

Spectroscopy and Kinetics of Wild-Type and Mutant Tyrosine Hydroxylase: Mechanistic Insight into O₂ Activation

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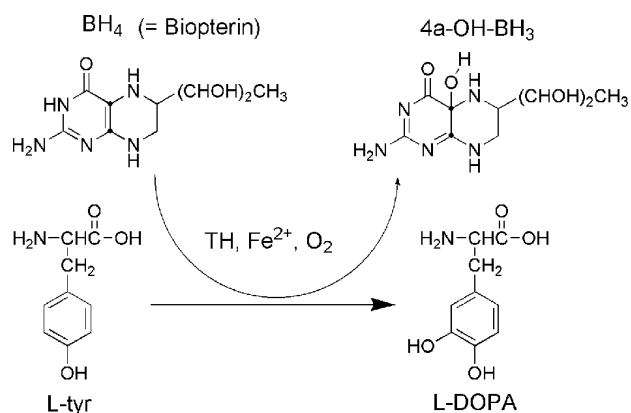
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Abstract: Tyrosine hydroxylase (TH) is a pterin-dependent nonheme iron enzyme that catalyzes the hydroxylation of L-tyr to L-DOPA in the rate-limiting step of catecholamine neurotransmitter biosynthesis. We have previously shown that the Fe^{II} site in phenylalanine hydroxylase (PAH) converts from six-coordinate (6C) to five-coordinate (5C) only when both substrate + cofactor are bound. However, steady-state kinetics indicate that TH has a different co-substrate binding sequence (pterin + O₂ + L-tyr) than PAH (L-phe + pterin + O₂). Using X-ray absorption spectroscopy (XAS), and variable-temperature-variable-field magnetic circular dichroism (VTVH MCD) spectroscopy, we have investigated the geometric and electronic structure of the wild-type (WT) TH and two mutants, S395A and E332A, and their interactions with substrates. All three forms of TH undergo 6C → 5C conversion with tyr + pterin, consistent with the general mechanistic strategy established for O₂-activating nonheme iron enzymes. We have also applied single-turnover kinetic experiments with spectroscopic data to evaluate the mechanism of the O₂ and pterin reactions in TH. When the Fe^{II} site is 6C, the two-electron reduction of O₂ to peroxide by Fe^{II} and pterin is favored over individual one-electron reactions, demonstrating that both a 5C Fe^{II} and a redox-active pterin are required for coupled O₂ reaction. When the Fe^{II} is 5C, the O₂ reaction is accelerated by at least 2 orders of magnitude. Comparison of the kinetics of WT TH, which produces Fe^{IV}=O + 4a-OH-pterin, and E332A TH, which does not, shows that the E332 residue plays an important role in directing the protonation of the bridged Fe^{II}-OO-pterin intermediate in WT to productively form Fe^{IV}=O, which is responsible for hydroxylating L-tyr to L-DOPA.

1. Introduction

Mononuclear nonheme iron enzymes catalyze a wide variety of reactions involving O₂. Tyrosine hydroxylase (TH, EC 1.14.16.2) belongs to the family of enzymes that utilize Fe^{II} and a pterin cofactor to activate dioxygen for aromatic amino acid hydroxylation (Scheme 1).^{1–3} In humans, TH is found in the central and peripheral nervous systems and catalyzes the rate-limiting step in catecholamine neurotransmitter biosynthesis by converting L-tyrosine into L-DOPA. L-DOPA is subsequently converted to dopamine, norepinephrine, and epinephrine, which play key roles in regulating motor-coordination, behavior, learning and memory, sleep–wake cycle, and the stress response. Dysfunction of this enzyme has been linked to several neurological disorders including Parkinson's disease,⁴ bipolar disorder,⁵ and Segawa's syndrome.^{6,7} The other two pterin-dependent hydroxylases also play key roles in proper neuro-

Scheme 1. Pterin-Dependent Reaction Catalyzed by TH



function: phenylalanine hydroxylase (PAH) converts phenylalanine into tyrosine, and its dysfunction results in severe mental retardation (phenylketonuria, PKU).^{8,9} Tryptophan hydroxylase

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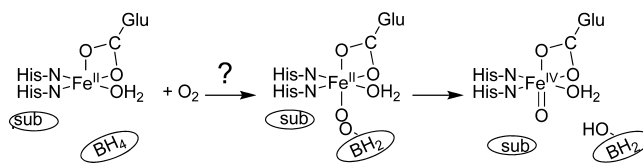
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makes 5-hydroxytryptophan in the first step of serotonin biosynthesis and plays an important role in mood regulation.¹⁰

In bacterial PAH the co-substrates are reported to bind in a specific order: phenylalanine, then pterin, and finally O₂, for productive enzyme turnover,¹¹ although there is some uncertainty in this.¹² Using magnetic circular dichroism (MCD) and X-ray absorption spectroscopies (XAS), we have found that the resting ferrous site of rat PAH is six-coordinate (6C).¹³ Binding of either phenylalanine or 5-deaza-6-methyltetrahydropterin (5-deaza-6MPH₄) only slightly perturbs the 6C Fe^{II} site, whereas binding of L-phe + 5-deaza-6MPH₄ converts the site to five-coordinate (5C) through the loss of a water ligand, and opens a coordination position for O₂ to react.^{14,15} This structural change at the PAH active site was subsequently observed by crystallography.^{16,17} The crystal structures showed that the resting 6C Fe^{II} site was coordinated by two histidines (His), one glutamate (Glu), and three waters. When substrate analogues (norleucine or thienylalanine) and cofactor are bound, the Fe^{II} site becomes 5C, with the Glu binding in a bidentate fashion.

A different sequence of co-substrate binding was observed with steady-state kinetics in TH, where the order is first pterin, followed by O₂ and then tyrosine before product release.¹⁸ In fact, the binding of tyrosine to Fe^{II}TH results in a dead-end complex. These findings suggest that the Fe^{II} active site in TH might undergo a different structural mechanism as compared to PAH, even though the enzymes share high sequence homology³ and perform similar reactions. However, there is limited structural information available on TH. The two crystal structures of TH are of the enzyme in the ferric form, one in resting state (1TOH) and the other with oxidized cofactor, 7,8-dihydrobiopterin, bound (2TOH).^{19,20} Both of these structures show a five-coordinate Fe^{III} site, with two His, one Glu, and two water ligands; the presence of oxidized pterin did not affect the structure of the active site. Mössbauer studies on TH confirmed that the Fe^{II} is high spin $S = 2$, and XAS studies on resting Fe^{II}TH and Fe^{II}TH + tetrahydrobiopterin (BH₄) indicate that both sites are 6C.²¹ The electron paramagnetic resonance (EPR) spectrum of Fe^{III}TH is characteristic of a high-spin,

Scheme 2. Proposed Mechanism for the Formation of Fe^{IV}=O Intermediate (BH₄ = Tetrahydrobiopterin)



predominantly rhombic site.²² At present, there is no structural information on catalytically relevant Fe^{II}TH with tyrosine or tyrosine + pterin bound.

