95% molecular mass cutoff of 20 kDa; catalog number 360-400-003, Spectrum Laboratories, Inc., Rancho Dominguez, CA) precoated inside with NuSerum (BD Biosciences). The cartridge was maintained on the outside in medium containing Dulbecco’s modified Eagle’s medium/nutrient mixture F-12 supplemented with 10% fetal calf serum, penicillin/streptomycin, HEPES, and Fungizone for 7 days. Cells were then rinsed twice with CSFM; 50 ml of spent medium was collected and replaced daily for 8 weeks. Secreted proteins were concentrated by precipitation with 65% (NH₄)₂SO₄; pellets were resuspended in 20 mM NaTES and 1.5 M NaCl (pH 7.0) and applied to a hydrophobic interaction column (HiTrap™ phenyl) equilibrated with the same buffer and eluted with 20 mM NaTES (pH 7.0). Fractions containing PALcc(Y654F) were identified by Western blot analysis, pooled, and concentrated using an Amicon stirred flow cell with a YM-30 membrane. The concentrated protein was loaded onto a Superdex™ 200 gel filtration column (25 × 350 mm) equilibrated and eluted with 20 mM NaTES and 100 mM NaCl (pH 7.0). Fractions containing PALcc(Y654F) were identified by Western blotting and pooled, and the buffer was exchanged using an Amicon stirred flow cell and 20 mM Tris-HCl (pH 9.0). Following application to a SOURCE™ 15Q ion exchange column (10 × 30 mm; Amersham Biosciences), PALcc(Y654F) was eluted with a gradient to 20 mM Tris-HCl and 1 M NaCl (pH 9.0). Fractions containing PALcc(Y654F) were pooled, concentrated using an Amicon stirred flow cell, and subjected to gel filtration on a Superose 6 column (10 × 300 mm) eluted with 20 mM NaTES and 100 mM NaCl (pH 7.0). The purity of the fractions containing the N-glycosylation site at Asn⁷⁶⁵ by mutating Ser⁷⁶⁷ to Ala. Each cDNA construct was sequenced in its entirety.
PALcc(Y654F) was evaluated by Coomassie Brilliant Blue staining after transfer to polyvinylidene difluoride membranes.

Metal Analysis, Spectrum Determination, and Reconstitution with Iron—Metal ion analysis was performed on a PerkinElmer Life Sciences Optima 2000 inductively coupled plasma optical emission spectrometer. Standard curves were linear over the range 40–2000 nm. The absorption spectrum of PALcc (260–800 nm) was determined using a Beckman DU800 spectrophotometer with a scan rate of 1200 nm/min; a buffer blank analyzed at the same time was subtracted. We attempted to load purified PALcc with additional iron. Purified PALcc was washed repeatedly with 50 mM Tris-HCl (pH 8.0) using an Ultrafree concentrator (10-kDa cutoff; Millipore Corp.). An aliquot of the concentrated protein was saved for metal analysis, and the spectrum was recorded. The concentrate was diluted 14-fold with the same buffer, and ferric nitrate (1 mM stock solution) was added slowly using a syringe pump. Insoluble material was removed by centrifugation, and the sample was again washed with buffer to remove unbound metals; the final filtrate was analyzed for metal content. The sample was concentrated for metal determination, and the spectrum was again recorded. Reconstitution with ferric ammonium citrate was attempted in a similar manner.

RESULTS

Purified PAL Is a Ureidoglycolate Lyase—The similarities in the reactions catalyzed by PAL and UGL suggested that PAL might accept ureidoglycolate as a substrate. To assess this possibility, purified PALcc was tested for its ability to generate glyoxylate from ureidoglycolate (Fig. 2). Based on SDS-PAGE, the purity of the PALcc used for these experiments was 92% (Fig. 2A). Production of glyoxylate was quantified using the phenylhydrazine assay for glyoxylate (30) and increased linearly as the concentration of PALcc was increased (data not shown). As the concentration of ureidoglycolate was increased, saturation was reached (data not shown). Using an Eadie-Hofstee plot (Fig. 2B), the $K_m$ of PALcc for ureidoglycolate is 0.65 ± 0.02 mM, and the $V_{max}$ is 0.27 ± 0.01 mmol/min. The $K_m$ value reported for purified B. cepacia UGL (0.5 mM) is quite similar, but the $V_{max}$ of the bacterial enzyme is 180 mmol/min, almost 700-fold higher than the $V_{max}$ of PALcc for ureidoglycolate (23). Consistent with the work of May and co-workers (23), PALcc can function as a UGL.

