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Evidence for a High-Spin Fe(IV) Species in the Catalytic Cycle of a Bacterial Phenylalanine Hydroxylase[†]

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ABSTRACT: Phenylalanine hydroxylase is a mononuclear non-heme iron protein that uses tetrahydropterin as the source of the two electrons needed to activate dioxygen for the hydroxylation of phenylalanine to tyrosine. Rapid-quench methods have been used to analyze the mechanism of a bacterial phenylalanine hydroxylase from *Chromobacterium violaceum*. Mössbauer spectra of samples prepared by freeze-quenching the reaction of the enzyme−^{57}Fe(II)−phenylalanine−6-methyltetrahydropterin complex with O_2 reveal the accumulation of an intermediate at short reaction times (20−100 ms). The Mössbauer parameters of the intermediate (δ = 0.28 mm/s, and $|\Delta E_Q|$ = 1.26 mm/s) suggest that it is a high-spin Fe(IV) complex similar to those that have previously been detected in the reactions of other mononuclear Fe(II) hydroxylases, including a tetrahydropterin-dependent tyrosine hydroxylase. Analysis of the tyrosine content of acid-quenched samples from similar reactions establishes that the Fe(IV) intermediate is kinetically competent to be the hydroxylating intermediate. Similar chemical-quench analysis of a reaction allowed to proceed for several turnovers shows a burst of tyrosine formation, consistent with rate-limiting product release. All three data sets can be modeled with a mechanism in which the enzyme−substrate complex reacts with oxygen to form an Fe(IV)=O intermediate with a rate constant of 19 mM^{-1} s^{-1}, the Fe(IV)=O intermediate hydroxylates phenylalanine with a rate constant of 42 s^{-1}, and rate-limiting product release occurs with a rate constant of 6 s^{-1} at 5 °C.

Phenylalanine hydroxylase (PheH)[1] is a non-heme iron-containing monooxygenase that catalyzes the hydroxylation of the aromatic amino acid phenylalanine to yield tyrosine (Scheme 1). The enzyme belongs to the family of pterin-dependent aromatic amino acid hydroxylases, along with tyrosine hydroxylase (TyrH) and tryptophan hydroxylase (*1*). PheH is found in organisms ranging from eukaryotes to prokaryotes. In mammals, PheH is present in the liver, where it catalyzes the first and rate-limiting step in the metabolism of the phenylalanine acquired in the diet; mutations that hamper the function of PheH have been linked to the disease phenylketonuria (*2*). The structures of the catalytic domains of the three eukaryotic enzymes and of a bacterial PheH from *Chromobacterium violaceum* (CvPheH) reveal a common fold in which the active site iron is coordinated by two histidines and a glutamate (*3*−*5*). A similar arrangement of ligands is found in the α-ketoglutarate-dependent dioxygenases, in which the non-heme iron is typically coordinated by two histidines and an aspartate (*6*).

The present understanding of the mechanism of the aromatic amino acid hydroxylases is based primarily on studies of the eukaryotic enzymes (*7*). In the absence of the substrates, the iron cofactor coordinates three water molecules, two histidines, and a monodentate glutamate. Binding of the amino acid substrate and the tetrahydropterin results in loss of two of the waters and conversion of the glutamate to bidentate binding, opening a site for oxygen (*8*−*10*). Reaction of oxygen with the iron and the tetrahydropterin cosubstrate is proposed to yield an Fe(IV)=O (ferryl) intermediate and the 4a-hydroxypterin product (Scheme 1) (*7*, *11*, *12*). Reaction of the amino acid with the ferryl intermediate by an electrophilic aromatic substitution mechanism would yield the hydroxylated amino acid (*13*−*17*), returning the iron to the Fe(II) form. In the case of the bacterial PheH, the structure of the ferric enzyme shows a bidentate glutamate (*3*), in contrast to the eukaryotic enzymes. Still, the reactivities of the hydroxylating intermediates in the eukaryotic and prokaryotic enzymes appear to be essentially identical on the basis of their similar abilities to catalyze aromatic, benzylic, and aliphatic hydroxylation (*17*−*19*).