When both pterin and tyrosine are present, TH catalyzes the dioxygen-dependent hydroxylation of both molecules (Scheme 1). The O₂ molecule is partitioned between the hydroxylated substrate and cofactor,^{23–25} leading to the proposed formation of a putative Fe^{II}–peroxy–pterin intermediate (Fe^{II}–OO–pterin),²⁴ which undergoes heterolytic O–O bond cleavage to give the hydroxylating intermediate Fe^{IV}=O and a 4a-hydroxy–pterin adduct (4a-OH–pterin). The Fe^{IV}=O intermediate in TH was recently trapped and studied using Mössbauer spectroscopy.²⁶ The 4a-OH–pterin subsequently dehydrates to produce quinonoid pterin.

Although a mechanism for the formation of the Fe^{IV}=O hydroxylating intermediate has been proposed and generally accepted,^{27–29} earlier steps in the pterin-dependent reaction, specifically the steps leading to the formation of the putative Fe^{II}–OO–pterin intermediate, are still poorly understood (Scheme 2). In a computational study, Siegbahn and colleagues have proposed that O₂ first reacts with Fe^{II} to form a Fe³⁺–O₂^{•−} complex.³⁰ However, this process is thermodynamically unfavorable by ~20 kcal/mol.³¹ In contrast, spectroscopic and kinetic studies on two PAH mutants suggested a different mechanism in which O₂ reacts first with pterin to form a peroxy–pterin species (HOO–pterin) before bridging to Fe^{II}.³²

In this study, Fe K-edge XAS edge and EXAFS (extended X-ray absorption fine structure) spectroscopy combined with near-IR MCD and variable-temperature variable-field MCD (VTVH MCD) are applied to investigate the geometric and electronic structure changes of the Fe^{II}TH active site and the effects of tyrosine and pterin binding. We also studied two mutants of TH that have different reactivity toward the pterin cofactor to correlate these differences in reactivity to active site structural differences. The homologous mutations in PAH have

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been shown to result in PKU.^{33,34} The S395A mutant produces 4a-OH-pterin at the same rate as wild-type (WT), but does so in predominantly uncoupled reaction (2% of WT tyrosine hydroxylation).³⁵ The S395 residue in TH hydrogen-bonds to the His336 Fe^{II} ligand and is in the vicinity of the tyrosine binding pocket. The E332A mutant hydroxylates less than 1% tyrosine compared to WT and does not produce 4a-OH-pterin.³⁶ The E332 residue is hydrogen-bonded to the first sphere ligand His331, as well as to the pterin cofactor. Together, these mutants probe the mechanism of the oxygen/pterin reaction in TH. These results are correlated to the results of new single-turnover kinetic studies on WT TH and the E332A mutant to evaluate the mechanisms of O₂ reaction, formation of a bridged species, and O–O cleavage.

2. Materials and Methods

2.1. Protein Expression: WT and Mutants. WT, E332A, and S395A rat TH were overexpressed in *Escherichia coli* and purified as previously described.^{35–37} After purification, the enzyme was precipitated with 70% ammonium sulfate and dissolved in deuterated MOPS/KCl buffer, pD = 7.3, (containing either 5 mM EDTA for WT and E332A or 1 equiv of ferrous ammonium sulfate, Fe(NH₄)₂SO₄, per monomer for S395A), followed by dialysis against the same buffer. Glycerol-*d*₃ was then added during concentration steps to give final buffer conditions of 100 mM MOPS, pD = 7.3, 65% glycerol-*d*₃ and 300 mM KCl. Iron contents were determined using a Perkin-Elmer Analyst 700 atomic absorption spectrophotometer.³⁸

2.2. Preparation of XAS and MCD Samples. All commercial reagents were of the highest grade and used without further purification. Solutions were made anaerobic either by 10 freeze–pump–thaw cycles or by purging with argon on a Schlenk line. Solids were purged with argon for 1–2 h. Anaerobic reagents were transferred into a nitrogen atmosphere maintained in a Vacuum Atmosphere Nexus-1 glovebox (<5 ppm oxygen). Fe(NH₄)₂SO₄ (J.T. Baker) and sodium dithionite (Sigma-Aldrich) were dissolved in anaerobic D₂O (99.9 atom % D). 6-Methyltetrahydropterin (6MPH₄, Schircks Laboratories) was dissolved in an anaerobic solution of 10 mM HCl. The concentration of the 6MPH₄ stock solution was determined from the absorbance at 266 nm ($\epsilon_{266\text{nm}} = 17.8 \text{ mM}^{-1} \text{ cm}^{-1}$). The redox inactive cofactor 5-deaza-6MPH₄ was synthesized according to literature procedures.³⁹ Concentrated solutions of L-tyrosine (L-tyr) were prepared in D₂O adjusted to pD = 10 using NaOD.

Enzyme samples were made anaerobic by purging with argon. In the glovebox, ~0.85 equiv of Fe(NH₄)₂SO₄ was added to the enzyme, followed by five reducing equivalents of dithionite solution. The Fe^{II}–TH samples were incubated for 10 min, then tyrosine and 6MPH₄ were added as needed, and the samples were incubated for another 10–20 min. Samples for XAS experiments were each syringed into Lucite 23 mm × 2 mm ×

3 mm pinhole type XAS cells with 37 μm Kapton windows. MCD samples were injected into an MCD cell, which consists of two Infrasil quartz disks (Esco Products) separated by a neoprene spacer (0.3 cm path length) and held in place by two fitted copper plates. After removal from the glovebox, the XAS and MCD samples were quickly frozen in liquid nitrogen and stored at 77 K.

Samples for spectroscopy included TH-[], TH-[L-tyr], TH-[6MPH₄], TH-[5-deaza-6MPH₄], and TH-[L-tyr, 6MPH₄] and typically contained 0.8–2 mM Fe^{II}, 2–5 mM tyrosine, 3–5 mM 6MPH₄ (or 5-deaza-6MPH₄), and ~60% v/v glycerol-*d*₃.