Purified PAL Contains Zinc, Calcium, and Iron—Both bifunctional PAM3 and PALcc contain almost stoichiometric amounts of zinc (26, 32). The lyase activity of both proteins is inhibited by metal ion chelators such as EDTA, EGTA, and α-phenanthroline (26, 32). A number of divalent metal ions restore catalytic activity to control levels after inactivation by EDTA, and site-directed mutagenesis of PALcc led to the prediction of two metal-binding sites, one structural and one catalytic (26). To identify other metal ions bound to PAL, several preparations of PALcc were analyzed using inductively coupled plasma optical emission spectrometry (Table 1). Close to stoichiometric amounts of zinc were again detected in purified PALcc (1.05 ± 0.06 mol/mol of PAL) (26). In addition, stoichiometric amounts of calcium were present (0.98 ± 0.14). Although the levels of manganese, cobalt, nickel, and copper were insignificant (Table 1), significant levels of iron were found in each sample analyzed; the average iron content of PALcc was 0.14 ± 0.01 mol/mol of PALcc.

| PALcc | Conc | Calcium | Manganese | Iron | Cobalt | Nickel | Copper | Zinc |
|-------|------|---------|-----------|------|--------|--------|--------|------|
| Sample A | 43.0 | 0.82    | 0         | 0.15 | 0      | 0      | 0      | 0.03 |
| Sample B | 77.5 | 1.05    | 0         | 0.14 | 0      | 0      | 0      | 0.05 |
| Sample C | 87.4 | 1.07    | 0         | 0.14 | 0      | 0      | 0      | 0.04 |

Mean ± S.D. | 0.98 ± 0.14 | 0.14 ± 0.01 | 1.05 ± 0.06

**FIGURE 3.** Iron availability affects the specific activity of PAL. Stably transfected CHO cells expressing PALcc were grown for 2 days in CSFM (control (Con)) or in CSFM containing the iron-nitriloacetic acid complex (Fe-NTA; 200 μM), holotransferrin (iron-loaded, 10 or 100 μM), or apotransferrin (iron-free, 10 or 100 μM). The spent medium (A) and cell lysates (B) were assayed for PAL activity (26), and PAL protein content was assessed by Western blot analysis. Relative specific activities were calculated, with the specific activity of PAL in CSFM set to 1.0. Data shown are from one experiment, which is representative of three similar experiments.

**TABLE 1**

Methylated bound to PALcc

Purified PALcc from three different purifications was subjected to metal analysis by inductively coupled plasma optical emission spectrometry. Protein concentrations were determined by measuring the absorbance at 280 nm. The molar ratio of metal to protein was calculated using a mass of 38.3 kDa for PALcc.

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Purified PALcc Is Purple—When samples of PALcc were concentrated for metal analysis, the solution became noticeably lavender-colored. Although zinc complexes with proteins are usually colorless, the presence of iron suggested an explanation for the lavender color. Zinc proteins known to exhibit a purple chromophore often contain an Fe(III)-Zn(II) binuclear center, as, for example, the purple acid phosphatases (PAPs) (33–36).

To assess this possibility, the UV-visible spectrum of PALcc was recorded (Fig. 2C). Consistent with the lavender color, a weak absorption peak occurred at 560 nm. To ensure that the 560 nm absorption peak was associated with PAL, the UV-visible spectrum of PALcc was recorded (Fig. 2D). Lyase activity peaked with both absorption peaks, leading us to conclude that PALcc, and not an impurity, is responsible for the 560 nm absorption peak. Based on three separate preparations, the molar extinction coefficient for the 560 nm peak is 750 ± 40; normalized to iron content, the molar extinction coefficient is 4600 ± 1400. Because absorption bands in the 400–600 nm range in non-heme systems are often associated with tyrosinate to metal charge transfer bands, we set out to explore this possibility (33–40).

The Ability of Stably Transfected CHO Cells to Load Iron into PAL May Limit Its Iron Content—Attempts to load additional iron into purified PALcc were uniformly unsuccessful. We first attempted to reconstitute PALcc with ferric ion using ferric ammonium citrate. Approximately 3 eq of ferric ion was added to PALcc over a period of 50 min; free iron was then removed by three cycles of ultrafiltration. Although analysis of the resulting ultrafiltrate was <1.5% of that bound to PALcc. Our inability to increase PAL activity by providing iron to purified PALcc led us to consider the possibility that proper incorporation of iron can occur only during biosynthesis.

