Phenylalanine Hydroxylase from *Chromobacterium violaceum*

**UNCOPPELED OXIDATION OF TETRAHYDROPTERIN AND THE ROLE OF IRON IN HYROXYLATION**

**Dawei Chen and Perry A. Frey‡**

*From the Institute for Enzyme Research, The Graduate School and Department of Biochemistry, College of Agricultural and Biological Sciences, University of Wisconsin-Madison, Madison, Wisconsin 53705*

A gene encoding phenylalanine hydroxylase has been cloned from *Chromobacterium violaceum* and expressed in *Escherichia coli*. The purified phenylalanine hydroxylase contains copper, which does not support enzymatic activity. Upon removal of copper by dithiothreitol (DTT), the enzyme contains substoichiometric amounts of calcium and zinc but little or no redox-active metal ions. The copper-depleted hydroxylase catalyzes the phenylalanine-dependent oxidation of 6,7-dimethyltetrahydropterin (DMPH₄) by O₂ in a reaction in which phenylalanine is not hydroxylated and does not appear to undergo a chemical change, and hydrogen peroxide is produced. Analogs of phenylalanine also activate the oxidation of DMPH₄. Both the copper-phenylalanine hydroxylase and the copper-depleted hydroxylase catalyze the hydroxylation of phenylalanine in the presence of DTT and FeSO₄ in a reaction in which hydrogen peroxide is not produced. The apparent values of *Kₗ* for Fe²⁺ and DTT are 0.28 μM and 1.1 mM, respectively, at 1.0 mM phenylalanine, 120 μM DMPH₄ and pH 7.4 and 28 °C. The apparent value of *Kₗ* is 14.3 s⁻¹ under these conditions. Glutathione, mercaptoethanol, and dihydrolipoate support the hydroxylation of phenylalanine essentially as well as DTT. Incubation of copper-depleted hydroxylase with FeSO₄, phenylalanine, and DTT followed by gel permeation chromatography leads to an iron-hydroxylase containing approximately 1 molecule of iron per molecule of enzyme. The iron-hydroxylase displays an optical absorption band extending from 300 to 600 nm, and it catalyzes the hydroxylation of phenylalanine at the same maximum rate as the iron-activated hydroxylase but does not require added Fe²⁺. We conclude that iron participates in the hydroxylation of phenylalanine. Iron is not required for the oxidation of DMPH₄, although it may exert a modest acceleration effect. A hypothetical mechanism is presented wherein the reaction of iron with the putative 4a-hydroperoxy-DMPH₄ leads to 4a-hydroxy-DMPH₄ and a high valent iron-oxy species. The iron-oxy species is postulated to react with phenylalanine in the hydroxylation process...

---

Phenylalanine hydroxylases are tetrahydropterin-dependent monooxygenases that catalyze the hydroxylation of phenylalanine by dioxygen to produce tyrosine, with the concomitant two-electron oxidation of a tetrahydropterin cofactor. The overall transformations of the cofactor are illustrated in Scheme 1. A quinonoid form of the dihydropterin can be observed spectrophotometrically as an intermediate but spontaneously undergoes isomerization to the corresponding 7,8-dihydropterin. Recycling of the cofactor between its dihydro- and tetrahydroforms can be brought about by reducing agents such as DTT.

Phenylalanine hydroxylases exist in many species from bacteria to humans and play important roles in aromatic amino acid metabolism. A genetic deficiency in phenylalanine hydroxylase activity in humans results in phenylketonuria, which is associated with mental retardation during development, as well as behavioral disorders (1, 2). Phenylalanine hydroxylases purified from mammalian sources are homotetrameric proteins that require iron for activity (3, 4). The precise mechanism by which iron-containing phenylalanine hydroxylases use dioxygen to hydroxylate an aromatic substrate is not completely understood, despite investigations extending over more than two decades (5).

A phenylalanine hydroxylase purified from *Chromobacterium violaceum* is a monomeric protein that contains approximately one Cu(II)/molecule (6, 7). However, copper does not support activity (8). Amino acid sequence alignments reveal an overall identity of approximately 22% to the catalytic domains of mammalian phenylalanine hydroxylases (9), which suggests similarities in three-dimensional structure. Among the mechanistic questions surrounding this enzyme are whether a reox-active metal center is required for its activity, how the molecular oxygen is activated to form a hydroxylating intermediate, and whether it shares a common mechanism with the mammalian enzymes.

We here describe the cloning of a gene encoding a phenylalanine hydroxylase from *C. violaceum* and the expression of this gene in *Escherichia coli*. The phenylalanine hydroxylase activity displayed by this enzyme requires iron but not other transition metal ions. The iron-free enzyme catalyzes phenylalanine-dependent oxidation of the tetrahydropterin with concomitant formation of hydrogen peroxide but not hydroxylation of the substrate. Thus, the oxidation of DMPH₄ can be uncoupled from the hydroxylation of substrates by the exclusion of iron. A mechanism in outline is proposed, in which the reaction of iron with the putative 4a-hydroperoxy-DMPH₄ leads to a high valent iron-oxy species and 4a-hydroxy-DMPH₄, which undergoes dehydration to DMPH₄. The iron-oxy species is postulated to react with phenylalanine in the hydroxylation process.