2.3. XAS. X-ray absorption spectra were recorded at the Stanford Synchrotron Radiation Lightsource (SSRL) on beamlines 7-3⁴⁰ and 9-3⁴¹ over an energy range of 6785–8000 eV ($k = 15 \text{ \AA}^{-1}$). Hutch slits were set at 2 mm × 10 mm and 1 mm × 4 mm on beamlines 7-3 and 9-3, respectively, with the first inflection point of the Fe foil set to 7111.2 eV as an internal calibration.⁴² Fluorescence data were collected using a solid-state 30-element Ge detector⁴³ with a Mn filter and Soller slits aligned at 90°, with sample orientated at 45° to incident beam. Sample cryogenic temperature of 10 K was maintained using an Oxford Instruments CF1208 continuous-flow liquid helium cryostat. Data collection was continuously monitored in order to ensure sample integrity by averaging blocks of two scans and comparing to the other previous averages. No sample degradation was observed in the edge or EXAFS data in any of the samples, thus all scans were used in the final average. Total scan numbers varied based on sample concentration and desired data quality resulting in 22, 31, 39, and 20 scans for TH-[], TH-[L-tyr], TH-[6MPH₄], and TH-[L-tyr, 6MPH₄] respectively. Reduction and normalization of the data was performed according to established methods^{44–46} using the program PySpline⁴⁷ for background subtraction and spline fitting. In order to compare multiple data sets, the full data set was truncated to $k = 9.5 \text{ \AA}^{-1}$ for pre-edge and edge analysis. The pre-edge data ($k = 9.5 \text{ \AA}^{-1}$) were normalized using a third-order postedge polynomial background and a two-segment spline. For the EXAFS data ($k = 15 \text{ \AA}^{-1}$) a second-order postedge polynomial and three-segment spline was used to appropriately normalize the data.

Using the fitting program EDG_FIT,⁴⁸ pre-edge features were successfully modeled using a pseudo-Voigt line shape in a 50:50 ratio of Lorentzian/Gaussian functions. The energy position, full width at half-maximum (fwhm), and peak intensity were

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(40) Source: SSRL Biological XAS beam line 7-3, 20-pole, 2-T wiggler, 0.8 mrad beam; SPEAR3 at 3 GeV, 85–100 mA. Optics: Si(220), liquid nitrogen cooled double crystal monochromator, fully tuned, with a flat, bent, harmonic-rejection vertically collimating Rh coated Si pre-monochromator M₀ mirror at 9 keV cutoff; energy resolution $1 \times 10^{-4} \Delta E/E$.

(41) Source: SSRL Biological XAS beam line 9-3, 16-pole, 2-T wiggler, 2.0 mrad beam, SPEAR3 at 3 GeV, 85–100 mA. Optics: Si(220), liquid nitrogen cooled double crystal monochromator, fully tuned. Pre-monochromator flat, bent, harmonic rejection vertically collimating Rh coated Si M₀ mirror; and post-monochromator bent, cylindrical, Rh coated Si focusing M₁ mirror; 10 keV cutoff, energy resolution $1 \times 10^{-4} \Delta E/E$.

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all allowed to vary throughout the fitting process. A function modeling the background was empirically chosen to give the best fit. For a fit to be acceptable it had to reasonably match both the pre-edge data as well as that of the second derivative. In all cases three acceptable fits with different fwhm (± 0.5 fixed from float) backgrounds were acquired over the energy ranges of 7108–7116, 7108–7117, and 7108–7118 eV resulting in a total of nine pre-edge fits per data set, which were averaged to get mean values. Standard deviations for the peak energies and intensities from the successful pre-edge fits were used to quantify the error.

EXAFS signals were calculated using FEFF (version 7.0) and a ferric tyrosine hydroxylase crystal structure truncated to an iron ligated by two waters, two His, and one Glu. The starting crystal structure (PDB ID 1TOH)⁴⁹ was then modified with either the addition of a water ligand to make a 6C model, or modification of the Glu to bind in a bidentate fashion in order to achieve a final fit. The models were fit using OPT as part of the program suite EXAFSPAK⁵⁰ by allowing the bond lengths (R) and bond variance (σ^2) to float. The shift in threshold energy ($k = 0, E_0$) was also varied but constrained as a common variable (ΔE_0) for all fit paths for a given data set. The amplitude reduction factors (S_0^2) was fixed to a value of 1.0 and coordination numbers (N) were varied systematically to accomplish the best fit to the data based on the model. All samples fit reasonably well using a combination of four or five paths except for TH[L-tyr, 6MPH₄] which required two additional paths. First coordination spheres were fit using a nitrogen backscatter, with a nitrogen/oxygen mix chosen in cases with a split first shell. As oxygen and nitrogen are indistinguishable by EXAFS, best first shell fits were chosen on the basis of normalized error (F) for all the available paths. Second and outer shell fits were again chosen on the basis of best normalized error (F) for all available paths resulting in the use of carbon single scattering and carbon multiple scattering through the His rings. σ^2 values were set to initial values of 0.005 Å² and then allowed to float in all fits, even for those where single scattering and multiple scattering paths were related to one another. The best overall fit was evaluated based on comparison of the normalized error (F) for each proposed fit and by inspection of the fit to the EXAFS to the data, Fourier transform and individual EXAFS wave components. On the basis of EXAFS studies of known complexes, the uncertainties in final distances are within 0.02 Å.

2.4. MCD Spectroscopy. Low-temperature near-IR MCD data were collected on a Jasco J200D spectropolarimeter (600–2000 nm) equipped with a liquid nitrogen-cooled InSb photodiode detector and fitted with an Oxford Instruments SM 4000–7T superconducting magnet along the beam path. MCD spectra were corrected for zero-field baseline effects either by subtracting the corresponding 0T scan or by averaging the positive and negative field data at each temperature. Gaussian fitting of the MCD spectra was performed using the program PeakFit (SPSS Science). VTVH MCD data were collected at 8 temperatures between 1.6 and 25 K and at 12 fields between 0 and 7 T.

2.5. Single Turnover Kinetics. Single turnover reactions of WT TH and E332A TH were monitored using an Applied Photophysics SX20 stopped-flow spectrophotometer in single wavelength absorbance mode. The stopped-flow instrument was made anaerobic by incubation with a solution of sodium dithionite for at least two hours. Anaerobic solutions of Fe^{II}TH (with or without substrates) were mixed with oxygen-saturated buffer (200 mM HEPES, pH 7.5, 10% glycerol and 0.1 M KCl) at 5 °C. Solutions of apoenzyme (100–300 μM in 200 mM HEPES, pH 7.5, 10% glycerol and 0.1 M KCl) were made anaerobic in a tonometer using at least 20 argon–vacuum cycles; ferrous ammonium sulfate and substrate solutions were then added under argon. Different oxygen concentrations in buffers were achieved by using a modified MaxBlend medical oxygen blender (Maxtec) to mix argon and oxygen in different ratios and then bubbling through buffer-containing syringes on ice for at least 15 min.