Although it has been proposed that ferryl complexes are the substrate-hydroxylating intermediates in the reactions of all the aromatic amino acid hydroxylases, only in the case of TyrH has direct spectroscopic evidence of such a complex been provided (*12*). The Mössbauer spectrum of that enzyme trapped at short reaction times by the freeze-quench method showed that it

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[1]Abbreviations: PheH, phenylalanine hydroxylase; CvPheH, *Chromobacterium violaceum* phenylalanine hydroxylase; TyrH, tyrosine hydroxylase; TauD, taurine:α-ketoglutarate dioxygenase; 6-$MePH_4$, 6-methyltetrahydropterin.

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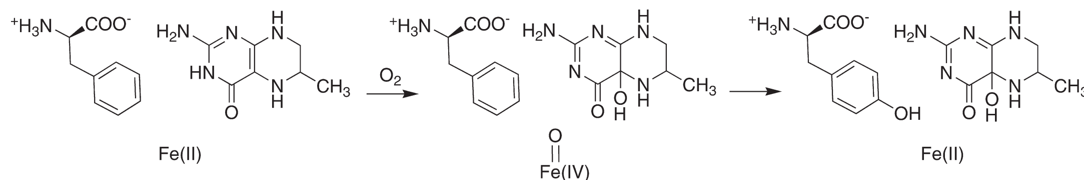
Scheme 1: Reaction Catalyzed by Phenylalanine Hydroxylase Showing (i) Generation of the 4a-Hydroxypterin Product and the Fe(IV)=O Intermediate and (ii) Hydroxylation of Phenylalanine by the Fe(IV)=O Species as Individual Steps (*7*)



contained iron in the +IV oxidation state; this was tentatively assigned as an Fe(IV)=O species on the basis of the similarity of its spectroscopic parameters to those of the extensively characterized high-spin Fe(IV)=O intermediate in taurine:α-ketoglutarate dioxygenase (TauD) (*20−24*). Here we describe the use of rapid-quench approaches to monitor the catalytic cycle of CvPheH. The results provide evidence that an Fe(IV) species, most likely a ferryl complex, is also the hydroxylating intermediate in this bacterial aromatic amino acid hydroxylase.

EXPERIMENTAL PROCEDURES

Materials. *Escherichia coli* strain BL21(DE3), used for protein expression, was from Novagen (Madison, WI). 6-Methyltetrahydropterin (6-$MePH_4$) was from Schircks Laboratories (Jona, Switzerland). L-Phenylalanine, HEPES, and ferrous ammonium sulfate were from Sigma-Aldrich Chemical Co. (Milwaukee, WI). Dithiothreitol was from Inalco (Milan, Italy).

Enzyme Expression and Purification. CvPheH was expressed in *E. coli* and purified as previously described (*17*). The ferric iron present in the purified enzyme was removed by incubation with 5 mM EDTA and 5 mM nitrilotriacetate on ice for 2 h followed by dialysis against 50 mM Hepes (pH 7.2) containing 50 mM NaCl.

Chemical-Quench Analyses. A solution of 100 mM HEPES buffer (pH 7.2) containing apo-CvPheH, 50 mM NaCl, and 5 mM phenylalanine was made anaerobic in a tonometer through 20 argon-vacuum cycles over a period of 1 h. A stoichiometric amount of ferrous ammonium sulfate from a 1.5 mM stock was then added to the tonometer under argon. An aliquot of 6-$MePH_4$ sufficient for a final concentration of 5 mM was placed in one arm of the tonometer. Additional argon-vacuum cycles were performed before the 6-$MePH_4$ was mixed with the CvPheH−Fe(II)−Phe complex in the main body of the tonometer. A solution of 1.9 mM O_2 was generated by bubbling oxygen into an ice-cold solution of 100 mM HEPES (pH 7.2) and 50 mM NaCl. Both oxygenated buffer and anaerobic enzyme solutions were loaded into an SFM-400/Q rapid-mixing instrument from Bio-Logic (Claix, France) calibrated using the reaction of 2,4-dinitrophenylacetate and hydroxide. The reactions were performed at 5 °C and quenched with an equal volume of 5 M HCl. The collected samples were chilled on ice until further analysis.

