An Examination of the Copper Requirement of Phenylalanine Hydroxylase from *Chromobacterium violaceum*†

Robert T. Carr and Stephen J. Benkovic*

Department of Chemistry, The Pennsylvania State University, University Park, Pennsylvania 16802

Received June 7, 1993; Revised Manuscript Received September 20, 1993®

ABSTRACT: Phenylalanine hydroxylase from *Chromobacterium violaceum* (CVPAH) was classified as a copper metalloenzyme by virtue of a 1/1 Cu/enzyme stoichiometry and its inhibition with various chelators [Pember, S. O., Villafranca, J. J., & Benkovic, S. J. (1986) *Biochemistry 25*, 6611]. We have prepared "copper-free" CVPAH by extraction with DTT. These preparations retained full activity though the Cu/enzyme ratio averaged 0.015. Reconstitution by extraneous copper was disproved by measuring a Cu/enzyme ratio of 0.09 in assay mixtures after the specific activity was determined to be within 85% of a fully copper-complexed control. Several copper chelators were examined and were not inhibitory. The "copper-free" enzyme had significant activity without a thiol or other reducing agent capable of reducing the copper center whereas copper-complexed CVPAH had minimal activity under these conditions. Copper-complexed CVPAH can be activated, however, by nonreducing copper ligands such as imidazole. From these results, we conclude that copper is not a requirement of activity. Iron, cobalt, nickel, manganese, molybdenum, and chromium were not found in the "copper-free" preparation, indicating that the active hydroxylating species may not require a redox-active metal. The K_ds for binding Cu^{2+} and Zn^{2+} were measured to be 0.48 and 0.85 μM, respectively. Both copper and zinc were found to be potent inhibitors of "copper-free" CVPAH in the absence of thiols. DTT reverses inhibition due to Cu^{2+} but not inhibition caused by Zn^{2+}. The product stoichiometry indicates the same tightly coupled turnover found with all other pterin-dependent hydroxylases when using natural substrates. The relevance of these results to mammalian pterin-requiring hydroxylases is discussed in terms of the metal requirement for the formation of active oxygen intermediates.

Phenylalanine hydroxylase (PAH)[1] (phenylalanine 4-monooxygenase) (EC 1.14.16.1) catalyzes the formation of tyrosine from phenylalanine and molecular oxygen concomitant with the hydroxylation of a tetrahydropterin cofactor to a 4a-hydroxytetrahydropterin. The mammalian form of the PAH enzyme is the best characterized member of the family of pterin-dependent monooxygenases that includes tyrosine hydroxylase and tryptophan hydroxylase. All of the mammalian forms of these enzymes have iron as a requirement for activity (Fisher et al., 1972; Gottschall et al., 1982; Dix et al., 1985; Kuhn & Lovenberg, 1985). The bacterial forms have not been extensively characterized, but iron has been implicated in the *Pseudomonas* PAH (Shiman, 1985) and copper in the PAH isolated from *Chromobacterium violaceum* (CVPAH) (Pember et al., 1986). It is thought that for activity the metal ion is required to be in its lower oxidation state (Fe^{2+}, Cu^{+}) (Wallick et al., 1984; Marota & Shiman, 1984; Pember et al., 1986).

Many hydroxylases fall into one of three general categories based on how they react with triplet oxygen to form an activated oxygen intermediate. In the flavin-dependent monooxygenases, this activated oxygen intermediate has been confirmed as the flavin 4a-hydroperoxide (Massey & Hemmerich, 1976, Beaty & Ballou, 1980), and no metal is required for either oxygen binding or activation. This is in contrast to the heme-containing cytochrome P-450 hydroxylases which contain no other organic cofactor, but instead activate oxygen by the binding of oxygen to the heme–iron center. The oxygen molecule then cleaves, forming an intermediate thought to be a hypervalent perferryl, (Fe^{V}—O), ion that is the hydroxylating species (White & Coon, 1980; Guengerich, 1990). In the pterin-dependent hydroxylases, the third class, neither a pterin hydroperoxide nor a metal oxo species, has been confirmed or excluded as the activated oxygen intermediate (Dix & Benkovic, 1988).

The actual role of the metal center in these latter hydroxylases has not been delineated, although several hypotheses have been proposed, including oxygen binding and electron transfer. Oxygen could bind to both the metal and cofactor, forming a (PAH–Fe^{2+})(O_2)(tetrahydropterin) μ-oxo-bridged structure which has been inferred from experiments that uncouple substrate hydroxylation from pterin oxidation in RLPAH (Dix & Benkovic, 1985). Oxygen binding to the copper in CVPAH has also been inferred from kinetic data that show oxygen as the first substrate to bind to the enzyme in an ordered mechanism (Pember et al., 1989). Any electron transfer must be cyclic since there is no requirement for reducing equivalents over those that are provided by phenylalanine and pterin, which provide two electrons each. Studies have suggested a role for the copper center of CVPAH in pterin binding (Pember et al., 1987a), and a role for iron in activity regulation in the mammalian PAH (Shiman & Jefferson, 1982).

† Supported by NSF Grant DMB-8614179 to S.J.B.

* To whom correspondence should be addressed.

® Abstract published in *Advance ACS Abstracts,* December 1, 1993.

