Identification of Metal Ligands in Cu(II)-Inhibited *Chromobacterium violaceum* Phenylalanine Hydroxylase by Electron Spin Echo Envelope Modulation Analysis of Histidine to Serine Mutations†

Shankar Balasubramanian,‡,§ Robert T. Carr,‡ Christopher J. Bender,∥ Jack Peisach,∥ and Stephen J. Benkovic*,‡

Department of Chemistry, The Pennsylvania State University, University Park, Pennsylvania 16802, and Department of Molecular Pharmacology, Albert Einstein College of Medicine of Yeshiva University, Bronx, New York 10461

Received February 9, 1994; Revised Manuscript Received May 3, 1994⊛

ABSTRACT: Phenylalanine hydroxylase from *Chromobacterium violaceum* (CVPAH) is known to bind an equivalent of divalent copper. The "metal-free" form of the protein is fully active, and Cu(II) is now shown to be an inhibitor of CVPAH rather than an activator of the enzyme [Carr, R. T., & Benkovic, S. J. (1994) *Biochemistry 32*, 14132–14138]. On the basis of amino acid sequence homology, the metal binding site may be related to those of rat liver PAH and other eukaryotic pterin-dependent hydroxylases, which require Fe(II) for activity. The conserved histidines at that site in CVPAH, histidines 138 and 143, were each mutated to serines. The mutant enzymes H138S and H143S were both catalytically inactive, but still able to bind Cu(II). Binding studies further demonstrated that both mutant enzymes still bind L-phenylalanine. Electron spin echo envelope modulation (ESEEM) studies on each of the mutants showed the presence of only a single copper-coordinating histidine, rather than the two histidine ligands suggested for the wild-type protein. This result supports a model in which Cu(II) is equatorially ligated to only two histidines in the Cu(II)-inhibited protein and allows us to unambiguously assign histidines 138 and 143 as these ligands. That the enzyme is inactive when these histidines are either bound with copper or when replaced with serines suggests that these histidines perform a catalytic function. Possible catalytic roles for these histidines in the hydroxylation mechanism of pterin-dependent monooxygenases are discussed along with potential future applications of the combination of ESEEM with site-directed mutagenesis.

Phenylalanine hydroxylase (PAH)[1] (phenylalanine 4-monooxygenase, EC 1.14.16.1) catalyzes the conversion of L-phenylalanine to L-tyrosine utilizing molecular oxygen and a tetrahydropterin cofactor [for review see Shiman (1986)]. PAH belongs to a very important class of mammalian pterin-dependent hydroxylases that includes tyrosine hydroxylase and tryptophan hydroxylase. PAH from rat liver is a highly regulated tetrameric enzyme requiring a non-heme iron, as Fe(II), for activity (Fisher et al., 1972; Wallick et al., 1984; Marota & Shiman, 1984). The function of the iron in RLPAH is not known, and no oxidation–reduction cycle is observed during enzyme turnover (Dix & Benkovic, 1985). It has been suggested that the iron is directly involved with formation of an activated oxygen intermediate (Dix & Benkovic, 1988; Siegmund & Kaufman, 1990), though no intermediates have been observed in any pterin-dependent hydroxylation. A bacterial PAH from *Chromobacterium violaceum* (CVPAH) is monomeric and binds an equivalent of Cu(II) instead of iron (Pember et al., 1986). Initially, it was thought that copper may play an analogous role in CVPAH to that of iron in RLPAH. Recent evidence, however, indicates that Cu(II) is actually an inhibitor of CVPAH rather than a required cofactor (Carr & Benkovic, 1993).

There is no crystal structure available for any pterin-dependent hydroxylase; however, there have been several studies designed to answer specific structural questions. The pterin binding site of RLPAH has been probed by both affinity labeling (Gibbs & Benkovic, 1991) and anti-idiotypic antibodies (Jennings & Cotton, 1990). EPR experiments have been performed on both the mammalian and bacterial forms of PAH to determine the nature of the metal binding centers. When Cu(II) binds to CVPAH as an inhibitor, it exhibits a type II copper EPR spectrum indicative of mixed nitrogen and/or oxygen ligation (Pember et al., 1986). ESEEM, a pulsed EPR method, was used to establish that at least two of the equatorial ligands were the imidazole side chains of histidine residues (McCracken et al., 1987). This observation has been supported by studies on copper–histidine model complexes of the active site (Kohzuma et al., 1989) and more recently by EXAFS analysis of Cu(II)-containing CVPAH (Blackburn et al., 1992). EPR spectra of RLPAH are indicative of a high-spin iron (Wallick et al., 1984), and visible ligand to metal charge-transfer bands of catecholate RLPAH complexes suggest that the iron is ligated by two histidines and a carboxylate (Cox et al., 1988). Two conserved histidines, 284 and 289, were recently identified as iron ligands in recombinant RLPAH by site-directed mutagenesis. The purified mutant proteins, H284S and H289S, lacked iron and were catalytically inactive (Gibbs et al., 1993).