The precipitated protein was removed by centrifugation for 5 min at 13,700*g*. An aliquot of the sample was diluted in water and injected onto a Waters high-performance liquid chromatography (HPLC) instrument equipped with a model 2475 fluorescence detector. Phenylalanine and tyrosine were separated using a Phenomenex C18 column using 10 mM sodium phosphate with 1% THF (pH 7.0) as the mobile phase (*25*). The intrinsic fluorescence of the amino acids was used for their detection with an excitation wavelength of 270 nm and an emission wavelength of 310 nm. The amount of tyrosine in each reaction mixture was determined by comparison to a tyrosine standard curve.

Freeze-Quench Mössbauer Spectroscopy. A solution of 100 mM HEPES buffer (pH 7.2) containing 1.6 mM apo-CvPheH, 50 mM NaCl, and 5 mM phenylalanine was made anaerobic in a round-bottom flask through several argon-vacuum cycles over a period of 1 h. This solution was placed in an MBraun (Peabody, MA) anoxic chamber with a nitrogen atmosphere, where it was made 5 mM in 6-$MePH_4$ by the addition of an aliquot from a stock solution prepared by dissolving the solid compound in anaerobic 100 mM HEPES buffer (pH 7.2) and 50 mM NaCl. A 50 mM stock solution of $^{57}Fe(II)$ was made by dissolving $^{57}Fe(0)$ in a volume of anaerobic 1 M H_2SO_4 sufficient to deliver 4 mol of H^+/mol of $^{57}Fe(0)$. To prevent precipitation of the enzyme, the stock solution of $^{57}Fe(II)$ was mixed with 0.5 volume equivalents of anaerobic 1 M Tris-HCl (pH 7.6) before its addition to the CvPheH−Phe−6-$MePH_4$ complex at a final concentration of 1.5 mM. An anaerobic control sample was prepared by taking an aliquot of the CvPheH−$^{57}Fe(II)$−Phe−6-$MePH_4$ complex and transferring it to a Mössbauer cell. The cell was sealed in a plastic vial, removed from the glovebox, frozen in liquid nitrogen, removed from the vial, and stored in liquid nitrogen.

The procedure for the preparation of freeze-quench Mössbauer samples has been described previously (*21*). The CvPheH−$^{57}Fe(II)$−Phe−6-$MePH_4$ complex was transferred to a rapid-quench syringe in the glovebox. The syringe was removed from the glovebox and positioned within the rapid-quench instrument. A second syringe filled with oxygen-saturated (at 0 °C) buffer (1.9 mM O_2) was also positioned within the instrument. These two solutions were mixed in a 1:1 ratio at 5 °C and allowed to flow through an aging line before being quenched by direct injection into 2-methylbutane at −150 °C. The total reaction time was calculated by adding 10 ms (the "quench time" that we have determined for a number of similar enzyme systems) to the time of transit through the aging line (*26*).

Mössbauer spectra were recorded on a spectrometer from WEB Research (Edina, MN) operating in the constant acceleration mode in a transmission geometry, equipped with an SVT-400 cryostat (Janis, Wilmington, MA). Spectra were recorded at 4.2 K without an externally applied magnetic field.

Data Analyses. KaleidaGraph (Synergy Software, Reading, PA) was used to analyze single kinetic traces. Global analyses of the kinetic data were performed using KinTek Explorer Pro (KinTek Corp., Austin, TX) (*27*). Mössbauer data analysis was conducted using WMOSS (WEB Research).

RESULTS

Rapid Freeze-Quench Mössbauer Spectroscopy. Mössbauer spectroscopy was used to determine directly if an Fe(IV) species is an intermediate in the CvPheH reaction. An anaerobic solution containing concentrated CvPheH−$^{57}Fe(II)$ complex, phenylalanine, and 6-$MePH_4$ was reacted with an equal volume of an O_2-saturated buffer solution, and the reaction was