[1] Abbreviations: PAH, phenylalanine hydroxylase; CVPAH, *Chromobacterium violaceum* phenylalanine hydroxylase; RLPAH, rat liver phenylalanine hydroxylase; ESEEM, electron spin echo envelope modulation; EXAFS, extended X-ray absorption fine structure; ESR, electron spin resonance; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; DTT, dithiothreitol; 7,8-$DMPH_2$, 6,7-dimethyl-7,8-dihydropterin; $DMPH_4$, 6,7-dimethyltetrahydropterin; $6MPH_4$, 6-methyltetrahydropterin; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetic acid; TRIS, tris(hydroxymethyl)aminomethane; HPLC, high-performance liquid chromatography; SDS, sodium dodecyl sulfate; BSA, bovine serum albumin.

0006-2960/93/0432-14132$04.00/0

To address the mechanistic question of metal ion function in pterin-requiring hydroxylases, we have focused on the copper center of the PAH from *Chromobacterium violaceum*. Pember et al. (1986) classified this hydroxylase as a copper metalloenzyme based on a 1/1 Cu–enzyme stoichiometry and its inhibition by various chelating agents. Further studies based on ESEEM (McCracken et al., 1988) and EXAFS (Blackburn et al. 1992) demonstrated that two histidines in the enzyme were important copper ligands. ESR spectra with [5-^{15}N]-6-methyltetrahydropterin implicated the pterin cofactor as a ligand (Pember et al., 1987a). Several basic questions concerning the copper center of CVPAH, however, have yet to be addressed. Specifically, what is the redox potential of the copper center, what is the binding affinity of the enzyme for copper in its two oxidation states, and why is activity stimulated instead of inhibited by thiols, which are potent Cu^+ chelators?

The present study was initiated to answer these questions; however, the results of a variety of experiments implied that the classification of CVPAH as a copper metalloenzyme might have been premature. To the contrary, the results presented here do not support a catalytic role of the copper center, but suggest it may perform a regulatory or protective function.

MATERIALS AND METHODS

Materials. Water in these experiments was obtained from a Milli Q (WATERS) system and had resistance greater than 18 MΩ·cm. HEPES, bathocuproinedisulfonate, catalase, and L-phenylalanine were from Sigma. Atomic absorption standards were from Aldrich, and DTT was from Boehringer Mannheim. $DMPH_4$ was synthesized by the procedure of Mager et al. (1967), with catalytic reduction over platinum. Reduction was confirmed by the UV spectrum with quantitation at 266 nm (ϵ = 16 000 M^{-1} cm^{-1}) in 0.1 N HCl. For "metal-free" work, HEPES and phenylalanine solutions were passed through a 20-cm Chelex-100 (Bio-Rad) column and stored in disposable plastic containers. Metals were removed from $DMPH_4$ by extraction with dithizone that had been recrystallized from acetone. All other reagents were of the highest grade commercially available.

Methods. Enzyme assays were performed on either a Gilford 240, a Cary 219, or a Cary 1 spectrophotometer. Atomic absorption measurements were made on a Perkin Elmer 1100B with an HGA graphite furnace atomizer. Fluorescence measurements were done on a SLM 8000c instrument. Oxygen was determined with an oxygen electrode (Yellow Springs Instruments). All HPLC was done with a Waters 625LC with a Model 990 photodiode array detector.

Recombinant CVPAH expressed in *Escherichia coli* was purified as described previously (Onishi et al., 1991). This scheme requires the addition of copper to a level of 0.6 mM. Protein was assayed by the BCA method (Pierce) using BSA standards. The activity of CVPAH was assayed at 25 °C by following the increase in absorbance at 275 nm (Miller et al., 1975; Pember et al., 1986). This assay mixture contained 1.0 mM phenylalanine, 180 μM $DMPH_4$, 90 μg/mL catalase (Sigma), and 6.0 mM DTT in 80 mM HEPES buffer, pH 7.4. A molar extinction coefficient of 2.6 mM^{-1} cm^{-1} was determined by end-point assays using limiting phenylalanine, and was confirmed by independent quantitation of tyrosine in the end-point assays by reverse-phase HPLC. This differs from the value of 1.7 mM^{-1} cm^{-1} which was originally determined with $6MPH_4$ in phosphate buffer at pH 6.8 for RLPAH. The difference is that under these but not the previous conditions DTT induces formation of 7,8-$DMPH_2$

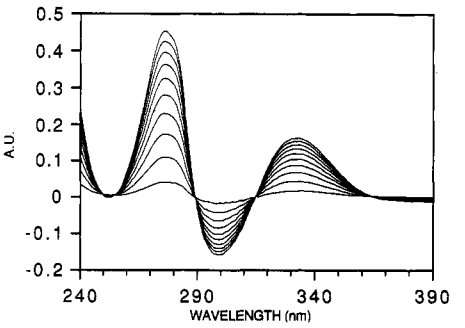

FIGURE 1: Absorbance changes of the CVPAH reaction. The assay mixture contained 30 mM HEPES, pH 7.4, 6.0 nM DTT, 1.0 mM phenylalanine, 180 μM $DMPH_4$, and 200 nM CVPAH. A background scan was taken immediately following completion of the reaction mixture and subtracted from all subsequent scans. The first scan was run immediately after the background. The remaining scans were at 1-min intervals.