To explore this possibility, stably transfected CHO cells expressing PALcc were maintained under conditions of altered iron availability. Holotransferrin or the iron-nitriloacetic acid complex was included in the serum-free culture medium to increase iron availability; apo transferrin was used to diminish iron availability (Fig. 3). Cells were maintained under these different conditions for 52 h; the spent medium collected over the final 24 h and the final cell extract were assessed for PAL activity and PAL protein. By normalizing PAL activity to PAL protein (quantiﬁed by Western blotting), relative specific activities could be compared. The specific activity of PALcc in both cell extracts and media was increased by growth in medium containing the iron-nitriloacetic acid complex or holotransferrin. Growth in medium containing apo transferrin resulted in a substantial decrease in relative specific activity. These data suggest that iron must be inserted into the active site of PAL during biosynthesis.

PALcc Is Inhibited by Trivalent Cations and Reducing Agents—Because the 560 nm band in the visible spectrum of PAL is characteristic of a phenolate-Fe(III) charge transfer complex, we speculated that a catalytic iron would be in the (III) oxidation state. To probe the relationship between metal content, oxidation state, and catalytic activity, we tested the effect on activity of preincubation of PALcc with trivalent cations (Fig. 4A). Both ferric nitrate and ferric chloride inhibited PAL, with sample revealed binding of 1.0 mol of iron/mol of PALcc (data not shown), catalytic activity was not altered. We next attempted reconstitution with ferric nitrate; both the metal content and spectra were compared before and after reconstitution (Fig. 2C). The iron content rose from 0.18 to 1.34 mol/mol, but the difference spectrum showed no increase in the absorption peak at 560 nm (Fig. 2C, inset). The altered spectral properties suggest that the additional iron is bound to a different site than in the native protein. In attempts at reconstitution, free iron concentrations in the filtrate were <2% of the concentration in the retentate, indicating tight binding of ~1 eq of ferric ion. Equimolar amounts of calcium were still present, with no significant amount of manganese, cobalt, nickel, or copper. The retention of calcium after the iron reconstitution protocol allowed us to conclude that calcium is tightly bound and is not substituted with trivalent cations; the concentration of calcium in the
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After treatment with ferrocyanide, PALcc lacked the 560 nm absorption peak (data not shown). Ascorbate and dithionite were less effective inhibitors, with both reductants producing half-maximal effects at $\sim 10$ mM.

**Substrate Protects PALcc from TNM-mediated Inactivation**—With evidence for a role for ferric iron in the reaction, we next sought evidence for involvement of a tyrosine residue. Exposed tyrosine residues in proteins can often be modified by reaction with TNM or N-acetyl-L-tyrosine (41). To determine whether the active site of PAL contains an essential tyrosine residue, the enzyme was exposed to increasing concentrations of TNM, which nitrates exposed tyrosine residues, or N-acetyl-L-tyrosine, which acetylates exposed tyrosine residues. Incubation with a 10-fold molar excess of TNM eliminated the catalytic activity of PAL (Fig. 5, upper panel). Incubation with a 500-fold molar excess of N-acetyl-L-tyrosine, a larger molecule, did not alter PAL activity (data not shown). We reasoned that an active-site tyrosine might be protected from TNM by inclusion of substrate (Fig. 5, middle and lower panels). Preincubation of PAL with α-hydroxyhippuric acid resulted in dose-dependent protection from TNM-mediated inactivation. Preincubation with ureidoglycolate also protected PAL from inactivation, although the effect was not dose-dependent. Taken together, the presence of bound iron, the 560 nm absorption peak, and the ability of TNM to inactivate PAL point to an essential role for an active-site tyrosine residue(s).

Although catalytic activity was eliminated following treatment with TNM, the 560 nm absorption peak attributed to the iron-tyrosinate charge transfer complex was unaltered (data not shown). The only nitrotyrosine residue that could be identified in endoprotease Lys-C or endoprotease Glu-C digests of TNM-modified PALcc was Tyr$^{585}$, which is situated near a conserved His residue (His$^{585}$). Mutation of His$^{585}$ to Ala was shown previously to result in a 50-fold reduction in $V_{max}$ (26). The protection from TNM modification afforded by substrate may reflect involvement of this region of PALcc in catalysis.