Single wavelength absorbance changes consisted of one or more exponential kinetic phases, which were analyzed by first fitting to single or multiple exponentials using the program KaleidaGraph (Synergy Software); results from these fits were then taken as initial estimates for global fits and simulations. Initial values for k_{on} and k_{off} of the reversible oxygen complex formation steps and estimates for unknown extinction coefficients were determined by global fitting of multiple traces (obtained at multiple wavelengths for varying O₂ concentrations) using the program DynaFit.⁵¹ Initial concentrations were allowed to float within 10% of the experimental values. Global fitting process was performed in an iterative fashion, in which one or two parameters were varied at a time and the rest of the parameters were kept constant. Best fit parameters obtained this way were then employed for the kinetic simulation (DynaFit) of the experimental data to appropriate mechanisms.

3. Results and Analysis

3.1. X-ray Absorption Spectroscopy. 3.1.1. Pre-edge. The Fe K pre-edge feature at ~7112 eV, while formally a 1s → 3d electric dipole forbidden transition, gains intensity through the weaker electric quadrupole mechanism, resulting in some pre-edge intensity for centrosymmetric complexes. In lower symmetry, the metal 3d and 4p orbitals mix, and the pre-edge feature increases in intensity through the much larger electric dipole allowed 1s → 4p character.⁵² As a result, XAS pre-edge features are very sensitive to coordination number and geometry.^{52,53} For a typical ferrous model complex in a 6C environment, pre-edge intensities average about 4 units, whereas 5C structures average 12–13 intensity units.⁵² For a protein environment as in TH, pre-edge intensities for a 6C structure are larger than in symmetric model complexes due to variations in ligation, bond lengths and angles.

The pre-edge and edge spectra of four TH complexes are shown in Figure 1. The pre-edge energies and intensities for the four TH complexes listed in Table 1. The normalized data for TH-[] (resting, in red), TH-[L-tyr] (substrate, in blue) and TH-[6MPH₄] (cofactor, in orange) all look very similar with respect to pre-edge shape and intensity. When both cofactor

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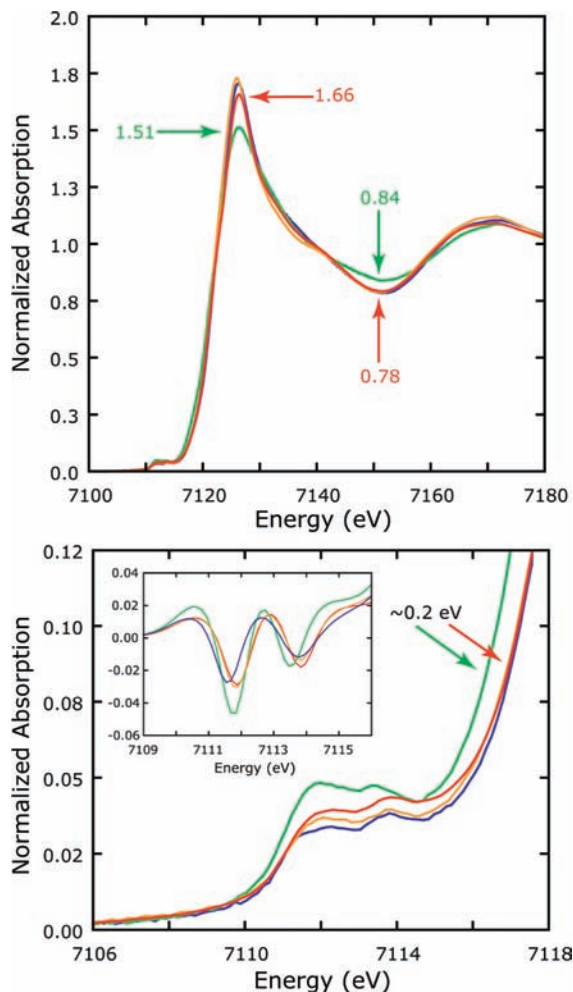


Figure 1. (Top) Fe K edge and pre-edge spectra of TH-[] (red), TH-[L-tyr] (blue), TH-[6MPH₄] (orange), and TH-[L-tyr, 6MPH₄] (green). (Bottom) Enlarged pre-edge region to show finer detail. Inset shows second derivative smoothed for clarity (smoothing = 0.5). A black and white version of this figure can be found in the Supporting Information, Figure S1.

Table 1. XAS Pre-edge Energies and Intensities^a

sample	peak (eV)	area	total intensity
TH-[]	7112.0	4.4	8.3 ± 0.6
	7113.8	3.9	
TH-[L-tyr, 6MPH ₄]	7111.8	6.4	12.5 ± 1.5
	7113.5	6.1	
TH-[6MPH ₄]	7111.9	4.6	8.2 ± 0.7
	7113.8	3.6	
TH-[L-tyr]	7111.9	4.4	8.5 ± 1.0
	7113.7	4.1	

^aPre-edge fit values obtained using EDG_FIT according to established methods.⁴⁸ Peak energies are listed at peak maximum, areas are multiplied by 100 for comparison to other data. Total intensity is the sum of both areas. Error values calculated from total intensity standard deviations across all nine fits per sample.

and substrate are present, TH-[L-tyr, 6MPH₄] (green), the pre-edge shows a notable increase in intensity as well as a shift to lower energy both of which are reflected in the second derivative (Figure 1, inset). A difference is also observed in the edge slope, as well as the maximum intensity of the rising edge. Small differences are also observed at higher energies above the edge (7150 eV in Figure 1), suggesting that a structural change has occurred when both substrate and cofactor are bound to TH.