† This work was supported by Grant DMB-8614179 from the National Science Foundation (S.J.B.), by Grants GM-40168 and RR-02583 from the National Institutes of Health (J.P.), and by a SERC/NATO Postdoctoral Research Fellowship (S.B.).

* Author to whom correspondence should be addressed at the Department of Chemistry, The Pennsylvania State University, University Park, PA 16802. Telephone: (814) 865-2882; Fax: (814) 865-2973.

‡ The Pennsylvania State University.

§ Present address: Cambridge Centre for Protein Engineering, University Chemical Laboratory, Lensfield Rd., Cambridge CB2 IEW, U.K.

∥ Albert Einstein College of Medicine of Yeshiva University.

⊛ Abstract published in *Advance ACS Abstracts,* June 15, 1994.

[1] Abbreviations: PAH, phenylalanine hydroxylase; CV, *Chromobacterium violaceum*; EPR, electron paramagnetic resonance; EXAFS, extended X-ray absorption fine structure spectroscopy; IPTG, isopropyl β-D-thiogalactopyranoside; DTT, dithiothreitol; PIT, phenyl isothiocyanate; BSA, bovine serum albumin; HPLC, high-performance liquid chromatography; ESEEM, electron spin echo envelope modulation; RL, rat liver.

0006-2960/94/0433-8532$04.50/0

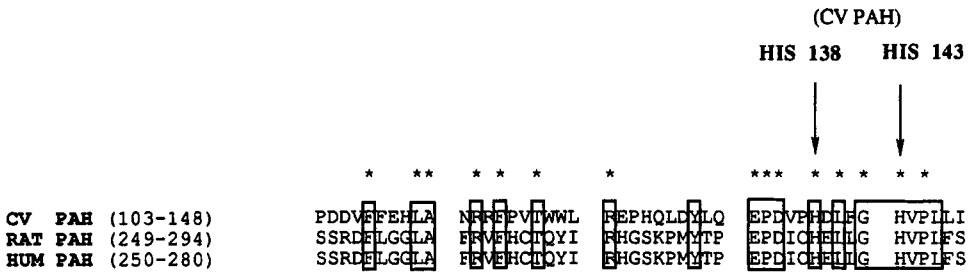

FIGURE 1: Alignment of the partial amino acid sequences of PAH from *C. violaceum*, rat liver, and human liver, showing a highly conserved region containing the histidines of interest. The asterisks denote homology that is extended to eukaryotic tyrosine and tryptophan hydroxylases.

There are 17 histidine residues in the amino acid sequence of CVPAH, making the assignment of the Cu(II) ligands difficult. However, Onishi et al. (1991) have shown that only histidines 138 and 143 are conserved in the sequence when aligned with those of rat liver (histidines 284 and 289) and human PAHs (Figure 1). The purpose of the present study is to determine by site-directed mutagenesis whether these histidine residues are indeed ligands to the copper when bound to CVPAH. In many instances the assignment of metal ligands purely on the basis of lost activity in point mutants can be ambiguous and is not applicable here since Cu(II) inhibits CVPAH activity. Metal binding affinities can be difficult to measure and may also be prone to artifacts arising from conformational changes induced by mutagenesis, and therefore cannot always be used reliably to identify metal ligands. To circumvent these difficulties, we have chosen to use ESEEM spectroscopy in conjunction with site-directed mutagenesis to assign the histidine ligands to copper in CVPAH. ESEEM spectroscopy can unambiguously determine whether there is one or more than one histidine coordinated to the copper center (McCracken et al., 1988). Since there is evidence of two histidine ligands to copper in CVPAH based on ESEEM spectral simulations, site-directed mutagenesis of either histidine ligand should result in distinctive changes in the ESEEM spectrum. Specifically, ESEEM studies on Cu(II)–imidazole complexes together with simulations have demonstrated that combination lines in the ESEEM spectrum result only when the Cu(II) is ligated to more than a single imidazole (McCracken et al., 1988). These combination frequencies have been observed in the ESEEM spectrum of Cu(II)–CVPAH and would be expected to disappear on removal of one the histidine ligands. Indeed, this takes place when either His 138 or His 143 is replaced by serine.