Tyr$^{564}$ Is Essential for PAL Activity—To test the hypothesis that the active site of PAL contains an essential phenolate-Fe(III) charge transfer complex, we assessed the effect of individually mutating three Tyr residues. Tyr$^{564}$, the only Tyr residue conserved in every PAL known to be catalytically active, is situated within the first disulfide loop of PALcc and is part of the second of four conserved NHL (NCL-1, HT2A, and LIN-41) repeats (Fig. 6A). In addition, we assessed the effect of mutating Tyr$^{582}$ (which is modified by TNM) and Tyr$^{595}$, non-conserved Tyr residues located in the first NHL repeat. Expression vectors encoding the three mutant PAL proteins, wild-type PAL, or a control protein (green fluorescent protein) were used to transiently transfect cells. PALcc secreted in a 24-h period was compared with PALcc remaining in cell extracts. Western blot analysis revealed similar levels of protein expression and secretion for wild-type PALcc and each of the three Tyr mutants (Fig. 6B). All three Tyr mutants of PALcc were efficiently secreted, with a 24-h collection of the spent medium containing $\sim 10$-fold more protein than the corresponding cell extract. Aliquots of the spent medium were then assayed for PAL activity. Mutation of Tyr$^{582}$ or Tyr$^{595}$ to Phe was without any discernible effect on the catalytic activity or secretion of PAL (Fig. 6B). In contrast, mutation of Tyr$^{564}$ to Phe resulted in the complete elimination of PAL activity.

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**FIGURE 5. Modification of tyrosine residues in PALcc.** Purified PALcc (0.65 mg/ml, 16.9 μM) was treated with the indicated molar excesses of TNM for 30 min on ice (upper panel). The reaction was stopped by dilution with standard assay diluent (20 mM NaTes, 10 mM mannitol, 1% Triton X-100, and 1.0 mg/ml bovine serum albumin (pH 7.4)). The ability of substrate to protect PAL from inactivation by TNM was assessed by preincubating PALcc with the indicated concentrations of α-hydroxyhippuric acid (middle panel) or ureidoglycolate (lower panel) for 1 h on ice before exposure to a 10-fold molar excess of TNM for 30 min on ice. Data shown are the mean of duplicates from a single experiment, which was replicated three times.

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half-maximal inhibition occurring at $\sim 0.50$ mM. Ferric ammonium citrate was less potent (half-maximal inhibition at 2−10 mM) (data not shown). Gallium chloride, which is not redox active, was also a potent inhibitor of PALcc (half-maximal effect at $\sim 0.4$ mM). The inhibition by gallium suggested that inhibition by trivalent ferric ions is not the result of oxidation of a protein residue. Neither nitrate nor citrate on its own (as the sodium salt) had any effect on activity (data not shown). These data suggest that trivalent cations interact with the enzyme at a site that leads to inhibition of catalytic activity, perhaps the same site at which iron was bound during our attempts at reconstitution.

A characteristic feature of the Fe(III)-Zn(II) dinuclear site of PAPs is inhibition by reducing agents capable of reducing Fe(III) to Fe(II). As a test of this chemistry, PALcc was preincubated with four different redox agents (ferricyanide, ferrocyanide ($E^0$ Fe(CN)$_6^{3-}/4^-$ = 0.36 V), dithionite ($E^0$ < 1 V), and ascorbate ($E^0$ = 0.28 V)) (Fig. 4, B and C). Ferricyanide, an oxidant, had no effect on activity, and neither did a potassium cyanide control (data not shown). Each reductant led to inhibition; ferrocyanide gave half-maximal inhibition at $\sim 0.4$ mM. After treatment with ferrocyanide, PALcc lacked the 560 nm absorption peak (data not shown). Ascorbate and dithionite were less effective inhibitors, with both reductants producing half-maximal effects at $\sim 10$ mM.

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PHM and PAL accept non-peptide substrates such as hippuric acid and α-hydroxybenzoylglycine, respectively (44). In addition to α-hydroxylation, PHM catalyzes sulfoxidation, amine N-dealkylation, and O-dealkylation (44). We have shown here that PALcc also functions as a UGL, producing glyoxylate from ureidoglycolate.

The $K_m$ for the PALcc for α-N-acetyl-Tyr-Val-α-hydroxyglycine (38 ± 13 μM for bovine PAL (1) and 33 ± 6 μM for rat PAL (26)) is 20-fold smaller than its $K_m$ for ureidoglycolate (650 ± 20 μM). The only UGL for which sequence data are available (B. cepacia) has a similar $K_m$ for ureidoglycolate (500 ± 10 μM) (23). The V max values determined for rat PALcc using α-N-acetyl-Tyr-Val-α-hydroxyglycine and ureidoglycolate as substrates are similar (37 and 16 mmol/mg/h, respectively) and are substantially below the V max reported for bacterial UGL (10,800 mmol/mg/h) (23, 26). UGL catalyzes the final step in the catalytic pathway leading from purines to urea (18), and activity has been described in many species (19, 20–22, 45–47, 49, 50). UGL activity is generally increased by divalent metals and decreased by chelators of divalent metals (21, 46). Like PAL, UGL is steriospecific for substrates with the (S)-hydroxyglycine configuration (5, 23).