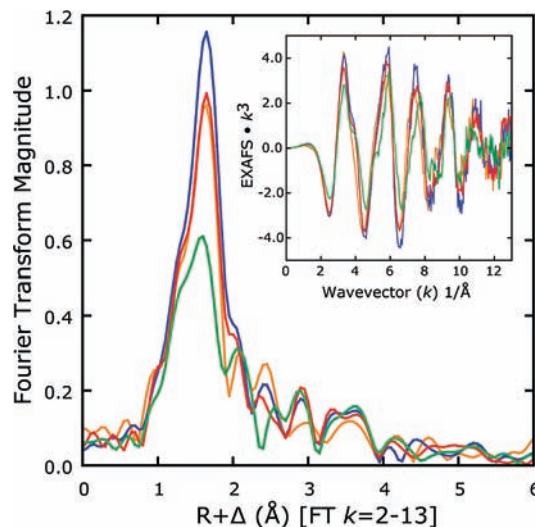


Figure 2. Fourier transforms to $k = 13 \text{ \AA}^{-1}$ for tyrosine hydroxylase. TH-[] (red), TH-[L-tyr] (blue), TH-[6MPH₄] (orange), and TH-[L-tyr, 6MPH₄] (green). The inset shows EXAFS data for the respective samples. A black and white version of this figure can be found in the Supporting Information, Figure S2.

For TH-[], the pre-edge region was accurately fit using two features at 7112.0 and 7113.8 eV with intensities of 4.4 and 3.9, respectively, for a total intensity of 8.3 units. TH-[L-tyr] and TH-[6MPH₄] were both fit with two features at 7111.9 and 7112.8 eV with total intensities of 8.5 and 8.2, respectively. While the total intensities for TH-[], TH-[6MPH₄], and TH-[L-tyr] are greater than that which is typical for O_h 6C model complexes with uniform ligation, they are similar to those found for 6C structures in Fe^{II} metalloenzymes.^{13,21,32,54}

When both substrate and cofactor are present, the total pre-edge intensity of TH-[L-tyr, 6MPH₄] increases to 12.5 units and is fit using two bands at slightly lower energies, 7111.8 and 7113.5 eV with 6.4 and 6.1 intensity units, respectively. This is similar to the 4 units of intensity increase observed in model complexes in going from 6C to 5C, which distorts the ligand field of the Fe^{II} and increases 4p mixing.

3.1.2. EXAFS. EXAFS data and the corresponding Fourier transform (FT) are shown in Figure 2. The intensity of the EXAFS beat pattern of TH-[] (red) and TH-[6MPH₄] (orange) are very similar with respect to the wave minima and maxima contributing to a first shell FT with the same intensity and position. When only substrate is present, an increase in the EXAFS signal is observed (TH-[L-tyr], Figure 2, blue). This increase in EXAFS amplitude is represented in the FT with an increase of about 20% (from 0.98 to 1.16 units) relative to TH-[] and TH-[6MPH₄]. Upon binding of both substrate and cofactor, TH-[L-tyr, 6MPH₄], shows both a decrease in the EXAFS amplitude as well as a slight shift to higher k . There are also subtle differences in the beat pattern including an additional feature at $k = 8.5 \text{ \AA}^{-1}$. The EXAFS amplitude reduction, and shift in k are also reflected in the FT as the first shell signal decreases from 0.98 in resting to 0.61, and shifts toward shorter bond distances.

EXAFS data for all samples were collected to $k = 15 \text{ \AA}^{-1}$; however, depending on data quality, fits were performed to $k = 14, 13, 14,$ and 12 \AA^{-1} for TH-[], TH-[L-tyr], TH-[6MPH₄],

(54) Pavlosky, M. A.; Zhang, Y.; Westre, T. E.; Gan, Q.-F.; Pavel, E. G.; Campochiaro, C.; Hedman, B.; Hodgson, K. O.; Solomon, E. I. *J. Am. Chem. Soc.* **1995**, *117*, 4316–4327.

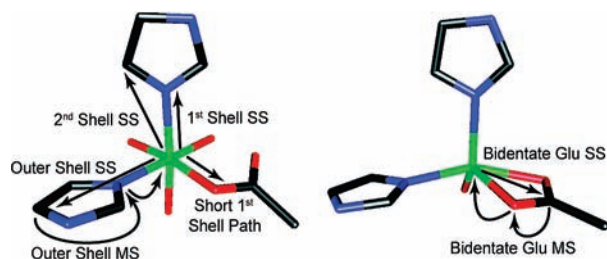


Figure 3. Tyrosine hydroxylase active site EXAFS models. The 6C model (left), based on the crystal structure of Fe^{III}TH (PDB 1TOH),⁴⁹ was used in fitting all EXAFS spectra with single (straight) and multiple (curved arrow) scattering paths shown. The 5C model (right), created from crystal structure of the ternary complex of PAH (PDB 1MMT)¹⁶ was required for successful fitting of TH-[L-tyr, 6MPH₄]. This includes additional single and multiple scattering paths associated with the presence of a bidentate bound Glu. The iron is shown in green, oxygen in red, carbon in black, and nitrogen in blue.

and TH-[L-tyr, 6MPH₄], respectively. All spectra were fit to both 5C and 6C models, which were built based on the ferric crystal structure of tyrosine hydroxylase (PDB 1TOH⁴⁹). Since a bidentate Glu has been observed in PAH-[L-phe, pterin],¹⁶ TH-[L-tyr, 6MPH₄] was also fit to a 5C model with a bidentate Glu. Both 6 and 5C EXAFS models and associated fitting paths are shown in Figure 3. Values for the most relevant EXAFS fits are presented in Table 2, with additional fits given in the Supporting Information.

The EXAFS spectrum for TH-[] is fit equally well using a 6C model with 4 or 5 paths in a 6 or 5 + 1 first shell distribution (fits 1 and 3 in Table 2). The pre-edge data for TH-[] (shown above) also indicate that the iron site is 6C, and this finding is supported by MCD data (vide infra). The outer shell was successfully fit using the remaining carbon single scattering at 3.12 and 4.08 Å and multiple scattering at 4.39 Å from the two histidine (His) rings.

Fits to the EXAFS data when only cofactor is bound (TH-[6MPH₄]) are very similar to those of TH-[]. The best fit to the data is with a 6C split first shell, in a 1:5 ratio (fit 9) with the shorter bond distance at ~2.1 Å and longer bond distances at ~2.2 Å. The outer shell is again successfully fit using only the single and multiple scattering from the His rings. When only substrate is bound, the short bond distance which is seen in TH-[] and TH-[6MPH₄] is no longer observed in the fit of the EXAFS signal of TH-[L-tyr] (fit 6). However, despite the lower error value which results with the addition of a split first shell, a path difference of 0.02 Å is beyond the resolution with data to $k = 13 \text{ \AA}^{-1}$. Thus, the best fit is 4 in which all ligands are incorporated into one 6C shell at 2.16 Å. A symmetric first shell with no resolvable short bond length is consistent with an increase in EXAFS amplitude, as the symmetric shell would provide for a stronger, more uniform signal. This increase is similar to the behavior observed for PAH when the substrate L-phe is present.¹³ For all three samples, when the data are fit using only five backscatters in the first shell rather than six, a decrease in σ^2 value is observed and the F factor increases, indicating a worse fit to the data, with the smallest effect observed for TH-[]. This result is similar to previously reported results for TH-[] and TH-[BH₄], where both forms of the enzyme were observed to contain a 6C first shell.²¹ Thus, TH-[], TH-[6MPH₄], and TH-[L-tyr] forms of tyrosine hydroxylase all contain a distorted 6C site.