EXPERIMENTAL PROCEDURES

Materials

Restriction endonucleases and other cloning enzymes were purchased from New England Biolabs or Boehringer Mannheim. Polymerase chain reactions were carried out using the GeneAmp kit from Perkin-Elmer Cetus Instruments. Oligonucleotides were purchased from American Synthesis Inc. DNA sequencing reactions utilized the Sequenase DNA sequencing kit from U.S. Biochemical Corp. [α-^{35}S]dATP and [U-^{14}C]-L-phenylalanine were obtained from Du Pont-New England Nuclear. The plasmid pLJL-I-213C was a generous gift of Dr. L. J. Liotta. IPTG was purchased from U.S. Biochemical Corp. HEPES, catalase, phenyl isothiocyanate, and L-phenylalanine were from Sigma. Cu(II) atomic absorption standards and dichloroethane were from Aldrich Chemicals, and DTT was from Boehringer Mannheim. 6,7-Dimethyltetrahydropterin was synthesized by the method of Mager et al. (1967) with catalytic reduction over platinum.

Methods

UV assays were carried out on a Cary 1 spectrophotometer. Atomic absorption measurements were taken on a Perkin-Elmer 1100B with an HGA graphite furnace atomizer. Fluorescence measurements were taken on an SLM 8000C instrument. Reversed-phase HPLC was done with a Waters Model 625 liquid chromatography system with a Model 990 photodiode array detector. The column was a 25-cm Bondpak C18 reversed-phase column.

Preparation of Mutants. Standard DNA manipulations were carried out as described in Maniatis et al. (1989). A 1.5-kb DNA fragment containing the CVPAH gene was isolated from pLJL-I-213C by restriction digestion with *Hin*dIII and *Aat*II. This 1.5-kb fragment was cloned between the *Hin*dIII and *Pst*I sites of pUC 19 using a 17 base pair double-stranded synthetic linker DNA (sequence 5′-CTG-ATGAGGGCCCTGCA-3′ and 5′-GGGCCCTCATCA-GACGT-3′) to adapt the *Aat*II site to a *Pst*I site. The resulting plasmid, pSB1, was used as a template for subsequent mutagenesis. Site-directed mutagenesis of His 138 and His 143 was carried out using the four primer overlap extension method of Ho et al. (1989). Flanking primers were complementary to pUC19 and were located approximately 400 base pairs from the polycloning region. The sequences of the mutagenic primers were as follows: H138S: 5′-GCCG-GACGTGTTCTCGGACCTGTTCGGCC-3′; and H143S: 5′-CGGACGTGTTCCACGACCTGTTCGGATC-3′ (and their complements). The PCR products formed at each stage were isolated by agarose gel electrophoresis prior to further manipulation. For each mutagenesis experiment the final overlap PCR product was digested with *Bal*I and *Sph*I, to afford a 245 base pair fragment, containing the desired His to Ser mutation. These 245 base pair *Bal*I/*Sph*I fragments containing the required single mutation were subcloned between the *Sma*I and *Sph*I sites of pUC19 to give the resulting mutated plasmids pSB143 and pSB138. Both strands of pSB138 and pSB143 were sequenced using the dideoxy chain-termination method of Sanger et al. (1977) to confirm the presence of the desired mutation and the absence of any spurious mutations. Finally, each 228 base pair mutated fragment obtained by digestion with *Bst*EII and *Sph*I was isolated from both pSB138 and pSB143 and was subcloned between these sites in pSB1 to give the plasmids pCV138 and pCV143 which contain the CVPAH gene carrying the H138S and H143S mutations, respectively.

The IPTG-induced cultures of pCV138, pCV143, and original wild-type clone in *Escherichia coli* DH5αF′ were grown and purified as described for the wild-type protein by Onishi et al. (1991). The enzyme purities for native recombinant CVPAH and both mutants were judged to be greater than 95% by sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

Metal Extraction and K_d Determinations. Bound metal was removed from wild-type and mutant proteins by repeated extractions (3 times) with 100 mM DTT, followed by the removal of DTT by gel filtration as previously described by Carr and Benkovic (1993). K_d values for copper were determined by the quenching of protein tryptophan fluorescence on addition of Cu(II) (as the nitrate salt). Excitation was at 290 nm, and emission was monitored at 340 nm. The sample contained 50 nM protein buffered at pH 7.4 (10 mM HEPES) in a volume of 2 mL. Fluorescence measurements were corrected for changes in volume upon addition of Cu(II). A tryptophan control was used to correct for any nonspecific quenching, which was found to be negligible. K_d values were obtained by fitting the data by nonlinear regression to the binding quadratic as described previously for the native recombinant enzyme (Carr & Benkovic, 1993).