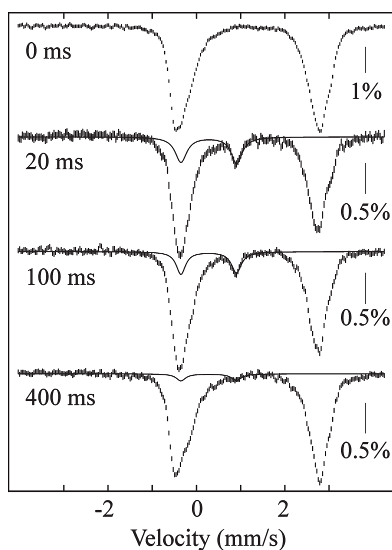


FIGURE 1: 4.2 K/zero-field Mössbauer spectra from the reaction of the CvPheH−^{57}Fe(II)−Phe−6-MePH$_4$ complex with O_2 [concentrations of 0.8 mM CvPheH, 0.75 mM ^{57}Fe(II), 2.5 mM 6-MePH$_4$, 2.5 mM Phe, and 0.95 mM O_2 after mixing] in 100 mM HEPES and 50 mM NaCl (pH 7.2) at 5 °C. The reaction times are indicated. The solid lines are quadrupole doublet simulations of the spectra of the Fe(IV) intermediate with the parameters quoted in the text.

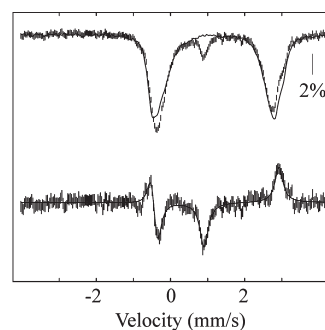


FIGURE 2: Determination of the Mössbauer parameters of the Fe(IV) intermediate observed in CvPheH. The top portion shows the 4.2 K/zero-field Mössbauer spectra of a sample of the CvPheH−^{57}Fe(II)−Phe−6-MePH$_4$ complex before (—) and after exposure to O_2 for 20 ms (vertical bars). The bottom portion shows the difference spectrum (vertical bars) analyzed as a superposition of two quadrupole doublets (—) with the following parameters: $\delta(1)$ = 1.20 mm/s, and $\Delta E_Q(1)$ = 3.45 mm/s (−13% intensity; this component is assigned to the O_2-reactive form of the enzyme); $\delta(2)$ = 0.28 mm/s, and $\Delta E_Q(2)$ = 1.26 mm/s [+17% intensity; this component is assigned to the Fe(IV) intermediate].

quenched when the mixture was injected into 2-methylbutane at −150 °C. Mössbauer spectra of the samples quenched at various reaction times are shown in Figure 1. The spectrum of the reactant CvPheH−Fe(II)−Phe−6-MePH$_4$ complex shows a broad quadrupole doublet with parameters typical of a high-spin Fe(II) (*28*). The asymmetry of the doublet suggests the presence of at least two different Fe(II) complexes that possibly arise from the conversion of a six-coordinate Fe(II) to a five-coordinate square-pyramidal Fe(II) site (*10*). The Mössbauer spectra of samples in which the reactant complex was exposed to O_2 for 20 or 100 ms exhibit a distinct peak at ~0.9 mm/s in addition to the contribution from the high-spin Fe(II) complexes. Because this peak has almost completely decayed after a reaction time of 400 ms, it is associated with a reaction intermediate. Analysis of the spectra of the 20 ms sample, in which the new peak has maximal intensity, and the anaerobic control reveals that the peak at ~0.9 mm/s is the high-energy line of a quadrupole doublet with an isomer shift (δ) of 0.28 mm/s and quadrupole splitting parameter (ΔE_Q) of 1.26 mm/s (Figure 2). These parameters are typical of high-spin Fe(IV) complexes (*29*). Deconvolution of the spectra reveals that the new iron species contributes 17 ± 3% of the absorption at 20 ms and 13 ± 3% at 100 ms. In the spectrum of the 400 ms sample, the upper limit of the new quadrupole doublet is 4% of the total intensity.

Similar substoichiometric accumulation of the Fe(IV) intermediate was previously described for TyrH (*12*), TauD (*21*), and a prolyl-4-hydroxylase (*30*). For the latter two enzymes, it was possible to increase the fraction of the Fe(IV) by taking advantage of the large deuterium kinetic isotope effect of ~50 for the C−H bond cleavage of the substrate (*30*−*32*). Such an approach is not practical for aromatic hydroxylation, because the deuterium kinetic isotope effects for this reaction are inverse at ~0.9 for the aromatic amino acid hydroxylases (*15*−*17*). However, CvPheH will also catalyze the hydroxylation of cyclohexylalanine. This aliphatic hydroxylation reaction does exhibit a large