from quinonoid $DMPH_2$, contributing to the change in absorbance at 275 nm. Figure 1 clearly demonstrates the formation of 7,8-$DMPH_2$, evidenced by the increase in absorbance at 335 nm and decrease at 300 nm, concomitant with tyrosine formation. The formation of 7,8-$DMPH_2$ is therefore responsible for 0.9 mM^{-1} cm^{-1} of the overall change in absorbance at 275 nm. The complete oxidation of $DMPH_4$ to 7,8-$DMPH_2$ along with tyrosine formation causes a $\Delta\epsilon$ at 275 nm of ca. 5.0 mM^{-1} cm^{-1} (Lazarus et al., 1981). Thus, for every enzyme turnover, 73% of the quinonoid $DMPH_2$ is recycled to $DMPH_4$ with the remaining 27% being transformed to 7,8-$DMPH_2$. That this transformation is not enzyme-dependent or operative via the 4a-hydroxypterin was established by the rapid generation of quinonoid $DMPH_2$ from $DMPH_4$ using H_2O_2 followed by addition of 6.0 mM DTT. The increases in absorbance at 275 and 334 nm upon DTT addition confirmed the DTT-mediated transformation of quinonoid $DMPH_2$ to 7,8-$DMPH_2$.

For "metal-free" activity determinations, catalase was omitted, and "metal-free" assay solutions were used. The pterin oxidation assay of Ayling et al. (1973) was used for measuring activity in the absence of DTT. This assay requires only enzyme, buffer, substrate, and cofactor, and is based on the change in absorbance as $DMPH_4$ is oxidized to $DMPH_2$ (ϵ = 3600 M^{-1} cm^{-1} at 347 nm). At high enzyme concentrations (greater than 50 nM), the activity of CVPAH progressively decreases, perhaps due to enzyme aggregation. For clarity, assays done at these higher enzyme concentrations have been normalized to reflect the activity of dilute solutions by use of a standard curve of specific activity versus enzyme concentration. All comparisons, however, are made between samples of the same enzyme concentration.

The 4a-hydroxypterin product was quantitated in 8.0 mM Tris buffer (pH 8.45) using 200 μM $6MPH_4$, 1.0 mM Phe, 6.0 mM DTT, and 0.5 μM CVPAH. The concentration of 4a-OH-$6MPH_4$ was determined at the point where the reaction was quenched with SDS for subsequent tyrosine assay by HPLC. A $\Delta\epsilon$ for conversion of $6MPH_4$ to 4a-OH-$6MPH_4$ of 7050 M^{-1} cm^{-1} at 257 nm was calculated from standard spectra (Lazarus et al., 1982; Dix & Benkovic, 1985). Oxygen was quantitated using an oxygen electrode (Yellow Springs Instruments Model 53). The assay contained 30 mM HEPES buffer, pH 7.4, with 6.0 mM DTT, 180 μM $DMPH_4$, 1.0 μM CVPAH, 100 μM Phe, and 300 μg of catalase in a volume of 3.0 mL. To quantitate the tyrosine produced in both of the above assays, the samples were treated with 10 mM H_2O_2 to oxidize any remaining pterin, and the protein was precipitated

with chloroform. A portion of the aqueous phase was then injected onto a C18 column and eluted isocratically with 100% H_2O. The peak area was integrated and compared to a standard curve.

Copper was removed from the enzyme by extraction with DTT. Routinely 20–30 mg of enzyme in 15 mL of 5 mM HEPES buffer (pH 7.4) was made 0.1 M in DTT and incubated for 10 min. This solution was then concentrated by a factor of 20 using a Centriprep 10 (Amicon) centrifugal ultrafilter, and then rediluted with fresh 0.1 M DTT to make up the original 15-mL volume. The concentration and redilution were repeated 2 more times, and the total protocol time was ca. 2 h. To remove the DTT, the final concentrate (~1 mL) was then applied to a 15-cm Sephadex G-25 (Pharmacia) column which had been washed extensively with "metal-free" 50 mM HEPES buffer. One-milliliter aliquots were collected, and those fractions having an absorbance at 280 nm greater than 1.5 AU were pooled. Removal of DTT was confirmed by DTNB reduction (Ellman, 1959).

The copper content of the enzyme was routinely determined by atomic absorption. Samples with low Cu/enzyme ratio were determined by the standard addition method (Fritz & Shenk, 1981). The copper content of the enzyme was periodically double-checked by Galbraith Laboratories (Nashville, TN) or colorimetrically using bathocuproinedisulfonate, ϵ_{483} = 12 250 M^{-1} cm^{-1}. For quantitation of copper using bathocuproine, 5–20 μM CVPAH in 5 mM HEPES buffer was made 50 mM in sodium ascorbate (pH 7.4), and bathocuproine was added to a final concentration of either 10 or 100 μM at 25 °C. The rate of copper removal was 0.13 s^{-1} and independent of bathocuproine concentration between 10 and 100 μM bathocuproine. Copper removal from CVPAH by bathocuproine was judged quantitative by comparison to the atomic absorption Cu determinations which were always in close agreement. Atomic absorption was also used to detect the presence of iron, cobalt, nickel, manganese, chromium, and zinc. Molybdenum was analyzed by Galbraith Laboratories.

The K_d for Cu^{2+} binding was determined by the quenching of the tryptophan fluorescence upon copper binding. For these measurements, excitation was at 290 nm, and the emission was monitored at 340 nm. A total volume of 2.0 mL of 20 mM HEPES buffer (pH 7.4) was made 0.2 μM in "metal-free" CVPAH and titrated with copper(II) nitrate. A tryptophan control was used to correct for any nonspecific quenching, which was found to be negligible. Readings were taken every 5 min after copper addition until no further change in fluorescence was observed. The K_d was determined by a nonlinear computer fit of the data to the quadratic equation for ligand binding (Pember et al., 1987a).