**EXPERIMENTAL PROCEDURES**

*Materials—* *C. violaceum* strain ATCC 12540 was obtained from the American Type Culture Collection. HEPES, catalase, and DTT were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Inst. for Enzyme Research, Dept. of Biochemistry, College of Agricultural and Biological Sciences, University of Wisconsin-Madison, 1710 University Ave., Madison, WI 53705.

*This work was supported by Grant DK 26807 from the NIDDK, National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.*

*The abbreviations used are: DTT, dithiothreitol; DMPH₄, 6,7-dimethyltetrahydropterin; DMPH₃, 6,7-dimethyltetrahydropterin; PCR, polymerase chain reaction; HPLC, high performance liquid chromatography.*
from Sigma. L-Phenylalanine and DMPH were from Fluka. All solutions of buffer and substrates were freed of adventitious metal ions by passage through a column of Chelex-100 (Bio-Rad, 1.5 × 20 cm) and storage in disposable plastic containers. The solutions were analyzed by inductively coupled plasma emission spectroscopy to verify the absence of transition metal ions. Distilled water was further purified by use of a NANOpure (Barnstead) ultrapure water system. Restriction endonucleases and other enzymes used in cloning and nucleotide sequence analysis were from commercial suppliers as indicated. All other chemicals were analytical reagent or molecular biology grade from commercial suppliers.

**DNA Amplification and Cloning—** Two oligonucleotide primers, 5'-ACAGGTACCGGATGCAGGTCGAAGCCG-3' and 5'-GCCCTAGCTTCTTATGCGAGGCTATG-3', were synthesized based on the deoxyribonucleotide sequences flanking the coding region of the *C. violaceum* phenylalanine hydroxylase gene described previously (9). Each of these primers was designed to have a restriction endonuclease site (KpnI or HinIII, respectively, as indicated by the underlined sequences) at the 5'-primer end to facilitate cloning. *C. violaceum* genomic DNA was isolated and purified as described (10). PCR amplification was carried out by the method of Saiki et al. (11) with minor modifications. The amplification reaction mixture (100 µl) contained 0.5 µg of genomic DNA template, 50 pmol each of the two primers, 2.5 units of Taq DNA polymerase (Life Technologies, Inc.) in 10 mM Tris-HCl, pH 8.8, 0.2 mM each of the four dNTPs, 50 mM KCl, 2.0 mM MgCl₂, and 0.01% bovine serum albumin. Twenty-eight PCR cycles (94 °C for 0.5 min, 55 °C for 0.5 min, and 72 °C for 2 min) were performed using a Perkin-Elmer GeneAmp PCR System 2400 thermocycler. A portion of the reaction mixture was analyzed by 1% agarose gel electrophoresis. Candidate products were purified using a GeneClean II kit (Bio 101, Inc.), digested with endonucleases KpnI and HinIII (Promega), and cloned into the KpnI and HindIII sites in plasmid vector pBluescript II KS (Stratagene) using standard procedures (12). The correctly amplified DNA fragments from several clones containing the gene were confirmed by DNA sequencing using the dyeoxy chain termination method (13).

**Gene Expression and Protein Purification—** To express the cloned phenylalanine hydroxylase gene, the amplified KpnI/HindIII DNA fragment was subcloned behind the hybrid tac promoter (14) in an expression vector construction described by Chen and Swenson (15). *E. coli* strains transformed with the expression vector were grown at 37 °C in LB medium containing 100 µg/ml ampicillin in the absence or presence of isopropyl-β-D-thiogalactoside. Expression of the recombinant phenylalanine hydroxylase was analyzed by SDS-polyacrylamide gel electrophoresis (16). Purification of phenylalanine hydroxylase to homogeneity was accomplished essentially by the published procedure, with minor modifications (9). The most significant changes were that *E. coli* cells were lysed by a single passage of the sample through a French pressure cell at 12,000–15,000 p.s.i. instead of by treatment with detergent and lysozyme. The DEAE-Sepharacel (Amershams Pharmacia Biotech) column was eluted with a linear gradient formed with 500 ml of 50 mM sodium acetate buffer at pH 6.0 containing 20 µM NaCl and 500 ml of the same buffer containing 500 mM NaCl, instead of by the gradient originally described. The purified enzyme was stored at −70 °C after being frozen in liquid nitrogen.

**Metal Analysis and Preparation of Copper-free Enzyme—** Enzyme preparations were analyzed for metal content by inductively coupled plasma emission spectroscopy (Soil Sciences Lab, University of Wisconsin-Madison). All components of assay mixtures were also assayed to verify the absence of adventitious metal contaminants. Copper was removed from the enzyme by several concentration/dilution cycles of the holoprotein in a Centriprep-10 concentrator (Amicon) with 50 mM HEPES buffer at pH 7.4 containing 100 mM DTT. Gel filtration chromatography to remove the excess DTT was carried out as described (8).

**Protein Analysis—** The purity of phenylalanine hydroxylase was judged by inspection of Coomassie Blue-stained gels from SDS-polyacrylamide gel electrophoresis. Routine measurements of the concentrations of phenylalanine hydroxylase solutions were determined by use of the BCA protein assay reagent (Pierce). The value of ε₂₈₀ for *C. violaceum* phenylalanine hydroxylase was calculated from measurements of A₂₈₀ on several enzyme preparations and the molar enzyme concentrations of these solutions calculated from amino acid compositions as deduced from the DNA nucleotide sequence of the cloned gene. The value of ε₂₈₀ was found to be 6.1 × 10⁴ M⁻¹ cm⁻¹. The N-terminal amino acid sequence of the recombinant *C. violaceum* phenylalanine hydroxylase was determined at the Macromolecular Structure Facility at Michigan State University. The overall molecular weight of the purified recombinant phenylalanine hydroxylase was measured by electrospray ionization mass spectrometry to an accuracy of ± 0.01% at the Biotechnology Center of the University of Wisconsin-Madison.