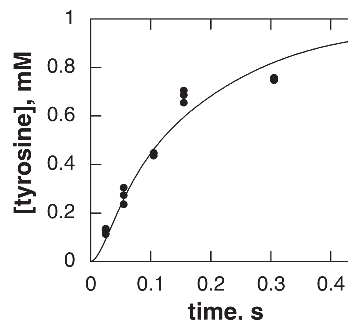
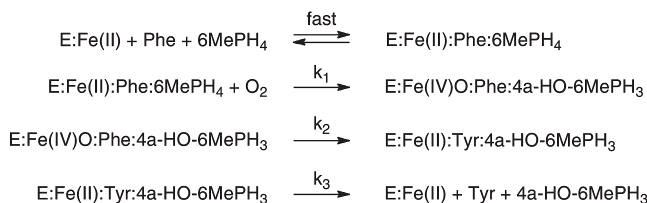


FIGURE 3: Time course for the formation of tyrosine during the reaction of the CvPheH−Fe(II)−Phe−6-MePH$_4$ complex with O_2 (concentrations of 0.8 mM CvPheH, 2.5 mM 6-MePH$_4$, 2.5 mM Phe, and 0.95 mM O_2 after mixing) at 5 °C. The line is from a simulation of the data using the mechanism of Scheme 2 and the following values: k_1 = 19 mM^{-1} s^{-1}, k_2 = 42 s^{-1}, and k_3 = 6 s^{-1}.

deuterium kinetic isotope effect (~15) (*19*), and amino acid hydroxylation is reported to be fully coupled to tetrahydropterin oxidation for this substrate (*33*). Similar rapid-quench Mössbauer experiments were conducted with deuterated cyclohexylalanine in an attempt to increase the amount of the Fe(IV) intermediate, but the intermediate could not be detected with this substrate. We attribute this to the much slower formation of the Fe(IV) intermediate with this unnatural substrate (*34*). A similar reduced substrate triggering efficacy was also observed in the related Fe(II)- and α-ketoglutarate-dependent halogenases (*35*).

Chemical-Quench Studies. The results shown in Figures 1 and 2 are consistent with the transient accumulation of an Fe(IV) species in the CvPheH reaction. To determine if the Fe(IV) intermediate is kinetically competent to be the hydroxylating intermediate, the time course of tyrosine formation was determined under the same conditions that were used to obtain the Mössbauer samples. An anaerobic solution of the CvPheH−Fe(II) complex with an excess of phenylalanine and 6-MePH$_4$ was mixed with O_2-containing buffer, and the reaction was quenched at times up to 300 ms when the solution was mixed with 5 M HCl. The amount of tyrosine produced at each time was then determined by HPLC. As shown in Figure 3, a stoichiometric amount of tyrosine was formed within the first 200−250 ms of the

Scheme 2: Minimal Mechanism for the Hydroxylation of Phenylalanine by PheH



reaction. The data in Figure 3 could be fit by a single-exponential increase in concentration with time to yield a rate constant of 10 $\pm$ 1.6 s^{-1} for the formation of tyrosine by CvPheH. This value is greater than the k_{cat} value at 5 °C of ~5 s^{-1}, suggesting that formation of tyrosine is not rate-limiting for turnover by CvPheH. To more directly probe whether a slower subsequent step limits the reaction, the chemical-quench analysis was repeated at a lower enzyme concentration (100 μM), so that multiple turnovers were possible. The results are shown in Figure 4. There is a clear burst of tyrosine formation in the first 200 ms, followed by a slower linear phase. This result is consistent with hydroxylation being faster than a subsequent step, most likely the release of the product(s) from the active site.

Global Analysis of the Kinetics. A minimal kinetic mechanism consistent with the chemical reaction of PheH and the results presented here is shown in Scheme 2. In the experiments described here, the initial concentrations of phenylalanine and 6-$MePH_4$ were in excess over the enzyme and 100 and 40 times the respective K_m values, so that the binding of these two substrates to the enzyme in the first step of the reaction to form the ternary complex was assumed to be rapid and saturating.[2] Similarly, in the absence of knowledge regarding which product is released first, the release of both products is incorporated into a single step.