The K_d for Zn^{2+} was measured by competition with Cu^{2+}. A 0.2 μM CVPAH solution in 20 mM HEPES buffer (pH 7.4) was made 1.0 μM in Cu^{2+} (2 × Cu^{2+} K_d). This solution was then titrated with Zn^{2+} while observing the increase in fluorescence as the Cu^{2+} is displaced from the enzyme by Zn^{2+}, which does not quench the tryptophan fluorescence at pH 7.4. The data were fitted to the quadratic for binding as above to obtain an apparent Zn^{2+} K_d. The Zn^{2+} K_d is calculated from the equation:

$$K_{d_{app}} = K_d(Zn^{2+})[1 + K_d(Cu^{2+})] \quad (1)$$

RESULTS

Preparation and Activity of "Copper-Free" CVPAH. The first indication that the copper in CVPAH might not be required for activity occurred when we found that DTT readily removed the copper from the enzyme, even though previous observations had shown that prolonged incubation with DTT caused no loss in activity. EDTA and bathocuproine removed copper from CVPAH, but were more difficult to remove from the enzyme solution. The extraction procedure gave "copper-free" enzyme with residual copper between 40 and 100 ppb, or an average Cu/enzyme ratio of 0.01 (Table 1). The overall yield of "copper-free" enzyme from this procedure is about 80% of the copper containing enzyme starting material.

Table 1: Activity of "Copper-Free" CVPAH

sample	Cu/enzyme[a] ratio	sp act. (units/mg)[b]
control[c]	1.18	12.2
1	0.014	11.3
2	0.010	11.0
3	0.021	12.4

[a] Copper content was determined by atomic absorption. [b] All assays were by the 275-nm protocol. [c] The control was a sample of CVPAH taken through the copper extraction procedure omitting the DTT chelator.

Table 2: Stoichiometry of "Copper-Free" CVPAH Reaction

	phenylalanine[a]	oxygen[b]	tyrosine[c]	4a-OH-pterin/tyrosine ratio[d]
concn consumed or produced (μM)	100	98 ± 3	99 ± 2	1.06 ± 0.05

[a] Phenylalanine was the limiting substrate at 100 μM. [b] Oxygen was quantitated by an O_2 electrode (YSI Model 53) in HEPES buffer, pH 7.4, with 6.0 mM DTT. [c] Tyrosine was quantitated by reversed-phase HPLC (see Methods) following O_2 determination (*b*) or 4a-OH-6MPH$_4$ quantitation (*d*) [d] 4a-OH-6MPH$_4$ quantitated by the UV absorbance change at 257 nm in pH 8.45 Tris buffer (see Methods).

The specific activity of the recombinant CVPAH is generally 10–19 units/mg[2] (Onishi et al., 1991). The specific activity of the "copper-free" preparations averaged ca. 11 units/mg (Table I), which is approximately 85% of a control taken through the Cu extraction steps but omitting the DTT chelator. These activities are stable during storage for several days at 4 °C or several weeks frozen at −70° C in 5 mM HEPES (pH 7.4). Earlier preparations which were stored at higher buffer (80 mM HEPES, pH 7.4) concentrations were not as active, due to trace impurities (probably Zn^{2+}, *vide infra*) in the HEPES buffer. With these preparations, the addition of Cu^{2+} to the copper-free enzyme stock before the assay produced an activation up to the level of the copper-complexed enzyme. Side-by-side comparison of "copper-free" and copper-complexed CVPAH assayed in the presence of DTT gave identical Michaelis constants for both phenylalanine and pterin substrates.

Table 2 shows the tight coupling between the substrates and products of the reaction with "copper-free" enzyme. For each phenylalanine molecule consumed, one oxygen molecule is consumed and one tyrosine molecule is formed. Also under conditions where the 4a-hydroxypterin can be measured, the ratio of its formation to tyrosine produced is unity. This is the same result as found for RLPAH with the normal phenylalanine substrate (Kaufman & Fisher, 1974).

Copper Concentration in Assay. Since this high level of activity was not expected for an enzyme thought to require copper, the actual copper content of the assay mixture was measured in order to determine if exogenous copper from the assay solution was partially reconstituting the enzyme. Table

[2] These values have been recalculated using our corrected molar absorptivity since they were done under conditions identical to ours.

Table 3: Concentration of Copper in CVPAH Enzyme Assay

sample	sp act.[a] (units/mg)	total copper[b] in assay (μM)	Cu/enzyme ratio
Cu^{2+}-complexed	10.6	0.227	1.26 ± 0.02
"copper-free"	9.2	0.019	0.09 ± 0.02

[a] Assay mixture contained 0.20 μM CVPAH, 180 μM $DMPH_4$, 1.0 mM phenylalanine, and 6.0 mM DTT in 15 mM HEPES, pH 7.4. [b] Reagents made "metal-free" as described under Methods. Copper content was determined by atomic absorption.

Table 4: Activity of CVPAH under Nonreducing Conditions

sample	sp/act. (units/mg)[a] in HEPES (pH 7.4)	sp/act. (units/mg)[a] in imidazole (pH 7.5)
Cu-complexed CVPAH	0.2	3.3
"copper-free"	5.9	3.4

[a] Activity was measured as the oxidation of $DMPH_4$ monitored by the change in absorbance at 348 nm (ϵ = 3600 M^{-1} cm^{-1}). CVPAH concentration was 0.3 μM in 30 mM HEPES, pH 7.4, with 1.0 mM phenylalanine and 200 μM $DMPH_4$.