The K_d values for L-phenylalanine were estimated by taking an equilibrium mixture containing PAH (20 μM, wild type or mutant) and L-phenylalanine (50 μM) with a trace of [U-^{14}C]-L-phenylalanine ($\sim 5 \times 10^4$ cpm) in 0.5 mL (pH 7.4, 30 mM HEPES), and then centrifuging in an ultrafilter (Centricon 10) until approximately 50% of the volume had passed through the filter. The difference in specific radioactivity between the retentate solution and the filtrate was used to determine the fraction of protein-bound L-phenylalanine and hence the K_d. Controls containing either BSA (20 μM) or simply buffer showed a negligible difference in specific radioactivity between the retentate and the filtrate.

Enzyme Assays. Assay mixtures contained 4.5 μM enzyme (wild type, H138S, or H143S), 1 mM L-phenylalanine, 6 mM DTT, 90 μg of catalase, and 180 μM 6,7-dimethyltetrahydropterin at pH 7.4 (30 mM HEPES) in a total volume of 1 mL. UV assays were monitored at 275 nm as described by Pember et al. (1987), using the value $\Delta\epsilon_{275} = 2600$ M^{-1} cm^{-1} calculated for these conditions (Carr & Benkovic, 1993). For the HPLC assays the protein was first precipitated with 1,2-dichloroethane (400 μL), and the aqueous portion was lyophilized and then derivatized with phenyl isothiocyanate (PIT) prior to analysis by reversed-phase HPLC. The HPLC assay was calibrated by the injection of PIT derivatives of phenylalanine and tyrosine standards.

Pulsed EPR Experiments. Samples of wild-type and H138S PAH contained 450 μM protein with 1 equiv of Cu(II); however, the sample of mutant H143S contained 700 μM protein and 410 μM Cu(II).

The pulsed EPR spectrometer has been previously described (McCracken et al., 1987), but several modifications have since been made. The microwave pulses are formed by two switches arranged in a parallel circuit. Each branch of the parallel circuit is isolated (40 dB) and consists of a variable attenuator and coaxial phase trimmer in addition to the switch. The phase trimmer is used to balance the two branches.

Electron spin echo modulation was collected at approximately 9 GHz. The microwave pump and echo observation is performed using a reflection cavity arrangement; the resonant element is a $\lambda/4$ strip that is coupled to the magnetic field mode of a rectangular TE_{101} cavity [cf. Lin et al. (1985) and Britt and Klein (1988)]. The Q of the stripline resonator is approximately 250, and the resultant minimum dead time during these experiments was 70 ns.

RESULTS

The yield of the mutants H138S and H143S was 1–5 mg of purified protein/L, which is comparable to the expression of native recombinant CVPAH using pSB1 in DH5α. Purification of these mutants proceeded exactly as for the native preparation, as has been described previously (Onishi et al., 1991). This suggests that the size, charge, and solubility properties of both mutants are very similar to those of wild-type CVPAH. Neither mutant retained stoichiometric amounts of bound copper throughout the purification, which is also true for the native recombinant CVPAH (Onishi et al., 1991). A parameter of enzyme function not significantly altered in these mutants is their ability to bind L-phenylalanine. This was determined by incubating ^{14}C-labeled L-phenylalanine with the enzyme, followed by ultrafiltration and counting both the filtrate and retentate as described under Methods. The results of several of these experiments all indicated that both the mutants bound phenylalanine with estimated K_ds within a factor of 3 of the wild-type controls (Table 1). These results suggest that the point mutations do not cause global changes to the protein structure.

Table 1: Binding Properties of Mutant Forms of CVPAH

	Cu(II) K_d (μM)[a]	phenylalanine K_d (μM)[b]
native CVPAH	0.5	60 ± 30
H138S	0.1	145 ± 30
H143S	6.0	130 ± 30

[a] Measured by fluorescence titration at pH 7.4 in 10 mM HEPES buffer (see Methods). [b] Measured by centrifugal ultrafiltration using ^{14}C-labeled phenylalanine (see Methods).

A striking difference between the mutants and native recombinant CVPAH is that neither mutant possessed any observable catalytic activity at any stage of the purification. The purified mutants showed no activity above background in the 275-nm assay even at an enzyme concentration of 4.5 μM. After a 16-h incubation of this assay, no tyrosine was detected by reversed-phase HPLC before or after derivatization with PITC. This latter method is capable of detecting 1 nmol of tyrosine, suggesting the activity of the mutants was less than 0.001 μmol min^{-1} mg^{-1}. This is less than 0.01% of the activity of native recombinant CVPAH.