For TH-[L-tyr, 6MPH₄], a fit using six ligands in the first shell results in a σ^2 value which is unreasonably large for bond

lengths averaging 2.13 Å (Supporting Information Table S2, fit 10) indicating that the data would be better fit with a 5C first shell. Changing the coordination from six to five also results in a better overall goodness of fit. A split first shell in a 1:4 ratio with bond lengths of 2.00 and 2.15 Å gives a reasonably good fit (Figure 4 and Table 2, fit 12). However, even with the 1:4 split shell, and the outer shell fit in a similar fashion to those for the other tyrosine hydroxylase samples (2 SS and 1 MS carbon paths at ~3.1, 4.1, and 4.4 Å, respectively; Table 2, fit 12), at least one wave is still present in the residual signal from the EXAFS fit (Figure 4). An additional single scattering wave at 2.53 Å and an accompanying multiple-scattering wave at 2.65 Å were needed to complete the fit, which now has a 2:3 split in the first shell (Figure 4 and Table 2, fit 15). The residual wave is available if the Glu has shifted into a bidentate coordination mode (Figure 4). None of the other TH complexes required an additional signal at ~2.5 Å to complete the fit. To test if a His ligand was lost to form the 5C complex, the outer shell signals from the His were halved. This led to unreasonably low σ^2 values, indicating that the data do not support His dissociation (Supporting Information Table S2, fits 7 and 8). The data indicate that the ligands lost are water, similar to the behavior observed in PAH.¹⁵ The k -space data and best fit for all complexes can be found in Supporting Information Figure S3.

3.2. MCD Spectroscopy. 3.2.1. WT Tyrosine Hydroxylase.

The high-spin Fe²⁺ (d^6) free ion has a ⁵D ground state, which splits in an octahedral (O_h) ligand field into a triply degenerate ⁵T_{2g} ground state and a doubly degenerate ⁵E_g excited state, which are separated by 10 Dq (~10 000 cm⁻¹ for O and N ligands). The transitions are very weak in absorption, but are intense at low temperature in MCD. The Fe sites in proteins have lower symmetry which removes the orbital degeneracy of these states. Hence LF transitions to the two components of the ⁵E_g excited state are observed. For a distorted 6C site, two transitions centered around 10 000 cm⁻¹ split by ~2000 cm⁻¹ are observed. In a 5C distorted square pyramidal site, the excited-state splitting (Δ^5E) increases, and transitions are observed at ~10 000 and 5000 cm⁻¹. Transitions in a 5C trigonal bipyramidal site are shifted to lower energy, and are typically observed at <10 000 and <5000 cm⁻¹. Due to lower values of 10 Dq , LF transitions of 4C distorted tetrahedral ferrous sites are observed at low energy, usually between 4000 and 7000 cm⁻¹. Thus, near-IR MCD spectra probe the ⁵E excited-state and provide information on the geometric and electronic structure of the ferrous active site.⁵⁵

Two ligand field (d-d) transitions are observed in the MCD spectrum of resting TH-[], shown in Figure 5A, that can be resolved into two Gaussian bands centered at 8720 and 10 750 cm⁻¹. The pattern of two bands centered around 10 000 cm⁻¹ split by 2030 cm⁻¹ is typical of 6C distorted octahedral ferrous sites. The MCD spectrum of TH-[L-tyr], shown in Figure 5B, appears to be slightly narrower than that of resting TH. The TH-[L-tyr] spectrum is best fit with two Gaussian bandshapes at 9390 and 10 950 cm⁻¹ with the higher energy band less intense. Figure 5C shows the MCD spectrum for TH-[6MPH₄].⁵⁶ This spectrum is also narrower than the resting TH spectrum. However, the spectrum cannot be fit with only one band, and is best fit with one band at 9250 cm⁻¹ and another less intense band at ~10 800 cm⁻¹ (the position of this band is dependent

(55) Pavel, E. G.; Kitajima, N.; Solomon, E. I. *J. Am. Chem. Soc.* **1998**, *120*, 3949–3962.

Table 2. EXAFS Fits^a

TH-[], Resting									
fit 1 (E ₀ = -4.0)			fit 2 (E ₀ = -3.8)			fit 3 (E ₀ = -3.8)			
	CN	R (Å)	σ ² (Å ²)	CN	R (Å)	σ ² (Å ²)	CN	R (Å)	σ ² (Å ²)
Fe-O/N	—	—	—	—	—	—	1	2.10	375
Fe-O/N	6	2.15	678	5	2.15	556	5	2.17	725
C SS	4	3.12	774	4	3.12	776	4	3.12	725
C SS	4	4.08	817	4	4.08	737	4	4.08	797
C-N MS	8	4.39	951	8	4.40	979	8	4.40	961
error			0.15			0.16			0.15
TH-[L-tyr], Substrate									
fit 4 (E ₀ = -3.5)			fit 5 (E ₀ = -3.4)			fit 6 (E ₀ = -3.8)			
	CN	R (Å)	σ ² (Å ²)	CN	R (Å)	σ ² (Å ²)	CN	R (Å)	σ ² (Å ²)
Fe-O/N	—	—	—	—	—	—	1	2.14	542
Fe-O/N	6	2.16	567	5	2.16	457	5	2.16	598
C SS	4	3.11	657	4	3.11	645	4	3.10	666
C SS	4	4.07	785	4	4.07	730	4	4.07	827
C-N MS	8	4.40	1168	8	4.41	1177	8	4.39	1132
error			0.22			0.31			0.21
TH-[6MPH ₄], Cofactor									
fit 7 (E ₀ = -4.5)			fit 8 (E ₀ = 4.3)			fit 9 (E ₀ = -3.7)			
	CN	R (Å)	σ ² (Å ²)	CN	R (Å)	σ ² (Å ²)	CN	R (Å)	σ ² (Å ²)
Fe-O/N	—	—	—	—	—	—	1	2.11	172
Fe-O/N	6	2.17	758	5	2.17	630	5	2.20	785
C SS	4	3.07	809	4	3.08	791	4	3.08	816
C SS	4	4.09	787	4	4.08	719	4	4.08	654
C-N MS	8	4.35	1045	8	4.36	1132	8	4.36	1210
error			0.29			0.34			0.25
TH-[L-tyr, 6MPH ₄], Active Monodentate Glu Model									
fit 10 (E ₀ = -4.1)			fit 11 (E ₀ = -3.8)			fit 12 (E ₀ = -4.3)			
	CN	R (Å)	σ ² (Å ²)	CN	R (Å)	σ ² (Å ²)	CN	R (Å)	σ ² (Å ²)
Fe-O/N	—	—	—	—	—	—	1	2.00	411
Fe-O/N	6	2.13	1127	5	2.13	932	4	2.15	567
C SS	4	3.11	663	4	3.11	602	4	3.10	664
C SS	4	4.07	886	4	4.06	818	4	4.06	867
C-N MS	8	4.37	778	8	4.38	771	8	4.37	769
error			0.27			0.20			0.19
TH-[L-tyr, 6MPH ₄], Active Bidentate Glu Model									
fit 13 (E ₀ = -4.9)			fit 14 (E ₀ = -4.6)			fit 15 (E ₀ = -4.3)			
	CN	R (Å)	σ ² (Å ²)	CN	R (Å)	σ ² (Å ²)	CN	R (Å)	σ ² (Å ²)
Fe-O/N	—	—	—	1	2.04	408	2	2.05	578
Fe-O/N	5	2.14	943	4	2.17	782	3	2.15	628
C SS	1	2.54	336	1	2.50	342	1	2.53	372
C-O MS	4	2.63	336	4	2.68	342	4	2.65	372
C SS	4	3.10	708	4	3.10	821	4	3.11	733
C SS	4	4.05	794	4	4.05	745	4	4.05	760
C-N MS	8	4.38	810	8	4.38	823	8	4.39	769
error			0.12			0.12			0.09