**Assays of Phenylalanine Hydroxylase Activity—** The phenylalanine-dependent DMPH oxidation activity was measured by monitoring the ΔA₄₇₅ with time. Rates were calculated based on the value of ε₄₇₅ = 3600 M⁻¹ cm⁻¹ (8, 17). The reaction mixtures contained 100 mM HEPES at pH 7.4, 0.5 µM copper-depleted phenylalanine hydroxylase, 120 µM DMPH, and phenylalanine at various concentrations at 23 °C. Hydrogen peroxide formation in DMPH dehydrogenation assays was measured by the horseradish peroxidase-dependent oxidation of 4-methoxy-1-naphthol (18).

**Assays for tyrosine production** were conducted by spectrophotometric and HPLC methods. In the spectrophotometric method, the increase in A₄₇₅ because of tyrosine formation was measured continuously in reaction mixtures containing 100 mM HEPES at pH 7.4, 0.5 µM phenylalanine hydroxylase, 6.0 mM DTT, 1.0 mM phenylalanine, 120 µM DMPH, and FeSO₄ at various concentrations at 23 °C. The rates were calculated assuming the value of ε₄₇₅ = 1700 M⁻¹ cm⁻¹ as described (7). In the spectrophotometric assays, the absorbance changes were measured using Hewlett-Packard model 8452A diode array spectrophotometer, equipped with a thermostatically controlled cuvette holder. In the HPLC assays, tyrosine formation was observed by timed point assays in reaction mixtures containing 100 mM HEPES at pH 7.4, 0.5 µM phenylalanine hydroxylase, 1.0 mM phenylalanine, and 120 µM DMPH, in the absence or presence of 6.0 mM DTT and/or 1.0 mM FeSO₄ at 23 °C. Timed aliquots were analyzed for tyrosine with a 0.21 × 25 cm C-18 reverse phase HPLC column eluted isocratically, using 80% acetonitrile and 0.1 mM sodium acetate at pH 6.4 containing 2.58 mM triethylamine. Dioxegen consumption was measured using a model 53 oxygen electrode (Yellow Springs Instrument Co.) calibrated with protocatechuate 3,4-dioxygenase and 3,4-dihydroxybenzoic acid. The reaction mixtures consisted of 100 mM HEPES at pH 7.4, 0.5 µM phenylalanine hydroxylase, 1.0 mM phenylalanine, and 120 µM DMPH at 23 °C in the DMPH oxidation reaction, that is, in DMPH oxidation uncoupled from phenylalanine hydroxylase. Dioxygen consumption in phenylalanine hydroxylation was measured in reaction mixtures containing 100 mM HEPES at pH 7.4, 0.5 µM phenylalanine hydroxylase, 6.0 mM DTT, 120 µM DMPH, and various concentrations of phenylalanine and FeSO₄ at 23 °C.

**Reconstitution of Phenylalanine Hydroxylase with Iron—** Reconstitution of phenylalanine hydroxylase with iron was carried out by incubating the metal-depleted phenylalanine hydroxylase with a stoichiometric excess of FeSO₄ in the presence of l-phenylalanine and DTT in 50 mM HEPES buffer at pH 7.4 and 0 °C for 30 min, followed by gel filtration chromatography to remove excess ligands. The reconstituted protein was then concentrated at 4 °C in a Centricron-10 concentrator (Amicon). Iron-containing phenylalanine hydroxylase with high activity was also obtained by adding FeSO₄ to the cell extract during the purification of the enzyme.

**RESULTS AND DISCUSSION**

**Gene Cloning and Expression—** A 1.2-kilobase DNA fragment was amplified by PCR from *C. violaceum* genomic DNA using the two oligonucleotide primers described under "Experimental Procedures." Nucleotide sequence analysis confirmed that this fragment contained an open reading frame that was similar to that reported to encode phenylalanine hydroxylase in this organism (9). Both strands of the fragment were sequenced, and 7-deaza-dGTP and dITP were used to expand GC-compressed regions. The nucleotide sequence within the open reading frame was found to be identical to that previously reported (9) from positions 1–690. The analysis revealed the presence of a C at position 691, a G at position 692, and a C at position 1000, which were absent from the published sequence. (A postulated
insertion of G at position 701 (19) was not confirmed by the present analysis.) These differences result in an altered amino acid sequence between residues 171 and 274, as compared with the published sequence (9).

The newly isolated gene contains an 894-base pair open reading frame, which encodes a polypeptide of 297 amino acids. The calculated molecular mass of the polypeptide is 33613.4 Da. The codon usage is uniform throughout the gene and displays a preference for G or C at the third base; 90.2% of the codons contain one of these bases at the third position. More than 90% of the amino acids of the C. violaceum phenylalanine hydroxylase are encoded by only 31 codons because of this biased codon selection.