3, however, shows that the total amount of copper in the entire reaction mixture amounted to only 9% of the moles of enzyme present, whereas the activity was 85% of that of the Cu^{2+}-complexed control.

Effects of Copper Chelators. The incubation of the copper chelators bathocuproine and neocuproine for a period of 15 min at room temperature using DTT as a reductant had no inhibitory effect on the activity of either "metal-free" or copper-complexed CVPAH up to a concentration of 20 μM chelator. Under these conditions, bathocuproine will sequester virtually all the free copper and quantitatively removes copper from the enzyme at a rate of 0.13 min^{-1} (see Methods). Pember (1987b and unpublished data) noted 40% inhibition by bathocuproine at a concentration of 260 μM. This level of inhibition may have been due to another form of inhibition (e.g., bathocuproine, like the pterin cofactor, is an aromatic nitrogen heterocycle). EDTA, listed by Pember et al. (1987b) as an inhibitor of CVPAH, must inhibit the enzyme by a mechanism other than copper removal since we find no activity restored by addition of 20 μM copper to enzyme (100 nM) completely inhibited by 10 μM EDTA. Though some metal-complexing reagents are inhibitory, the facts that some chelating agents show no inhibition (neocuproine and bathocuproine) and others are actually activators (DTT, cysteine, and imidazole) do not support a requirement for copper.

Reductive Activation and "Copper-Free" CVPAH. Pember et al. (1986) reported that the copper center of CVPAH must be reduced for activity, requiring a relatively strong reducing agent such as dithionite, methyl viologen, or a thiol. Ascorbate, ferrocyanide, and $DMPH_4$ were not effective. DTT, which is used in the normal assay mixture, provided an additional activation that increased the rate of hydroxylation about 10-fold over enzyme prereduced with dithionite. Though not well understood, this activation does not require that DTT be consumed in the reaction. This is evidenced by our observation that 50 nmol of DTT can stimulate the enzyme to hydroxylate over 200 nmol of phenylalanine.

If "copper-free" enzyme is indeed active without the need for copper, then a copper reducing agent would not be necessary. In the pterin oxidation assay which does not include DTT, the specific activity is very low at low enzyme concentrations and increases to ca. 50% of full activity relative to the DTT-containing assay (compare Tables 1–4). This is more than 20 times the activity of Cu^{2}-complexed enzyme measured without DTT. When the assay is run in 20 mM imidazole buffer, the rate is the same for both "Cu-free" and Cu-complexed CVPAH (Table 4). These rates collectively (Table 4) are slower than those measured by the 275-nm assay that includes DTT (specific activity 11.5) by a factor of 2–3. Imidazole, therefore, is a more potent activator than dithionite but not as potent as DTT. This activation correlates better with the ability of these to remove copper rather than reduce copper.

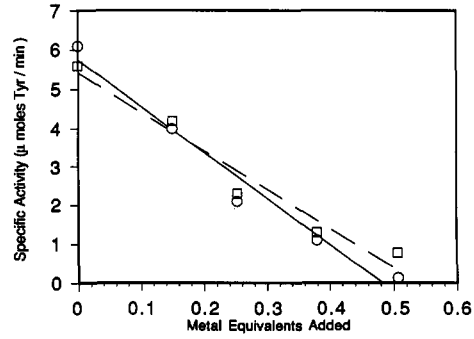

FIGURE 2: Inhibition by Cu^{2+} and Zn^{2+}. The rate is based on the change in absorbance due to $DMPH_4$ oxidation. Enzyme (300 nM) was incubated with either copper (○) or zinc (□) for 10 min prior to addition to the complete assay mixture of 200 μM $DMPH_4$, 1.0 mM phenylalanine, and 30 mM HEPES buffer, pH 7.4, to give a final enzyme concentration of 150 nM.

Inhibition by Cu^{2+} and Zn^{2+}. Also in the absence of DTT, Zn^{2+} and Cu^{2+} strongly and about equally inhibit the "copper-free" enzyme. Figure 2 shows a linear relationship between inhibition and metal concentration for both copper and zinc. The inhibition is complete after addition of half an equivalent of metal. This observation could be related to the half-maximal specific activity seen in the absence of DTT in that one or more inhibitory metals such as zinc, already present in the solution, could be causing the reduced activity in the pterin oxidation assay. In this case, ca. 50% of the specific activity of "copper-free" enzyme is observed with no addition of exogenous metal ion. Otherwise, it would be difficult to rationalize the stoichiometry of metal inhibition. The linear inhibition by copper and zinc implies that the binding is significantly tighter than the enzyme concentration used (300 nM). When assays include DTT, only zinc is inhibitory, suggesting that 6.0 mM DTT effectively removes copper but not zinc from the enzyme.

Metal Binding. The tryptophan fluorescence of "copper-free" CVPAH is quenched by binding to Cu^{2+}. On the basis of this spectroscopic property, the titration of "Cu-free" CVPAH with Cu^{2+} was monitored and produced a binding curve that follows simple saturation behavior (Figure 3). At pH 7.4, computer fit gives a K_d value of 480 ± 30 nM. At pH 7.4, the zinc does not quench the tryptophan fluorescence; however, the Zn^{2+} K_d could be determined by its competition with Cu^{2+}, showing an increase in fluorescence as copper is displaced from the enzyme (Figure 4). The Zn^{2+} K_d was calculated as 850 ± 50 nM at pH 7.4.