Residual copper was removed from the mutant proteins by the DTT extraction procedure described previously (Carr & Benkovic, 1993). The copper binding affinity was measured by the quenching of tryptophan fluorescence upon binding Cu(II) (Carr & Benkovic, 1993). The titration curves (data not shown) indicate copper binding to a single site as reported previously for the native enzyme. The Cu(II) K_ds are given in Table 1. The copper affinity of H143S decreased an order of magnitude from that of the native recombinant CVPAH, which is consistent with alteration of the metal binding site; however, this decrease could be caused by conformational changes rather than loss of a copper ligand. Furthermore, the H138S mutant had a slightly enhanced affinity for divalent copper. Studies on peptide–Cu(II) complexes have previously shown serine to be a poor Cu(II) ligand (Siegel et al., 1977); therefore, this enhanced affinity for Cu(II) in H138S is not thought to be due to ligation by the serine replacement. The increased affinity for copper in this mutant probably results from a combination of the enhancement of the geometry of the remaining ligands with additional ligation from a residue not available when the imidazole side chain of His 138 is present. Since we could not unambiguously assign these histidines as copper ligands on the basis of the above data, another method was needed.

EPR spectroscopy of the mutant proteins was performed on samples with substoichiometric amounts of copper added to the concentrated "metal-free" proteins. The K_ds in Table 1 were used to compute the amount of copper required to

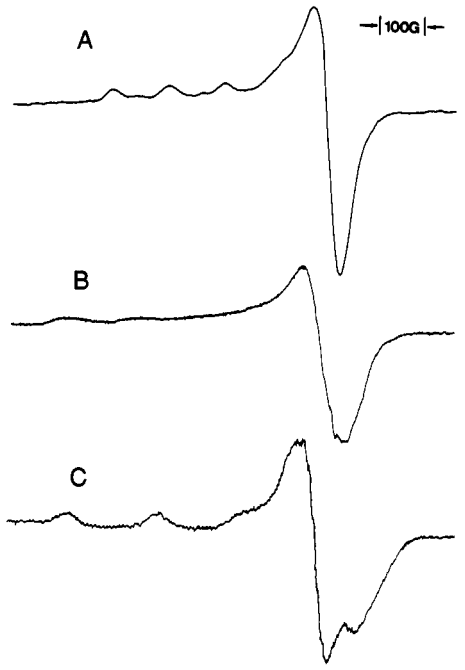

FIGURE 2: Continuous-wave EPR spectra of Cu(II) bound to wild type (A), H143S (B), and H138S (C) *C. violaceum* PAHs. Instrument parameters: modulation, 6.3 G; power, 10 mW; sweep, 100g/8 min; temperature, 77 K. In (A), $g_{\|} = 2.32$, $g_{\perp} = 2.06$, $A_{\|} = 150$ G; in (B), $g_{\|} = 2.29$, $g_{\perp} = 2.07$, $A_{\|} = 170$ G; and in (C), $g_{\|} = 2.25$, $A_{\|} = 240$ G.

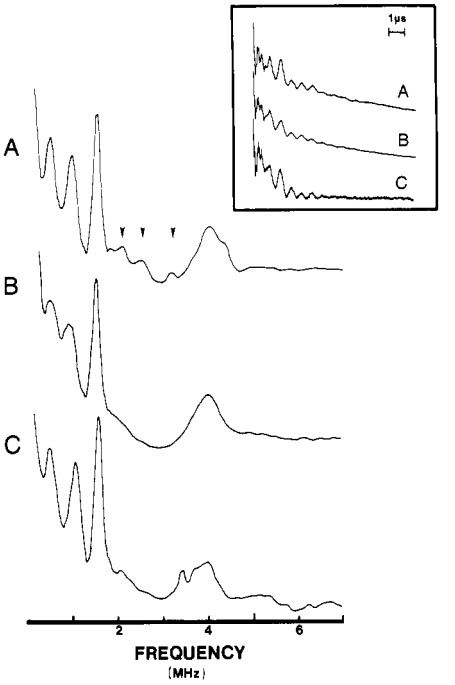

FIGURE 3: ESEEM traces (insert) and Fourier transform spectra (main figure) of Cu(II) bound to wild-type (A), H143S (B), and H138S (C) *C. violaceum* PAHs. Measurement conditions were the following: microwave frequency, 9.0210 GHz; magnetic field strength, 3100 G; microwave pulse power, 30 W; τ value, 152 ns; sample temperature, 4.2 K; pulse sequence repetition rate, 100 Hz; each time point an average of 80 events.

ensure that the concentration of free copper was less than 10% of the enzyme-bound copper. Continuous-wave EPR spectra of native and mutant proteins are depicted in Figure 2. All three spectra are consistent with a type II copper center with N and/or O ligation (Peisach & Blumberg, 1974). The EPR spectra of the mutants (Figure 2B,C) show significant alteration in the g and A values (Figure 2) relative to those of native recombinant protein (Figure 2A), indicative of altered ligand structure. The change in line shape exhibited in the EPR spectrum of H138S (Figure 2C) is also suggestive of a change in ligand environment toward a more rhombic geometry. It was not possible, therefore, to unambiguously define the structural differences on the basis of these EPR spectra, as can sometimes be carried out when ligand geometry remains regular (Peisach & Blumberg, 1974).