The translated amino acid sequence of the gene described here is homologous with those of the liver and Pseudomonas aeruginosa phenylalanine hydroxylases. The amino acid sequence of C. violaceum phenylalanine hydroxylase (Cv) is aligned with those of the catalytic domains of phenylalanine hydroxylases from human (Hs) and rat (Rn) liver and Drosophila melanogaster (Dm). Sequences were aligned by use of the Pileup program from the GCG package (Genetics Computer Group, Inc.). The potential pterin-binding regions (29) are underlined, and asterisks designate the amino acid residues that are conserved in all four sequences. Dots indicate gaps inserted to optimize the alignments.

The amplified gene was placed behind the hybrid tac promoter of an expression vector for E. coli. Among several E. coli strains transformed, high level expression was observed in strain DH5α without induction by isopropyl-β-D-thiogalactoside. The expressed phenylalanine hydroxylase was purified to homogeneity essentially by the published procedure (9). Approximately 15 mg of purified enzyme was obtained per liter of cell culture grown to stationary phase. Evidence that the purified protein is the cloned C. violaceum phenylalanine hydroxylase is based on N-terminal amino acid sequence analysis and the molecular mass of the purified protein determined at high resolution. The N-terminal sequence was found to be NDRADFV, which was in agreement with the sequence translated from the nucleotide sequence of the gene except for the absence of N-terminal methionine. Apparently the methionine was removed in a post-translational modification, perhaps by the methionine-specific aminopeptidase of E. coli (20). The molecular mass of the recombinant phenylalanine hydroxylase was found by electrospray mass spectrometry to be 33487.7 ± 1.7 and 33,482.9 ± 1.6 Da in two determinations on samples that had been prepared to remove metal ions. These values are virtually identical and agree very well with the molecular mass calculated based on the cloned DNA nucleotide sequence (33613.4), after subtracting the mass of the N-terminal methionine (131.2 Da). The measured mass is not in agreement with that of the polypeptide encoded by the gene originally described (9).

Metal Content of Various Forms of Phenylalanine Hydroxylase from C. violaceum—Analyses of the purified recombinant
phenylalanine hydroxylase by inductively coupled plasma emission spectroscopy, shown in Table I, indicated the presence of copper and zinc as reported for the enzyme described previously. An approximate 1:1 stoichiometry of (copper + zinc): hydroxylase was consistently observed when CuSO₄ was added to the cell extract at an early stage of purification. The copper bound to the enzyme could be removed by extraction with DTT, followed by removal of excess DTT by gel filtration chromatography, as described previously. The copper-depleted hydroxylase contains calcium and zinc but few redox-active divalent metal ions (Table I). Zinc-free phenylalanine hydroxylase can be prepared by incubating the protein with excess CuSO₄ to replace zinc with copper and then removing the copper by DTT extraction. These observations are consistent with previous reports on phenylalanine hydroxylase from C. violaceum (7, 9).

Uncoupled DMPH₄ Oxidase Activity of Phenylalanine Hydroxylase—The copper-depleted hydroxylase catalyzes the phenylalanine-dependent oxidation of DMPH₄ by O₂ in reaction mixtures containing 90–100 mM HEPES buffer, copper-depleted hydroxylase, DMPH₄, and phenylalanine. Although the phenylalanine requirement was absolute, as shown in Fig. 2, no tyrosine formation could be detected by the spectrophotometric or HPLC assays for tyrosine. Absorbance changes during the course of the reaction were consistent with the formation of DMPH₂ but not tyrosine, as shown in Fig. 3. If tyrosine had been produced, the absorbance increase at 275 nm would have been much greater. The phenylalanine analogs 3-cyclohexyl-L-alanine and D-phenylalanine also promoted the oxidation of DMPH₄, as judged from their unchanged reverse phase HPLC chromatographic behavior. One mole of O₂ was consumed for each mole of DMPH₄ oxidized in these uncoupled DMPH₄ oxidations. The kinetic parameters derived from the data in Fig. 2 are $K_{\text{m,app}} = 2.29 \pm 0.74$ mM and $k_{\text{cat,app}} = 0.96 \pm 0.30$ s⁻¹. Under the same conditions, assays by O₂ consumption yielded values of $K_{\text{m,app}} = 2.63 \pm 0.98$ mM and $k_{\text{cat}} = 1.05 \pm 0.38$ s⁻¹.

Hydrogen peroxide was detected as a product of tetrahydropterin oxidation by copper-depleted phenylalanine hydroxylase when reaction mixtures lacking ferrous ion were assayed by use of the horseradish peroxidase-catalyzed oxidation of 4-methoxy-1-naphthol. Typical data are given in Table II, which shows that hydrogen peroxide formation is maximal in the absence of ferrous ions and DTT. It will be shown in a later section that the hydroxylation of phenylalanine requires ferrous iron and DTT, which presumably couple the oxidation of DMPH₄ by dioxygen to the hydroxylation of substrates. Experiment 1 was a complete hydroxylation reaction in which no H₂O₂ was produced. In Experiment 2 only enzyme and phenylalanine were present, and no H₂O₂ was produced. In Experiment 3 the exclusion of enzyme, DTT, and ferrous iron led to rous iron and DTT, which presumably couple the oxidation of DMPH₄ by dioxygen to the hydroxylation of substrates. Experiment 1 was a complete hydroxylation reaction in which no H₂O₂ was produced. In Experiment 2 only enzyme and phenylalanine were present, and no H₂O₂ was produced. In Experiment 3 the exclusion of enzyme, DTT, and ferrous iron led to