^a Select fit parameters for TH XAS EXAFS samples. All distances are in Å. σ² values are multiplied by 10⁵ for convenience. SS and MS indicate single and multiple scattering pathways, respectively. Error (F) is defined as $F = \frac{\sum[(\chi_{\text{exp}} - \chi_{\text{obsd}})^2 k^6]}{\sum(\chi_{\text{exp}}^2 k^6)}$.

on the intensity distribution of the two Gaussians used to fit the spectrum). Thus, the MCD spectra of TH-[L-tyr] and TH-[6MPH₄] are consistent with both species having distorted 6C geometries with slightly stronger ligand fields (10 Dq ≈ 400

cm⁻¹ higher in energy) and less axial distortion (smaller Δ⁵E) compared to TH-[]. These results are consistent with the XAS results (vide supra) which show that resting, substrate, or cofactor bound forms of TH are 6C.

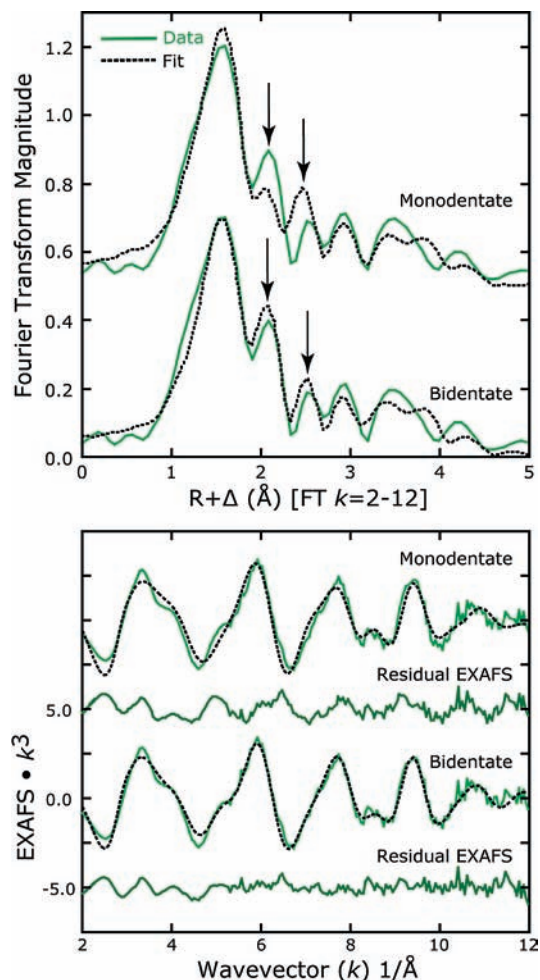


Figure 4. (Top) Fourier transform plots comparing best fits (12 and 15) for TH-[L-tyr, 6MPH₄] using a monodentate vs bidentate Glu model. The monodentate Glu lacks the ~ 2.5 Å feature (black arrows) as shown by the residual component from the monodentate EXAFS fit (below). A bidentate model and fit including a ~ 2.5 Å signal with associated MS path is required to complete the fit. (Bottom) k -space data, fit, and residual corresponding to the above Fourier transforms of fits 12 ($F = 0.19$) and 15 ($F = 0.09$) (Table 2). In all plots, data (—), fits (•••).

In contrast, TH-[L-tyr,6MPH₄] shows only one band at 8960 cm^{-1} (Figure 5D),⁵⁷ suggesting that the second band of the 5E has shifted to below the cutoff of the MCD instrument, and the Fe^{II} site has gone 5C when both substrate and cofactor are present. This is supported by the variable-temperature-variable-field (VTVH) MCD results in the next section and is consistent with the XAS results (vide supra). The $<10\,000$ cm^{-1} energy of the band would support a trigonal bipyramidal site; however, analysis of VTVH MCD data gives a +ZFS system (vide infra) which indicates that the site is closer to square pyramidal.^{55,58,59} The shift of the transition to less than 10 000 cm^{-1} suggests a strong axial perturbation to the iron site which would decrease the equatorial ligand field.

3.2.2. VTVH MCD. While MCD spectra probe the Fe^{II} 5E excited state, the splittings of the 5T_2 ground-state can be obtained

(56) The MCD spectrum of TH-[5deaza-6MPH₄] is shown in the Supporting Information (Figure S4). This normalized spectrum overlays with that of TH-[6MPH₄], indicating that the spectral changes are independent of the nature of the pterin cofactor. This also allows the comparison of the TH[cofactor] spectra to the PAH-R[cofactor] spectrum (ref 13), which shows much less perturbation compared to PAH-T[].