For the reasons described above, we turned to electron spin echo modulation spectroscopy as a means of unambiguously determining if histidines 138 and 143 are copper ligands. Three-pulse ESEEM spectra are illustrated in the insert of Figure 3 for wild-type protein (A) and mutants H143S and H138S (B and C, respectively). The dead time data were reconstructed using a method described by Mims (1984), and the resultant Fourier transform spectra are presented in the main figure. The major features found in each spectrum consist of three sharp lines near 0.55, 1.00, and 1.55 MHz and a broader line near 4 MHz. These spectral features are characteristic of the coupling between the electron spin of the Cu(II) with the remote ^{14}N of coordinated histidyl imidazole (Mims & Peisach, 1978; Jiange et al., 1990). For one of the spin manifolds, under conditions where the nuclear Zeeman and the electron–nuclear hyperfine interaction for ^{14}N are comparable, the ESEEM spectrum is dominated by the three low-frequency lines that arise primarily from the nuclear quadrupole interaction. The 4-MHz line arises from the second manifold and constitutes a $\Delta M = 2$ transition; the $\Delta M = 1$ transitions in this manifold are highly orientation dependent (Colaneri et al., 1990, 1992) and are not resolved for powder samples.

In addition to these spectral features, one also observes a number of low-intensity lines at 2.1, 2.6, and 3.2 MHz in the spectrum of the wild-type protein (indicated by the arrows in Figure 2A), but which are absent in the spectra of the mutants. The frequencies of these lines are combinations of frequencies of the nuclear quadrupole lines. These combination lines have also been identified in the Fourier transform spectra of galactose oxidase (Kosman et al., 1980), amine oxidase (McCracken et al., 1987), Cu–isopenicillin synthase (Jiang et al., 1991), and laccase (Lu et al., 1992). The presence of these lines can be taken as evidence of there being more than one nucleus of a given type coupled to the electron spin. Further, the relative intensity of the combination lines with respect to other spectral components is dependent on the number of such interacting nuclei. For Cu(II)–imidazole complexes, one can clearly differentiate the case of a single coordinating imidazole from those in which there are more than one (McCracken et al., 1988). Quantifying the exact number of imidazoles when there is more than one requires greater analysis through spectral simulations (Cornelius et al., 1990). In the previous ESEEM study of *C. violaceum* PAH (McCracken et al., 1988), analysis of ESEEM data sets with those obtained for several copper–imidazole complexes indicated that the combination lines in PAH were in all likelihood attributable to a pair of equatorially coordinated imidazoles, although some ambiguity existed in the analysis.

The origin of combination lines has been described by Mims in his analysis of electron spin echo modulation patterns of three-pulse data (Mims, 1972). In summary, the modulation observed in a given spin echo experiment is the product of individual terms of the type $\cos \omega_i(\tau + T)$, where ω is the ENDOR frequency. It should therefore be recalled that in a three-pulse experiment τ, the time interval between first

and second pulses, is a fixed value and T, the time interval between pulses 2 and 3, is varied. If a single ^{14}N nucleus is characterized by an echo envelope containing two frequencies, $\cos \omega_i(\tau + T)$ and $\cos \omega_j(\tau + T)$, then the modulation of two identical ^{14}N nuclei will in addition contain the harmonic terms $\cos 2\omega_i(\tau + T)$ and $\cos 2\omega_j(\tau + T)$ and the combination term (a cross product) $\cos \omega_i(\tau + T) \cos \omega_j(\tau + T)$. It follows that if we make single deletions of histidine in PAH, the combination lines will disappear if the deletion corresponds to one of the two copper-coordinating imidazole moieties.

The two lower Fourier transform spectra of Figure 3 were obtained from the mutants H143S (B) and H138S (C). It can be seen from the spectra that combination lines present in the wild-type CVPAH are absent in the spectra of the two mutants. The absence of these peaks in the mutant CVPAH clearly indicates that only a single histidine remains in coordination with the copper. This can be taken as evidence that the Cu(II) of CVPAH is equatorially ligated to two and to only two histidine imidazoles as predicted from initial analysis of ESEEM (McCracken et al., 1988) and EXAFS data (Blackburn et al.,1992) and favors the structural assignment of His 138 and His 143 as the copper ligands.

DISCUSSION

Ambiguities often plague the assignment of structural or catalytic features to specific amino acids in enzymes lacking crystal structures. When assigning amino acids as metal ligands, it is necessary to remember that loss of activity or metal affinity can be caused either by a direct loss of ligation or by a change in the coordination geometry. In this study the mutant proteins retained a significant portion of their metal affinity but exhibited a complete loss of catalytic activity. This presented a confusing picture of the function of these residues in CVPAH. Therefore, we employed ESEEM for the assignment of His 138 and His 143 as two of the ligands to Cu(II) in the inactive CVPAH–Cu(II) complex.