Sequence data are available only for B. cepacia UGL (23). Despite the fact that PAL and UGL can catalyze the same
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Although zinc complexes are usually colorless, the presence of iron accounts for the lavender color of concentrated solutions of PAL. Iron is essential for many metabolic processes, including oxygen transport, DNA synthesis, and electron transport. As for copper, iron must be bound to protein to prevent tissue damage. Every preparation of PALcc examined contained substoichiometric amounts of iron. This may reflect the limited ability of CHO cells to incorporate iron into newly synthesized secretory pathway proteins. The growth of PAL-producing lines in medium containing holotransferrin or the iron-nitrolyacetic acid complex produced a modest increase in PAL specific activity. The magnitude of the increase was less than the 3–4-fold increase expected if PALcc were stoichiometrically loaded with iron and therefore fully active; purification of PALcc from cells grown in medium containing holotransferrin or the iron-nitrolyacetic acid complex will be required to determine whether iron loading has actually been increased. Endocytic uptake of the transferrin-transferrin receptor complex provides iron to Nramp2 for transport to its site of utilization or storage (54–56). *Arabidopsis* glyoxalase II, with two metal-binding sites, contains different mixtures of iron, zinc, and manganese when grown in media of different metal composition, and its $k_{cat}$ and $K_m$ values vary with metal content (57).

The fact that reductants inhibit PAL suggests that the bound iron functions as Fe(III). Of the reducing agents tested, K$_4$Fe(CN)$_6$ was the most potent inhibitor of PAL (half-maximal effect at 0.4 mM); higher doses of ascorbate and dithionite (10 mM) were required to produce 50% inhibition. Inactivation of tartrate-resistant acid phosphatase requires exposure to 100 mM dithionite (58). Reduction of Fe(III) to Fe(II) also inhibits the PAPs (59, 60). Reductive inhibition of PAL may reflect reduction of Fe(III) to Fe(II) at a Fe(III)-Zn(II) dinuclear center or reduction of a critical disulfide bridge. Loss of the 560 nm absorption peak following exposure to K$_4$Fe(CN)$_6$ may reflect a direct effect on the iron-tyrosinate complex or reduction of the neighboring disulfide bond. Based on our mutagenesis studies, 3 of the 10 residues thought to bind iron and zinc are located within the two disulfide loops of PAL (26).

**Tyr$^{564}$ Plays an Essential Role in Forming the Active Site in PAL**—Mutation of the only conserved Tyr residue in PAL (Tyr$^{564}$) to Phe reduced catalytic activity by >10,000-fold, eliminated the absorption peak at 560 nm and the presence of bound iron, and greatly reduced the amount of bound zinc and bound calcium. Our data are consistent with the hypothesis that the active site of PAL includes a Fe(III)-Zn(II) bimetallic center with a bridging Tyr residue. The loss of zinc, calcium, and iron from PALcc(Y654F) suggests roles for all three metals in the active site. The ability of TNM, but not the slightly larger modifier N-acetylimidazole, to inactivate PAL suggests limited access to a catalytically essential Tyr moiety. Surprisingly, TNM-modified PALcc retained its 560 nm absorption peak. Consistent with this observation, the only nitrotyrosine residue identified was Tyr$^{582}$. The ability of substrate to protect PAL from inactivation by TNM is consistent with the essential role of a nearby, highly conserved His residue (His$^{585}$) in determining $V_{max}$ (26).

**Comparing PAL with Other Iron-Zinc Enzymes**—Our data enabled us to identify the 560 nm band associated with PAL as
a Fe(III)-Tyr654 charge transfer complex with an essential role in catalysis. The absorption spectrum of PAL bears a striking similarity to that of the PAPs (33, 34, 36, 39, 40). These proteins generally contain a dinuclear active site with Zn(II), Fe(II), or Mn(II) accompanying a Fe(III) center. Their phenolate-iron charge transfer bands occur in the 530–570 nm range, very similar to the \( \lambda_{\text{max}} \) observed for PAL. The molar extinction coefficient estimated for PAL (4600 \( \mu \)mol/mg/mol) is similar to the extinction coefficients measured for the PAPs (1800–4000) (35–40, 61, 62).