DISCUSSION

It is not surprising that CVPAH, a small protein with 17 histidines, would bind copper or other metals with an affinity in the range we report here (i.e., a K_d of 500 nM). This is in fact a rather modest affinity compared to most other copper enzymes. For example, the mammalian copper transport proteins albumin and ceruloplasmin have dissociation constants between 10^{-17} and 10^{-22} M (Lindler, 1992). Since the concentration of free copper is less than micromolar in most

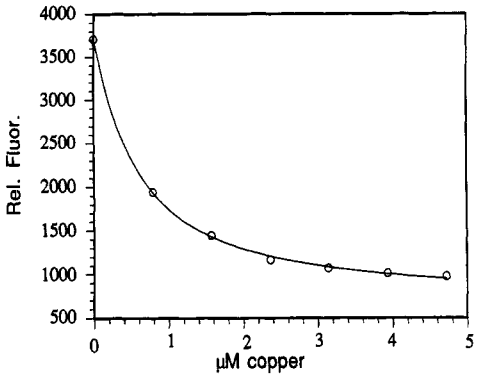

FIGURE 3: Cu^{2+} K_d. The "copper-free" enzyme concentration was 0.2 μM in a 2.0-mL volume of 30 mM HEPES, pH 7.4. Corrected fluorescence values are used (see Methods). The line is a computer fit of the data with a K_d of 0.48 μM.

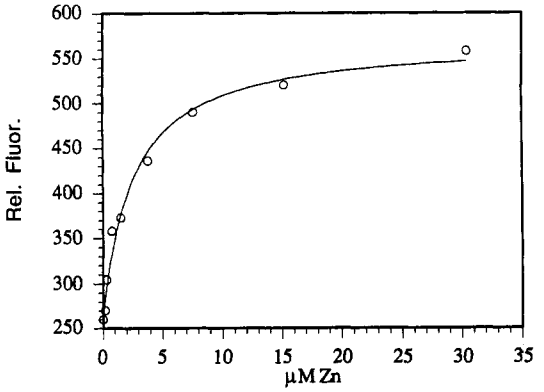

FIGURE 4: Zn^{2+} K_d. 500 nM CVPAH with 1.0 μM Cu^{2+} in 20 mM HEPES buffer, pH 7.4, was titrated with Zn^{2+}. Tryptophan fluorescence was monitored as in Figure 1. The line is a computer fit using a Cu^{2+} K_d of 0.48 μM, an apparent Zn^{2+} K_d of 2.55 μM, and eq 1. This produces a value of 0.85 ± 0.05 μM for the Zn^{2+} K_d.

cells and there is no evidence that Cu(I) has greater affinity for CVPAH than Cu(II), one would guess that *in vivo* much of the enzyme would be uncomplexed.

The results presented here in fact do not support a requirement for copper. Inhibition with chelators is evidence for metal requirement only if other possible modes of inhibition by the chelator can be excluded. The chelators used in this study, however, had if anything a slightly stimulating effect (markedly stimulating if DTT is considered as a chelator). A more definitive experiment to establish a metal requirement is to follow the progressive loss of activity as the metal is removed from the enzyme, or conversely to observe the restoration of activity as "metal-free" enzyme is reconstituted. The evidence reported here is that CVPAH with a Cu/enzyme ratio of 0.01 is nearly as active (>85%) as enzyme with a Cu/enzyme ratio of 1.2. The possibility that "metal-free" CVPAH is being reconstituted by copper in the assay solutions is disproved by the finding that the ratio of total copper to enzyme in the assay mixture was only 0.09, whereas the activity was within 85% of a fully Cu-constituted control. Therefore, 0.09 would be the upper limit for the proportion of copper-bound enzyme.

In the absence of DTT, "copper-free" CVPAH has (about half-maximal) activity whereas the copper-complexed enzyme does not. As copper(II) is titrated into a stock solution of CVPAH, the progressive loss of activity confirms the inhibitory nature of copper(II). The inhibition does in fact appear greater than that calculated from the K_d for copper measured by fluorescence in the absence of substrates. Two likely explanations for this would be either that substrate binding alters the metal binding site to increase copper affinity or that the substrate(s) actually contribute(s) to the ligation of copper in a ternary complex. There is evidence for the latter hypothesis from ESR investigations showing that the N-5 of the pterin is a ligand for copper in the copper–enzyme–pterin complex (Pember et al., 1987a). Figure 3 also shows zinc inhibiting the enzyme in much the same way as copper. In both cases, the enzyme is completely inactivated by less than 1 equiv of metal. In the absence of DTT, the specific activity of "copper-free" CVPAH is very low below about 150 nM enzyme concentration and increases with increasing enzyme concentration. The stoichiometry of inhibition relates to the specific activity of CVPAH at a given enzyme concentration. Thus, under the conditions of Figure 3, the enzyme is half-active and is inhibited by half an equivalent of added metal. When the enzyme concentration is doubled to 600 nM final concentration, more than 400 nM metal is required for complete inactivation. We believe the most likely explanation of this is that inhibitory metals (approximately 150 nM) are carried into the assay mixture with the assay solutions.

The inhibition of zinc is different from copper in that zinc-complexed enzyme is inactive even when assayed in the presence of 6.0 mM DTT. The difference could be due to the ability of DTT to reduce copper(II) to copper(I) which has less affinity for the enzyme relative to DTT than copper(II). Zinc of course has to leave the enzyme as zinc(II) and, therefore, is removed only slowly by DTT (DTT will eventually remove zinc from CVPAH especially at higher DTT concentrations than used in the assay). Enzyme that is purified without the addition of copper is often only 40–60% active. This inactivation has also been traced to inactivation by other metals (mostly zinc) and can be reversed by displacing the inhibitory metals with copper and then removing the copper with DTT.