---

**Table I**

| Metal       | Copper-hydroxylase | Copper-depleted hydroxylase | Iron-reconstituted hydroxylase |
|-------------|--------------------|-----------------------------|-------------------------------|
| Calcium     | 7.0                | 22.5                        | 4.1                           |
| Chromium    | 0.13               | <0.11                       | 0.01                          |
| Manganese   | <0.01              | 0.01                        | 0.43                          |
| Iron        | <1.6               | <1.2                        | 118                           |
| Cobalt      | <0.01              | <0.01                       | 0.02                          |
| Nickel      | 0.27               | 0.52                        | 0.49                          |
| Copper      | 79.9               | 0.20                        | 1.1                           |
| Zinc        | 24.6               | 28.1                        | 3.9                           |
| Myoglobin   | <0.01              | <0.01                       | <0.01                         |

*a Copper was removed by extraction with DTT followed by gel permeation chromatography.

*b Phenylalanine hydroxylase was reconstituted by incubating copper-depleted phenylalanine hydroxylase with FeSO₄, phenylalanine, and DTT, followed by gel permeation chromatography.
significant H₂O₂ production, because of nonenzymatic oxidation of DMPH₄. In Experiment 4 the exclusion of phenylalanine, DTT, and ferrous iron resulted in a small amount of H₂O₂, less than the nonenzymatic control (Experiment 3), indicating that phenylalanine hydroxylase partially protected DMPH₄ from nonenzymatic oxidation. The most H₂O₂ was produced in Experiment 5, which included enzyme, phenylalanine, and DMPH₄, as well as O₂ from the air. These were the components for the uncoupled enzymatic oxidation of DMPH₄.

The H₂O₂ produced in Experiment 5 was stoichiometrically less than the amount of DMPH₄ initially present, and the reaction was essentially complete. Substoichiometric H₂O₂ production was confirmed in other experiments. The imperfect stoichiometry was attributed to side reactions of H₂O₂. A side reaction that we verified in separate experiments was the nonenzymatic oxidation of DMPH₄ by H₂O₂. Under conditions comparable with the enzymatic reaction in Experiment 5 with the exclusion of enzyme, 100 μM H₂O₂ nonenzymatically oxidized DMPH₄ to a significant extent. Most of the apparent deficit in H₂O₂ production in Experiment 5 could be attributed to this side reaction.

The formation of hydrogen peroxide in the absence of hydroxylation is consistent with the intermediate formation of the pterin-4a-hydroperoxide adduct as the initial pterin-O₂ species (21). However, the observations of 1:1 DMPH₄/O₂ stoichiometry and H₂O₂ production in the uncoupled oxidation of DMPH₄ are contrary to what was observed in the uncoupled tetrahydropterin oxidation catalyzed by rat liver phenylalanine hydroxylase with p-chlorophenylalanine as the substrate, in which two tetrahydropterins were oxidized per O₂ consumed, and no H₂O₂ formation was apparent (22). These discrepancies suggest that either the decomposition of the pterin-4a-OOH species followed different mechanisms when the uncoupling was triggered under different conditions or the O₂ itself was activated by different mechanisms.

**Requirement for Iron in the Hydroxylase Activity—**Both the copper-containing phenylalanine hydroxylase and the copper-depleted hydroxylase catalyze the tetrahydropterin-dependent hydroxylation of phenylalanine when catalase and DTT are included in the reaction mixtures. However, the effect of catalase in promoting phenylalanine hydroxylation can be replaced by FeSO₄. Fig. 4 shows the concentration dependence of the FeSO₄-dependent phenylalanine hydroxylation catalyzed by the copper-containing and copper-depleted hydroxylase in the absence of catalase. The values of Kₕₚ₈ for FeSO₄ are 0.27 ± 0.01 and 0.30 ± 0.03 μM for the copper-containing and copper-depleted phenylalanine hydroxylase, respectively, under the conditions of Fig. 4. The values of kₕₚ₈ are 14.5 ± 0.6 and 14.1 ± 1.0 s⁻¹, respectively. Specific activities in the absence or presence of iron for both types of phenylalanine hydroxylase are given in Table III. No hydrogen peroxide could be detected under these conditions, whereas the amount of O₂ consumed, as calculated from the rate of O₂ uptake, was comparable with that of tyrosine produced. When the rate of the iron-dependent hydroxylation of phenylalanine was measured with O₂ consumption, it was found to be the same as the rate of tyrosine production, that is kₕₚ₈ = 15.0 ± 2.4 s⁻¹. These results prove that substrate hydroxylation catalyzed by the C. violaceum phenylalanine hydroxylase described here requires iron. The specific activities reported earlier for tyrosine formation were 12.2 units mg⁻¹ for the copper-containing enzyme and 11.6 units mg⁻¹ for the copper-depleted enzyme (8), somewhat lower values than those in Table III for the iron-reconstituted enzyme prepared in this laboratory.