The CvPheH−Fe(II)−Phe−6-$MePH_4$ complex reacts with oxygen to form the Fe(IV)=O species and the hydroxypterin; this reaction was treated as a single step in the absence of evidence of an intermediate. It was necessary to model this step explicitly as a second-order reaction, because the complex was mixed with oxygen directly to start the reactions, and the concentrations of enzyme and oxygen were comparable in the experiments shown in Figures 1 and 3. The Fe(IV)=O intermediate reacts with the amino acid substrate to yield tyrosine, converting the iron back to the ferrous form. Finally, the products tyrosine and hydroxypterin dissociate from the enzyme. The results of all the analyses could be reasonably well simulated using this kinetic mechanism and a single set of rate constants (k_1 = 19 mM^{-1} s^{-1}, k_2 = 42 s^{-1}, and k_3 = 6 s^{-1}). The simulations are shown as the lines in Figures 3−5. A change in the value of any of the rate constants of >25% resulted in a visibly worse fit to the data.

DISCUSSION

The results of the rapid-quench analyses presented here establish that an Fe(IV) species accumulates during the reaction of CvPheH. Its kinetics are consistent with the hypothesis that it

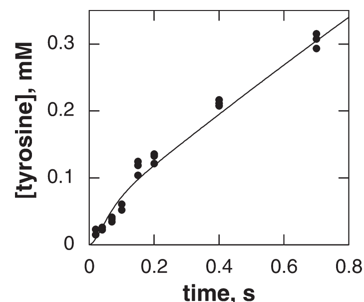


FIGURE 4: Time course for the formation of tyrosine during the reaction of the CvPheH−Fe(II)−Phe−6-$MePH_4$ complex with O_2 (concentrations of 0.1 mM CvPheH, 2.5 mM 6-$MePH_4$, 2.5 mM Phe, and 0.95 mM O_2 after mixing) at 5 °C. The line is from a simulation of the data using the mechanism of Scheme 2 and the following values: k_1 = 19 mM^{-1} s^{-1}, k_2 = 42 s^{-1}, and k_3 = 6 s^{-1}.

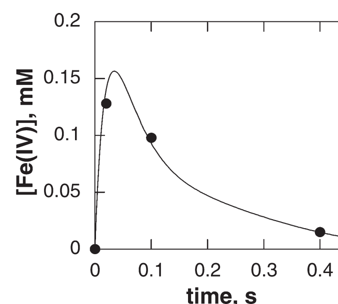


FIGURE 5: Time course for the Fe(IV) intermediate during turnover of CvPheH. The data are from Figure 1, and the line is from a simulation using the mechanism of Scheme 2 and the following values: k_1 = 19 mM^{-1} s^{-1}, k_2 = 42 s^{-1}, and k_3 = 6 s^{-1}.

is the oxygen donor for hydroxylation of the amino acid substrate. The Mössbauer spectral parameters for this Fe(IV) intermediate are similar to those previously reported for the Fe(IV) intermediates in TyrH (*12*) and in several Fe(II)- and α-ketoglutarate-dependent hydroxylases (*21*, *30*) and halogenases (*35*, *38*, *39*). These all have high-spin (S = 2) Fe(IV) centers with similar spectroscopic parameters (isomer shifts of ~0.3 mm/s, quadrupole splittings of −1.3 to −0.8 mm/s, axial zero-field splitting parameters of 10−15 cm^{-1}, and small rhombic zero-field splitting parameters). For the extensively studied Fe(IV) intermediate from TauD, the presence of the ferryl group was conclusively shown by resonance Raman spectroscopy (*20*), X-ray absorption spectroscopy (*22*), and computational studies (*40*). The similar Mössbauer parameters of the Fe(IV) intermediates in CvPheH and TyrH and their demonstrated kinetic competency as the oxygen donors for aromatic hydroxylation support their assignment also as Fe(IV)=O species.