Copper then, instead of being a requirement for activity, should be thought of as an inhibitor that is removed by the thiol for enzyme activation. The hysteretic product accumulation seen when the assay is initiated by DTT was thought to be due to reductive activation of the copper center (Pember et al., 1986).[3] It now appears that the lag in product accumulation is related to the activation of the enzyme by the ability of DTT to remove and sequester the copper from the enzyme. Binding of the substrates blocks this site, causing a much slower activation when the thiol is added after the substrates. This hypothesis fits very well with the observation that the "metal-free" enzyme is active without a thiol or other reducing agent whereas the copper-complexed form is not, and is further confirmed by the finding that imidazole, a nonreducing metal ligand, is capable of activating copper-complexed CVPAH (Table 4).

To briefly summarize, the "metal-free" active form of the enzyme is inactivated by complexing with either Zn^{2+} or Cu^{2+}. When the enzyme is inhibited by bound copper, it can be quickly reactivated by the addition of DTT. This most likely occurs in two rapid steps, where first the DTT reduces bound Cu^{2+} to Cu^{+}, followed by dissociation of E–Cu^{+} into active enzyme and the formation of a copper tetrathiolate complex. The rate of copper removal is slowed considerably by the presence of substrates, which in turn causes the lag in product accumulation when the assay is initiated by the addition of DTT or copper-complexed CVPAH. When the inhibitory

[3] Briefly, when the reaction is initiated by the addition of either enzyme (copper-complexed) or DTT, there is a lag period where the rate increases to a maximum after 40–60 s. There is no lag when the reaction is initiated by substrate addition to an enzyme–DTT mixture.

Table 5: Metal Content of "Metal-Free" CVPAH[a]

metal	mol % of CVPAH	metal	mol % of CVPAH
copper	1–3	nickel	<3
iron	<3	chromium	<3
cobalt	<3	molybdenum	<3
manganese	<3	zinc	~25

[a] All metals measured by atomic absorption.

site is bound with zinc, DTT is not effective in activating the enzyme under conditions of the assay. Adding an excess of copper to an E–Zn^{2+} complex shifts the equilibrium toward the E–Cu^{2+} complex which can be activated by DTT addition. The remaining Zn^{2+} is at first mostly sequestered from the enzyme by the DTT, and once the enzyme is turning over, it is not susceptible to inhibition (data not shown). This explains the observation that under the usual conditions, adding copper to zinc-inhibited enzyme reactivates the enzyme to nearly normal activity. Other metals might also have some affinity for this site. Iron and cobalt, for example, are inhibitory at concentrations comparable to zinc.

If CVPAH is not a copper enzyme, is it in fact a metalloenzyme at all? None of the metals most likely to perform an electron-transfer-type role proposed originally for the copper in CVPAH were found in significant proportions in "metal-free" CVPAH (Table 5). There is also complete lack of an ESR signal in the "metal-free" CVPAH. The simplest conclusion then is that this enzyme can perform the hydroxylation reaction without the need of any redox-active metal. This immediately excludes any oxygen–metal intermediate including a hypervalent "cupryl" ion, (Cu^{III}=O), or a metal–pterin peroxo species. For this enzyme then, a pterin hydroperoxide remains as the most likely activated oxygen intermediate.

The initial formation of the pterin hydroperoxide would also have to be metal-independent in this enzyme. This means that the initial transformation of triplet oxygen to the singlet manifold must be only a function of the cofactor and enzyme. It has been demonstrated that the rate of reaction of reduced pterins with molecular oxygen is insufficient to support the rate of turnover observed for PAH (Eberlein et al., 1984). We therefore propose that the CVPAH enzyme itself must activate the pterin cofactor to facilitate its initial interaction with molecular oxygen.

There is an apparent contradiction between these results and a previous study based on initial rate and pre-steady-state data that indicated that oxygen was the first ligand to bind to CVPAH in a partially ordered mechanism (Pember et al., 1989). Interpretation of the type of steady-state data used in that experiment is often plagued with ambiguities (Rudolph & Fromm, 1979), and other mechanisms are possible. Using stopped-flow spectrophotometry, we have failed to observe any burst of product under conditions similar to those used previously that implied (but did not directly demonstrate) a burst of product corresponding to an E–O_2 complex. This, along with the lack of precedence for oxygen binding to a single copper site in an enzyme, lead us to propose that the pterin cofactor is primarily responsible for oxygen binding, probably at the 4a carbon in analogy with oxygen binding to flavoproteins.