Catalase, the use of which was initially suggested in traditional hydroxylation assays (23), does not stimulate hydroxylation when FeSO₄ is included in reaction mixtures. The requirement for iron in substrate hydroxylation and the
uncoupling of hydroxylation from tetrahydropterin oxidation in the absence of iron indicate that iron participates in the hydroxylation process but is not required for the oxidation of tetrahydropterins. In the absence of iron, the release of hydrogen peroxide could arise by its elimination from the putative intermediate 4a-hydroperoxy-DMPH₄, resulting in the direct formation of a dihydropterin according to Scheme 2, without the intermediate formation of 4a-hydroxy-DMPH₄. In the absence of iron, its reaction with 4a-hydroperoxy-DMPH₄ could lead to 4a-hydroxy-DMPH₄ and a high valent iron-oxy species.

The iron-oxy species may react with phenylalanine in the hydroxylation process according to Scheme 3 to produce tyrosine. The direct oxidation of tetrahydropterin to the corresponding quinonoid dihydropterin was observed in the uncoupled tetrahydropterin oxidation catalyzed by rat liver phenylalanine hydroxylase (22), consistent with the above interpretations.

Addition of Cu²⁺ to assay mixture in the absence of Fe²⁺ does not promote hydroxylation of phenylalanine, nor did other redox-active transition metal ions, including Cr³⁺, Mn²⁺, Co²⁺, and Ni²⁺ (data not shown). The presence of high concentrations of Cu²⁺ in assay mixtures exerted only a modest inhibition effect when Fe²⁺ and DTT were present, indicating that Cu²⁺ was neither required for hydroxylation nor acting as an effective inhibitor.

**The Thiol Requirement for Hydroxylation**—In addition to iron, both the copper-containing and the copper-depleted phenylalanine hydroxylases display an absolute requirement for a thiol such as DTT in the hydroxylation reaction. No tyrosine formation could be detected in the absence of DTT, even in prolonged incubations and in the presence of high concentrations of DMPH₄. The effect of increasing DTT concentrations on the rate of phenylalanine hydroxylation catalyzed by the copper-depleted hydroxylase in the presence of FeSO₄ was hyperbolic. The apparent value of $K_m$ for DTT was found to be 1.1 ± 0.1 mM in assay mixtures containing 100 mM HEPES at pH 7.4, 0.5 mM hydroxylase, 1.0 mM phenylalanine, 120 μM DMPH₄ and 1.0 mM FeSO₄ at 25 °C. Glutathione, mercaptoethanol, and dihydrolipoate supported the hydroxylation as well as DTT, with apparent $K_m$ values of 2.2 ± 0.3, 1.4 ± 0.1, and 1.5 ± 0.2 mM, respectively, under the same conditions.

The absence of hydroxylation activity in the absence of a thiol compound cannot be explained by decreased recycling of DMPH₄ during turnover because initial rates were measured, and no tyrosine was produced even in assays with DMPH₄ concentrations as high as 1.0 mM. The results indicated that DTT played a mechanistic role in addition to recycling the oxidized pterin to its reduced state during the catalytic turnover. Reductive activation by a cofactor or dithionite to form the catalytically active enzyme species was reported for the phenylalanine hydroxylase from rat liver (24, 25). Spectroscopic studies linked the reductive activation to the conversion of iron bound to the enzyme from Fe(III) to Fe(II) (25).

The thiol compounds required for the C. violaceum phenylalanine hydroxylase-catalyzed hydroxylation may play a role in maintaining the active site in a reduced state that is necessary for catalytic activity. However, the specific site and nature of this postulated reduction has yet to be determined. Further studies will be required to elucidate the complete role of the thiol compound in the phenylalanine hydroxylase-catalyzed hydroxylation.

**Reconstitution with Iron**—Incubation of copper-depleted phenylalanine hydroxylase with FeSO₄, phenylalanine, and DTT followed by gel permeation chromatography leads to an iron-hydroxylase containing approximately one iron molecule per molecule of enzyme (Table I). The iron-hydroxylase displays a visible absorption band extending from 300 to 600 nm, as shown in Fig. 5, in addition to the prominent UV absorption band due to the protein. The iron-phenylalanine hydroxylase catalyzes the hydroxylation of phenylalanine at the same maximum rate as the ferrous activated phenylalanine hydroxylase but does not require added FeSO₄ (Table III). The absence of any distinctive electronic transition in the visible wavelength region indicates that the bound iron is a non-heme iron. Because no distinctive ligand to metal charge transfer bands in the 420–550 nm region characteristic of thiolate or phenolate ligands are present (26, 27), the absorption spectrum gives no indication of the presence of cysteine or tyrosinate coordination in the iron-binding sites. The UV-visible absorption spectrum of iron-phenylalanine hydroxylase from C. violaceum is very similar to that reported for the mammalian hydroxylase (24).

Addition of FeSO₄ to the cell extract in the course of purifying the recombinant phenylalanine hydroxylase from C. violaceum also led to the iron-phenylalanine hydroxylase as the purified protein. Although ammonium sulfate precipitation of the protein following the addition of FeSO₄ caused some loss of bound iron, the addition of FeSO₄ followed by gel permeation chromatography restored the iron content. The iron-phenylalanine hydroxylase purified with supplemental FeSO₄ exhibits the same specific activity in hydroxylation of phenylalanine as the ferrous-activated or iron-reconstituted enzyme but without the need for added FeSO₄.