The electron spin echo experiments confirm that the CVPAH-bound Cu(II) is coordinated by two and only two equatorial histidine ligands. The combination lines observed in the spectrum for the native recombinant CVPAH could have resulted from Cu(II) coordination by more than one histidine ligand. However, the complete loss of the combination lines in the ESEEM spectra when either His 138 or His 143 is mutated to a serine confirms that they could be originally attributed to two rather than three or four histidines. For this same reason we can now assign His 143 and His 138 as these two equatorial histidine ligands. The ability of ESEEM to distinguish between one or two histidines in the coordination sphere of a copper center has allowed us to make these assignments without the need to mutate other histidine residues or to make a series of different substitutions at positions 138 and 143. The combination of ESEEM with site-directed mutagenesis will undoubtedly be useful in the future study of copper–histidine interactions in proteins.

That histidines H138 and H143 are critical for catalytic activity is not surprising in light of their strict conservation in the amino acid sequences of all known pterin-dependent monooxygenases. While it is true that we cannot rule out a subtle, yet significant, conformational alteration leading to total loss of catalytic activity in the mutant proteins, our observations during the protein preparations and the L-phenylalanine binding studies argue against significant structural changes due to the mutagenesis. The homologous histidines for RLPAH (His 295 and His 291) were also recently shown to be required for activity (Gibbs et al., 1993). In that study, however, it was concluded that the loss of activity was due to loss of iron binding ability by the mutant proteins lacking a histidine ligand, though other explanations were not totally ruled out. An analogous explanation is untenable for CVPAH since copper or other metals are not required by this enzyme. Copper, zinc, iron, and cobalt are inhibitory when bound to this site in CVPAH (Carr & Benkovic, 1993). CVPAH then is inactive when either these histidines are bound by metal or the side-chain imidazole is changed to a hydroxyl. These results present the interesting possibility that histidines 138 and 143 are performing a catalytic function in the CVPAH mechanism.

There are several steps in the reaction mechanism of PAH where histidines might be necessary for proton transfer, or involved in stabilizing an enzyme-bound intermediate, so that Cu(II) binding would be inhibitory. These histidines could be important for the initial formation of a pterin–oxygen intermediate such as a 4a-hydroperoxytetrahydropterin proposed in analogy to the flavin 4a-hydroperoxide observed in flavin-dependent hydroxylases (Beaty & Ballou, 1980). It has been shown that the rate of oxygen addition to tetrahydropterins in solution is not competent to support the catalytic rate of PAH. Therefore, some feature of the enzyme must facilitate this reaction (Eberlein et al., 1984). Since the reaction of tetrahydropterin with oxygen would be favored by deprotonation at N-3 (pK_a 11.4), these two histidines could potentially be involved in stabilizing this form to promote the electron transfer reaction with oxygen. There is precedent for a histidine acting on a high pK_a substrate in flavin enzymes. Specifically, His 373 of flavocytochrome b_2 is thought to facilitate removal of the α proton of the substrate (an α keto acid) which has a pK_a value of about 15 (Lederer, 1992). If an analogous situation is occurring in the PAH reaction, then it is likely that other enzyme residues would contribute in the optimization of the geometrical arrangement of these histidines, since free imidazole had no effect in activating either histidine mutant.

These histidines could also be necessary for stabilization of an intermediate formed as oxygen adds to the aromatic ring of phenylalanine. One proposed intermediate is a cation formed either directly by electrophilic addition of oxygen or from opening of an intermediate arene oxide (Dix & Benkovic, 1988). If the function of these histidines were only important subsequent to the formation of an activated oxygen intermediate, one would expect that in their absence uncoupled pterin oxidation would be observed as this intermediate decays. However, no uncoupled pterin oxidation was observed for either mutant, which suggests involvement of the histidines prior to the formation of the hydroxylating intermediate.

In this study we have used site-directed mutagenesis to identify two histidine residues that appear to be critical for catalytic activity. The use of ESEEM has allowed us to assign the same two histidines as equatorial Cu(II) ligands, and to propose a model for the inhibition of CVPAH by copper and other metals. ESEEM together with site-directed mutagenesis forms an efficient means of assigning histidine ligands to primary protein structure and will undoubtedly find future applications in this area of study.

ACKNOWLEDGMENT

We thank Drs. J. D. Stewart and M. W. Crowder for helpful suggestions.