How much can we extrapolate these findings to the mammalian iron-containing hydroxylases? The relevance of this study to those enzymes rests on the structural and functional similarity abetween CVPAH and its mammalian counterpart, RLPAH. Structurally the bacterial enzyme is smaller and less complex but has a sequence homology that would indicate that the two enzymes share important structural elements. There is, for example, a conserved region that could be the metal binding site (Onishi et al., 1991). Functionally both enzymes perform the same reaction on the same substrates with only slight differences in binding affinities. The enzymatic rates are nearly the same when the two are compared on a subunit basis, though the regulation of RLPAH is more complex (Shiman, 1985). The tight coupling of tyrosine formation to oxygen consumption and pterin hydroxylation (Table 2) is the same as what has been found for RLPAH (Kaufman & Fisher, 1974), but is different than the cyt P-450 systems that most often undergo significantly more redox cycling than product formation (Nordblom & Coon, 1977). It is likely then that both the bacterial and the mammalian forms of these enzymes share a common mechanism. Experiments to directly compare the mechanisms of CVPAH and RLPAH are underway to test this and to determine how the presence of iron in the mammalian enzymes might affect the hydroxylase reaction. However, until experimental evidence is produced that defines the role of the iron in the mammalian pterin-dependent hydroxylases, our precedent of a metal-independent pterin-mediated aromatic hydroxylation would question the need to invoke the intermediacy of iron–oxygen species for both the bacterial and mammalian forms of these enzymes, but would not eliminate this optional pathway.

REFERENCES

Ayling, J., Pirson, R., Pirson, W., & Boehm, G. (1973) *Anal. Biochem. 51*, 80–90.

Beaty, N. B., & Ballou, D. P. (1980) *J. Am. Chem. Soc. 255*, 3817.

Blackburn, N. J., Strange, R. W., Carr, R. T., & Benkovic, S. J. (1992) *Biochemistry 31*, 5298–5303.

Dix, T. A., & Benkovic, S. J. (1985) *Biochemistry 24*, 5839–5846.

Dix, T. A., & Benkovic, S. J. (1988) *Acc. Chem. Res. 21*, 101–107.

Dix, T. A., Bollage, G. E., Domanico, P., & Benkovic, S. J. (1985) *Biochemistry 24*, 2955–2958.

Eberlein, G., Bruice, T. C., Lazarus, R. H., & Benkovic, S. J. (1984) *J. Am. Chem. Soc. 106*, 7916–7924.

Ellman, G. L. (1959) *Arch. Biochem. Biophys. 82*, 70–75.

Fisher, D. B., Kirkwood, R., & Kaufman, S. (1972) *J. Biol. Chem. 247*, 5161–5167.

Fritz, J. S., & Shenk, G. H. (1974) in *Quantitative Analytical Chemistry*, Allyn and Bacon, Boston, MA.

Gottschall, D. W., Dietrich, R. F., Benkovic, S. J., & Shiman, R. (1982) *J. Biol. Chem. 257*, 845–849.

Guengerich, F. P. (1990) in *Biological Oxidation Systems* (Reddy, C., Hamilton, G. A., & Madyastha, K. M., Eds.) Vol. 1, pp 51–67, Academic Press, New York.

Kaufman, S., & Fisher, D. B. (1974) in *Molecular Mechanisms of Oxygen Activation* (Hayaishi, O., Ed.) pp 285–369, Academic Press, New York.

Kuhn, D. M., & Lovenberg, W. (1985) in *Folates and Pterins* (Blakley, R. L., & Benkovic, S. J., Eds.) Vol. 2, pp 363–382, Wiley-Interscience, New York.

Lazarus, R. A., Dietrich, R. F., Wallick, D. E., & Benkovic, S. J. (1981) *Biochemistry 20*, 6834–6841.

Lazarus, R. A., DeBrosse, C. W., & Benkovic, S. J. (1982) *J. Am. Chem. Soc. 104*, 6869–6871.

Lindler, M. L. (1991) *Biochemistry of Copper*, Plenum Press, New York.

Mager, H. I. X., Addink, R., & Berends, W. (1967) *Recl. Trav. Chim. Pays-Bays 86*, 833.

Marota, J. J. A., & Shiman, R. (1984) *Biochemistry 23*, 1303–1311.

Massey, V., & Hemmerich, P. (1976) *Enzymes (3rd Ed.) 12*, 191.

McCracken, J., Pember, S. O., Benkovic, S. J., Villafranca, J. J., Miller, R. J., & Peisach, J. (1988) *J. Am. Chem. Soc. 110*, 1069–1074.

Miller, M. R., McClure, D., & Shiman, R. (1975) *J. Biol. Chem. 250*, 1132.

Nordblom, G. D., & Coon, M. J. (1977) *Arch. Biochem. Biophys. 186*, 343–347.

Onishi, A., Liotta, L. J., & Benkovic, S. J. (1991) *J. Biol. chem. 266*, 18454–18459.

Pember, S. O., Villafranca, J. J., & Benkovic, S. J. (1986) *Biochemistry 25*, 6611–6619.

Pember, S. O., Benkovic, S. J., Villafranca, J. J., Pasenkiewicz-Gierula, M., & Antholine, W. E. (1987a) *Biochemistry 26*, 4477–4483.

Pember, S. O., Villafranca, J. J., & Benkovic, S. J. (1987b) *Methods Enzymol. 142*, 50–56.

Pember, S. O., Johnson, K. A., Villafranca, J. J., & Benkovic, S. J. (1989) *Biochemistry 28*, 2124–2133.

Rudolph, F. B., & Fromm, H. J. (1979) *Methods Enzymol. 63*, 138.

Shiman, R. (1985) in *Folates and Pterins* (Blakley, R. L., & Benkovic, S. J., Eds.) Vol. 2, p 179, Wiley-Interscience, New York.

Shiman, R., & Jefferson, L. S. (1982) *J. Biol. Chem. 257*, 839–844.

Wallick, D. E., Bloom, L. M., Gaffney, B. J., & Benkovic, S. J. (1984) *Biochemistry 23*, 1295–1302.

White, R. E., & Coon, M. J. (1980) *Annu. Rev. Biochem. 49*, 315.