Previous studies of the reactions of CvPheH and the three eukaryotic aromatic amino acid hydroxylases have established that the iron centers in these enzymes have similar reactivities (*17*, *18*). The similar Mössbauer parameters of the Fe(IV) intermediates in TyrH and CvPheH further confirm the similarities of the bacterial and eukaryotic enzymes. In contrast, the structures of the eukaryotic enzymes in the absence of both substrates show the glutamate ligand to the iron being monodentate (*5*, *41*), while the structure of CvPheH shows a bidentate glutamate in the absence of substrates (*3*). A similar arrangement of a bidentate glutamate and two waters as iron ligands has been described for the PheH from *Colwellia psychrerythraea* 34H (*42*). For both TyrH and the eukaryotic PheH, the glutamate becomes

[2]While phenylalanine and the tetrahydropterin necessarily bind in separate steps, a mechanism involving two rapid binding steps is kinetically indistinguishable from one with a single rapid step. Moreover, there is some discrepancy with respect to the order of binding of substrates to CvPheH (*36*, *37*); the mechanism of Scheme 2 is not meant to imply any order of binding of phenylalanine and the tetrahydropterin.

a bidentate ligand only when both an amino acid substrate and a pterin are bound (*10*, *43*, *44*). The importance of this difference in the resting forms of the bacterial and eukaryotic enzymes is not clear. In all cases, the iron site is hexacoordinate in the absence of substrate, with three waters as the remaining ligands in the eukaryotic enzymes and two waters in CvPheH. A productive reaction with oxygen by the pterin-dependent enzymes requires conversion of the iron to a pentacoordinate site, opening a site for oxygen (*10*), so that the arrangement of ligands in the reactive ferryl enzyme is likely the same for the eukaryotic and prokaryotic enzymes. The difference in the type of interaction that the glutamate ligand forms with the iron in prokaryotic PheHs versus the eukaryotic enzymes in the absence of substrates may reflect relatively minor differences in the stability of the two interactions that can be affected by crystal packing forces. Alternatively, it may reflect tighter control of the reactivities of the iron in the larger eukaryotic enzymes in the absence of substrates.

The Fe(IV) intermediate in CvPheH does not accumulate to more that 20% of the total iron present. The nearly stoichiometric formation of tyrosine occurs over the same time period as the Fe(IV) is seen (Figure 3) and more rapidly than a single turnover, as product release limits turnover, establishing that at least 90% of the enzyme is active under the conditions of these analyses. Consequently, the substoichiometric levels of the Fe(IV) complex are not due to low enzyme activity at the high (~1 mM) concentrations used. Instead, the substoichiometric amount can be attributed to the rate constant for the decay of the Fe(IV) being comparable to the rate constant for its formation (*32*, *45*). This conclusion is supported by the global analysis, in that $k_1[O_2] \sim k_2$. A similar, more rapid decay of the Fe(IV) complex has previously been described for TyrH (*12*) and a prolyl-4-hydroxylase (*30*).

Previous analyses of kinetic isotope effects in the CvPheH reaction suggested that formation of the hydroxylated amino acid is followed by a step that is ~9-fold slower (*17*). These results support that conclusion and identify product release as the most likely slow step. The value of k_3, the rate constant for product release obtained from the global analysis, is ~7-fold lower than the value of k_2, the rate constant for tyrosine formation, consistent with the previous analysis. Analyses of the single-turnover kinetics of TyrH (*11*) and tryptophan hydroxylase (*46*) have established that product release is slower than hydroxylation also for those eukaryotic enzymes.

The single-turnover kinetics of the eukaryotic TyrH and our kinetic analysis of the CvPheH reaction make possible a comparison of the intrinsic rate constants for eukaryotic and prokaryotic amino acid hydroxylases. With TyrH, the rate constants for formation of the Fe(IV)=O intermediate and for hydroxylation of tyrosine (*12*) are within error of the values of k_1 and k_2 for CvPheH. This is consistent with the similar reactivities of the iron center in the eukaryotic and prokaryotic enzymes, in support of a similar conclusion based on kinetic isotope effects and the contribution of tunneling to hydrogen abstraction by these enzymes (*18*).

In summary, this study provides spectroscopic evidence of an Fe(IV) intermediate in the catalytic cycle of a bacterial aromatic amino acid hydroxylase with characteristics similar to those observed in the eukaryotic TyrH and other mononuclear non-heme iron enzymes with facial 2-His 1-carboxylate triads (*12*, *21*, *30*). The similar spectroscopic properties and abilities to catalyze both aromatic and aliphatic hydroxylation suggest that the pterin-dependent and α-ketoglutarate-dependent oxygenases represent two mechanisms for forming a common hydroxylating intermediate using a mononuclear iron center.

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