The chemical signature of the PAPs provides a framework for comparison with PAL. The best characterized members in the PAP group are mammalian uteroferrin, bovine spleen PAP, and red kidney bean PAP. The mammalian enzymes contain a Fe(III)-Fe(II) dinuclear center, whereas red kidney bean PAP has a Fe(III)-Zn(II) center. When oxidized to the Fe(III)-Fe(III) state, the mammalian enzymes are inactive. Incubation with dithionite (5 mM, 15 min) leads to reduction to the Fe(II)-Fe(II) state, which results in destruction of the metal center and release of Fe(II) (59).

Although Ga(III) might be expected to substitute for Fe(III) in enzymes in which the metal acts solely as a Lewis acid and does not change its oxidation state during catalysis, the only effect of exogenous Ga(III) on PAL was inhibitory. Unlike Fe(III), Ga(III) is a main group element and always diamagnetic. Ga(III) is the only biologically accessible redox state for gallium, whereas Fe(II), Fe(III), and Fe(IV) have all been detected in biological systems (63). Ga(III) and Fe(III) have similar ionic radii (0.62 Å versus 0.65 Å), charges, and coordination preferences (64). Complexes of Fe(III) and Ga(III) show similar ligand exchange rates and \( pK_a \) values for coordinated water. Gallium has been used as a structural analog of Fe(III) in iron-binding proteins such as transferrin (65–67), lactoferrin (68), and ovotransferrin (48, 69), and the invariant ferric site iron in bovine spleen PAP can be functionally substituted with Ga(III) (59). Because the Fe(III)-tyrosinate site of PALcc is only partially filled, we expected the addition of trivalent metals such as Ga(III) to increase activity. The fact that both Fe(III) and Ga(III) salts inhibited PAL with similar potency suggests binding to an inhibitory site or displacement of an essential metal ion.

Analysis of the spectrum of PALcc loaded with ferric nitrate (1.34 mol of iron/mol) revealed the presence of an alternative binding site. Difficulty accessing the Fe(III) site in the fully folded enzyme or the formation of stable inert Fe(III)-O(H)-Fe(III) (iron-oxygen-gallium) species may contribute to difficulty in reconstituting the active site (51). Although spectroscopically similar, the active site of PAL uses reaction chemistry distinct from that of the PAPs.

The presence of such a complex active site in an enzyme catalyzing an apparently simple reaction was not anticipated. Although our data are most consistent with an essential iron-zinc center with calcium playing an important role in its assembly, we cannot discount an alternative hypothesis, that iron and calcium compete for the same site. In this scenario, zinc and calcium might form the active site with iron acting in an inhibitory fashion. Further work will be necessary to fully explore the

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### TABLE 2

Comparison of metals bound to PALcc and PALcc(Y654F)

| PALcc          | Protein conc (mg/ml) | Specific activity (mol/mg/h) | Zinc (µM) | Iron (µM) | Zinc/protein | Iron/protein |
|----------------|----------------------|-----------------------------|-----------|-----------|--------------|--------------|
| Wild-type      |                      |                             |           |           |              |              |
| Sample D       | 0.23                 | 6.11                        | 307       | 0.47      | 0.07         | 0.17         |
| Sample E       | 0.65                 | 16.9                        | 300       | 12.3      | 0.98         | 0.14         |
| Sample F       | 0.61                 | 15.9                        | 330       | 13.6      | 1.04         | 0.09         |
| Average        |                      |                             |           |           | 0.93 ± 0.14  | 0.18 ± 0.04  |

| Y654F          |                      |                             |           |           |              |              |
| Sample A       | 0.23                 | 6.30                        | <0.03     | 0.47      |              |              |
| Sample B       | 0.15                 | 4.03                        | <0.03     | 0.69      | 0.096        | 0.01         |
| Range          |                      |                             |           |           | 0.12 ± 0.05  | 0.01 ± 0.01  |

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**FIGURE 8.** PALcc(Y654F) lacks an absorption peak at 560 nm. The absorption spectra of PALcc(Y654F) and wild-type (WT) PALcc (both at 0.23 mg/ml) were compared; a separate preparation of PALcc(Y654F) (0.15 mg/ml) yielded similar data (not shown). Data from 350 to 800 nm are shown in the inset; a buffer blank analyzed at the same time was subtracted.
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assembly of metals in the active site of PAL and their catalytic roles.

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