**Possible Role of Histidine in Binding Iron**—The amino acid sequence spanning residues 111–160 of the C. violaceum phenylalanine hydroxylase is 40% identical with that of the aligned sequence of the human enzyme. Similarities within this region extend to approximately 60% when conservative replacements are considered. Histidine residues 138 and 143 of the C. violaceum sequence are conserved in the rat, human, Drosophila, and P. aeruginosa phenylalanine hydroxylases. Variants of the previously described C. violaceum hydroxylase lacking either His¹³⁸ or His¹⁴³ were found to be inactive but retained the capacity to bind copper (28). Site-directed mutagenesis of either of the corresponding histidine residues in the rat liver enzyme to serine resulted in a protein that lacked both iron and enzymatic activity (29).

Addition of Fe²⁺ and DTT failed to restore activity to the variant proteins, suggesting that these two histidine residues may participate in binding iron. The activity loss upon replacement of these two histidines correlated with a possible loss of iron binding capacity. The amino acid sequences conserved between C. violaceum and mammalian phenylalanine hydroxylases and the similar electronic absorption spectra of the iron-containing proteins suggest that they share a common iron coordination environment. Of further interest, these two conserved histidine residues are located within the potential pterin-binding domain identified by Jennings et al. (30), suggesting that the iron-binding site...
Mechanism of Oxygenation by Phenylalanine Hydroxylase—
The present results show that substrate-dependent oxidation of
DMPH₄ by phenylalanine hydroxylase does not require iron
and does not involve the hydroxylation of the substrate. In
contrast, the hydroxylation of substrates requires iron. These
facts, together with the cofactor requirements for hydroxyla-
tion, lead us to suggest Scheme 3 as a unification of the DMPH₄
oxidation mechanism with that of hydroxylation. In this mech-
anism, O₂ reacts with DMPH₄ to produce 4a-hydroperoxy-
DMPH₄ as an intermediate, which in turn reacts with Fe²⁺
to
produce a high valent oxy-iron species (Fe⁴⁺=O) and 4a-hy-
droxy-DMPH₄. The oxy-iron species hydroxylates the sub-
strate, whereas 4a-hydroxy-DMPH₄ undergoes dehydration to
DMPH₂ and water. In the case of tyrosine hydroxylase, evi-
dence has been presented in support of an electrophilic hy-
droxylation mechanism (31). An oxy-iron species is potentially
an electrophilic hydroxylation intermediate.

The iron-containing liver phenylalanine hydroxylase also
may be in the vicinity of the pterin-binding site.

Mechanism of Oxygenation by Phenylalanine Hydroxylase—
The present results show that substrate-dependent oxidation of
DMPH₄ by phenylalanine hydroxylase does not require iron
and does not involve the hydroxylation of the substrate. In
contrast, the hydroxylation of substrates requires iron. These
facts, together with the cofactor requirements for hydroxyla-
tion, lead us to suggest Scheme 3 as a unification of the DMPH₄
oxidation mechanism with that of hydroxylation. In this mech-
anism, O₂ reacts with DMPH₄ to produce 4a-hydroperoxy-
DMPH₄ as an intermediate, which in turn reacts with Fe²⁺
to
produce a high valent oxy-iron species (Fe⁴⁺=O) and 4a-hy-
droxy-DMPH₄. The oxy-iron species hydroxylates the sub-
strate, whereas 4a-hydroxy-DMPH₄ undergoes dehydration to
DMPH₂ and water. In the case of tyrosine hydroxylase, evi-
dence has been presented in support of an electrophilic hydroxylation mechanism (31). An oxy-iron species is potentially
an electrophilic hydroxylation intermediate.

The iron-containing liver phenylalanine hydroxylase also
displays fully uncoupled oxidation of tetrahydropterin under a
different condition, that is, in the presence of tyrosine in place
of phenylalanine (32). Presumably, the hydroxylation reaction
is diverted to H₂O₂ production in the presence of the hydroxy-
lated product. In the present work, the iron-free C. violaceum
enzyme catalyzes the oxidation of DMPH₄ by dioxygen to form
DMPH₂ and H₂O₂, proving that iron is not required for this
reaction. The iron-containing enzyme from C. violaceum reacts
more rapidly with dioxygen and DMPH₄ in the hydroxylation of
phenylalanine than does the iron-free enzyme in the uncoupled
oxidation of DMPH₄. Therefore, iron could accelerate the reac-
tion of dioxygen, but it is not essential for the oxidation of
DMPH₄.

ADDENDUM

The structure of the gene encoding the C. violaceum phenyl-
alanine hydroxylase described here differs from that reported
earlier from the laboratory of Prof. S. J. Benkovic (9). The
properties of the purified enzyme also differ from those de-
scribed earlier (7, 8, 33). Prof. Benkovic has compared our
results with those from his laboratory and concluded that two
phenylalanine hydroxylases are present in C. violaceum. His
personal communication follows.

The C. violaceum phenylalanine hydroxylase studied previously
has a completely different amino acid sequence after position 172 and
also is 1 amino acid shorter, 296 versus 297, than that described here.
We not only sequenced the plasmid in both strands but also deter-
mined the amino acid sequence at the N- and C-terminal ends (9).
This sequence difference in the region of amino acids 172–272 would
explain most if not all of the differences in the kinetic properties
reported by us and in this paper.