REFERENCES

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.
Britt, R. D., & Klein, M. P. (1988) *J. Magn. Reson. 74*, 535–537.
Carr, R. T., & Benkovic, S. J. (1993) *Biochemistry 32*, 14132–14138.
Colaneri, M. J., & Peisach, J. (1992) *J. Am. Chem. Soc. 114*, 5335–5341.
Colaneri, M. J., Putenza, J. A., Schugar, H. J., & Peisach, J. (1990) *J. Am. Chem. Soc. 112*, 9451–9458.
Cornelius, J. B., McCracken, J., Clarkson, R. B., Belford, R. L., & Peisach, J. (1990) *J. Phys. Chem. 94*, 6977–6982.
Cox, D. D., Benkovic, S. J., Bloom, L. M., Bradley, F. C., Nelson, M. J., Que, L., Jr., & Wallick, D. E. (1988) *J. Am. Chem. Soc. 110*, 2026–2032.
Dix, T. A., & Benkovic, S. J. (1985) *Biochemistry 24*, 5839.
Dix, T. A., & Benkovic, S. J. (1988) *Acc. Chem. Res. 21*, 101–107.
Eberlein, G., Bruice, T. C., Lazarus, R. H., & Benkovic, S. J. (1984) *J. Am. Chem. Soc. 106*, 7916–7924.
Fisher, D. B., Kirkwood, R., & Kaufman, S. (1972) *J. Biol. Chem. 247*, 5161–5167.
Gibbs, B. S., & Benkovic, S. J. (1991) *Biochemistry 30*, 6795–6802.
Gibbs, B. S., Wojchowski, D., & Benovic, S. J. (1993) *J. Biol. Chem. 268*, 8046–8052.
Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., & Pease, L. R. (1989) *Gene 77*, 51–59.
Jennings, I., & Cotton, R. (1990) *J. Biol. Chem. 265*, 1885.
Jiang, F., McCracken, J., & Peisach, J. (1990) *J. Am. Chem. Soc. 112*, 9035–9044.
Jiang, F., Peisach, J., Ming, L. J., Que, L., & Chen, V. J. (1991) *Biochemistry 30*, 11437–11445.
Kohzuma, T., Masuda, H., & Yamauchi, O. (1989) *J. Am. Chem. Soc. 111*, 3431–3433.
Kosman, D., Peisach, J., & Mims, W. B. (1980) *Biochemistry 19*, 1304–1308.
Lederer, F. (1992) *Protein Sci. 1*, 540–548.
Lin, C. P., Bowman, M. K., & Norris, J. R. (1985) *J. Magn. Reson. 65*, 369–374.
Lu, J., Bender, C. J., McCracken, J., Peisach, J., Severns, J. C., & McMillin, D. R. (1992) *Biochemistry 31*, 6265–6272.
Mager, H. I. X., Addiuk, R., & Berends, W. (1967) *Recl. Trav. Chim. Pays-Bays 86*, 833–851.
Maniatis, T., Fritsch, E. F., & Sambrook, J. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Marota, J. J. A., & Shiman, R. (1984) *Biochemistry 23*, 1303–1311.
McCracken, J., Peisach, J., & Dooley, D. M. (1987) *J. Am. Chem. Soc. 109*, 4064–4072.
McCracken, J., Pember, S. O., Benkovic, S. J., Villafranca, J. J., Miller, R. J., & Peisach, J. (1988) *J. Am. Chem. Soc. 110*, 1069–1074.
Mims, W. B. (1972) *Phys. Rev. 5*, 2409–2419.
Mims, W. B. (1984) *J. Magn. Reson. 59*, 291–306.
Mims, W. B., & Peisach, J. (1978) *J. Chem. Phys. 19*, 4921–4970.
Onishi, A., Liotta, L. J., & Benkovic, S. J. (1991) *J. Biol. Chem. 266*, 18454–18459.
Peisach, J., & Blumberg, W. E. (1974) *Arch. Biochem. Biophys. 165*, 691–708.
Pember, S. O., Villafranca, J. J., & Benkovic, S. J. (1986) *Biochemistry 25*, 6611–6619.
Pember, S. O., Villafranca, J. J., & Benkovic, S. J. (1987) *Methods Enzymol. 142*, 50–56.
Sanger, F., Miliken, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463–5467.
Shiman, R. (1985) in *Folates and Pterins*, p 179, Wiley-Interscience, New York.
Siegmund, H., & Kaufman, S. (1990) *J. Biol. Chem. 266*, 2903–2910.
Sigel, H., Naumann, C. F., Prijs, B., McCormick, D. B., & Falk, M. C. (1977) *Inorg. Chem. 16*, 790–796.
Wallick, D. W., Bloom, L. M., Gaffney, B. J., & Benkovic, S. J. (1984) *Biochemistry 23*, 1295.