Nevertheless, Drs. Jae Hoon Shim and Zhigang Wang reexamined
the key results reported in Refs. 7 and 8 and reconfirmed: 1) in the
absence of DTT and catalase, there was 20% coupling of the pterin
oxidation to tyrosine formation; 2) in the presence of DTT with metal
free phenylalanine hydroxylase (0.04 iron, 0.0 copper, and 0.2 zinc
equivalents) full coupling of O₂ consumption to tyrosine production
was observed; and 3) Cu²⁺ and Zn²⁺ at μM levels strongly inhibited
“metal free” phenylalanine hydroxylase. The addition of Fe²⁺ up to 1
equivalent gave no change in specific activity; at higher levels (>2
equivalents) a higher specific activity was found that plateaued at a
ratio of 5 iron/hydroxylase. At all iron levels the recovered enzyme
was found to have less than 0.2 equivalent of iron incorporated.
In conclusion, this enzyme does not bind iron with a high affinity,
and the formation of tyrosine is not a consequence of the trace iron
present. The removal of Zn²⁺ and Cu²⁺ from the active site is essen-
tial for activity. Fe²⁺ may act as an activator in an undefined mech-
anism but is not an integral part of the enzyme. Two pathways, one
metal ion-independent and the other metal ion-dependent, appear to
exist for the hydroxylation process.
Role of Iron in the Reaction of Phenylalanine Hydroxylase

REFERENCES

1. Shimam, R. (1985) in *Polates and Pyrines* (Blakely, R. L., and Benkovic, S. J., eds) Vol. 2, pp. 179–249, Wiley Interscience, New York
2. Goodman, B. L. (1979) in *Aromatic Amino Acid Hydroxylases and Mental Disease* (Youdin, M. B. H., ed) pp. 5–79, Wiley Interscience, New York
3. Fisher, D. B., and Kaufman, S. (1972) *J. Biol. Chem.* 247, 2250–2252
4. Gottschall, D. W., Dietrich, R. F., Benkovic, S. J., and Shimam, R. (1982) *J. Biol. Chem.* 257, 845–849
5. Kappock, T. J., and Caradonna, J. P. (1996) *Chem. Rev.* 96, 2659–2756
6. Nakata, H., Yamauchi, T., and Fujisawa, H. (1979) *J. Biol. Chem.* 254, 1892–1933
7. Pember, S. O., Villafranca, J. J., and Benkovic, S. J. (1987) *Methods Enzymol.* 142, 50–56
8. Carr, R. T., and Benkovic, S. J. (1993) *Biochemistry* 32, 14131–14138
9. Ouishi, A. Littta, L. J., and Benkovic, S. J. (1991) *J. Biol. Chem.* 266, 18454–18459
10. Schleif, R. F., and Wensink, P. C. (1981) *Practical Methods in Molecular Biology*, pp. 98–99, Springer-Verlag, New York
11. Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K., and Ehrlich, H. A. (1988) *Science* 239, 487–490
12. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
13. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U. S. A.* 74, 5463–5467
14. Amman, E., Brosius, J., and Ptashne, M. (1983) *Gene (Amst.*) 25, 167–178
15. Chen, D., and Swenson, B. P. (1994) *J. Biol. Chem.* 269, 32120–32130
16. Laemmli, U. K. (1970) *Nature* 227, 680–685
17. Ayling, J., Pirson, R., Pirson, W., and Boehm, G. (1973) *Anal. Biochem.* 51, 80–90
18. Guilbaud, G. G., and Kramer, D. M. (1964) *Anal. Chem.* 36, 2485–2500
19. Zhao, G., Xin, T., Song, J., and Jensen, R. A. (1994) *Proc. Natl. Acad. Sci. U. S. A.* 91, 1366–1370
20. Vogt, V. M. (1970) *J. Biol. Chem.* 245, 4760–4769
21. Dix, T. A., and Benkovic, S. J. (1988) *Acc. Chem. Res.* 21, 101–107
22. Dix, T. A., and Benkovic, S. J. (1985) *Biochemistry* 24, 5839–5845
23. Kaufman, S. (1962) *Oxygenases* (Hayashi, I., ed) pp. 129–180, Academic Press, New York
24. Marota, J. J. A., and Shimam, R. (1984) *Biochemistry* 23, 1303–1311
25. Wallick, D. E., Bloom, L. M., Gaffney, B. J., and Benkovic, S. J. (1984) *Biochemistry* 23, 1295–1304
26. Ainscough, E. W., Brodie, A. M., Plwman, J. E., Brown, K. L., Addison, A. W., and Gainsford, A. R. (1988) *Inorg. Chem.* 19, 3655–3663
27. Maesil, E. E., Millar, M. M., and Koch, S. A. (1992) *Inorg. Chem.* 31, 4594–5600
28. Balasubramanian, S., Carr, R. T., Bender, C. J., Peisach, J., and Benkovic, S. J. (1994) *Biochemistry* 33, 8532–8537
29. Gibb, R. S., Wojchowski, D., and Benkovic, S. J. (1993) *J. Biol. Chem.* 268, 8046–8052
30. Jennings, I. G., Kemp, B. E., and Cotton, R. G. H. (1991) *Proc. Natl. Acad. Sci. U. S. A.* 88, 5734–5738
31. Hilla, F. J., and Pitzpatrick, P. P. (1996) *Biochemistry* 35, 6969–6975
32. Davis, M. D., and Kaufman, S. (1993) *Arch. Biochem. Biophys.* 304, 9–16
33. Pember, S. O., Villafranca, J. J., and Benkovic, S. J. (1986) *Biochemistry* 25, 6611–6619