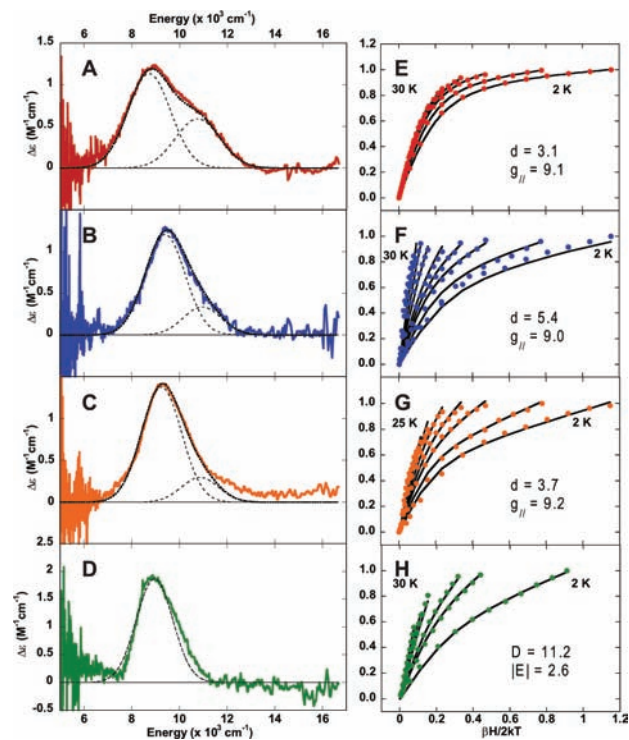


Figure 5. Near-IR MCD spectra and saturation behavior for TH-[] (A and E, in red), TH-[L-tyr] (B and F, in blue), TH-[6MPH₄] (C and G, in orange), and TH-[L-tyr, 6MPH₄] (D and H in green). In A, B, and C, the sum of the Gaussian-resolved bands (dashed lines) yield the generated spectrum (dotted lines), which reproduce the observed spectrum well. VTVH data (points) and their best fits (—) collected at 7950 cm^{-1} for TH-[] (E), 8855 cm^{-1} for TH-[L-tyr] (F), 8230 cm^{-1} for TH-[6MPH₄] (G), and 8245 cm^{-1} for TH-[L-tyr, 6MPH₄] (H).

by VTVH MCD. The field and temperature dependence of the normalized MCD intensity of the TH complexes are plotted against $\beta H/2kT$ (where β is the Bohr magneton, H is magnetic field in T, k is Boltzmann's constant, and T is temperature in K) in Figure 5E–H. The resulting isotherms are nested (nonsuperimposed) and this nesting behavior can be used to gain insight into the ground-state electronic structure of the Fe^{II} sites.^{28,58,60} The 3-fold orbitally degenerate $^5T_{2g}$ ground-state will split in energy in the low-symmetry protein environment. This splitting can be defined in terms of an axial splitting of the d_{xy} from the $d_{xz,yz}$ orbitals by an amount Δ , and rhombic splitting of the d_{xz} and d_{yz} orbitals by an amount $|V|$. The resultant orbitally nondegenerate ground-state is still 5-fold degenerate in spin ($S = 2$), and undergoes a zero-field splitting (ZFS), due

(57) We also attempted to fit the MCD spectrum of Fe^{II}TH[tyr,6MPH₄] with two bands (Figure S6 in the Supporting Information), but the best resultant does not improve the fit to data. In addition, the two Gaussian bands used in the best fit have widths of ~ 1500 cm^{-1} , narrower than the ~ 2000 cm^{-1} required to fit all other ferrous d–d bands. Moreover, the two Gaussian bands used in the fit have an energy separation of ~ 1000 cm^{-1} , which is too small to be associated with a single ferrous center, where the minimum splitting is the ~ 1400 cm^{-1} that would occur in a very symmetric 6C environment, (Jones, G. D. Phys. Rev. 1967, 155, 256–261).

(58) Solomon, E. I.; Pavel, E. G.; Loeb, K. E.; Campochiaro, C. *Coord. Chem. Rev.* **1995**, 144, 369–460.

(59) A recent Mossbauer study on Fe^{II} TH-[tyr,6MPH₄] shows an asymmetric signal, indicating that two distinct Fe^{II} complexes ($\sim 80:20$ ratio) are present (ref 26). However, the second minor Fe^{II} species is not detectable in MCD, which suggests that the ligand field and geometry of the proposed minor second Fe^{II} TH-[tyr,6MPH₄] complex is very similar to the major Fe^{II} complex observed in the MCD samples.

(60) Solomon, E. I. *Inorg. Chem.* **2001**, 40, 3656–3669.

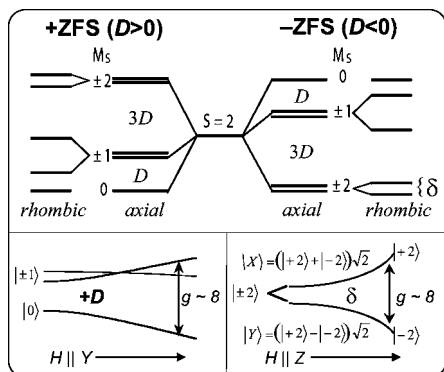


Figure 6. Energy splittings of the $S = 2$ ground state for positive ZFS (top left) and negative ZFS (top right). Rhombic and magnetic field splitting and mixing of the $M_S = 0$ and one $M_S = \pm 1$ component (bottom left), $M_S = \pm 2$ non-Kramers doublet (bottom right).

to spin-orbit coupling of the low symmetry split components of the 5T_2 state, to yield $M_S = \pm 2, \pm 1$, and 0 sublevels split by $3D$ and D , where D is the axial ZFS parameter. Systems with weak axial ligand fields have negative ZFS ($D < 0$) and the $M_S = \pm 2$ doublet is the lowest energy (Figure 6, top right). This will further split by an amount δ due to the rhombic ZFS splitting of the 5T_2 . When a magnetic field is applied, the splitting of the $M_S = \pm 2$ non-Kramers doublet increases due to the Zeeman effect ($g_{\parallel}\beta H$) and shows nonlinear dependence on magnetic field due to a change of its wave function (Figure 6, bottom right). Simulations show that magnitude of δ is directly proportional to the degree of nesting of MCD isotherms.⁶¹ For positive ZFS systems ($D > 0$, Figure 6, top left) which corresponds to strong axial LF perturbation of the 5T_2 , the $M_S = 0$ sublevel is lowest in energy. When a perpendicular magnetic field is applied, it interacts with one component of the $M_S = \pm 1$, which is D cm⁻¹ above it in energy. The resulting pseudodoublet will Zeeman split and behave similarly to the $M_S = \pm 2$ doublet in the $-ZFS$ case to yield similar nonlinear field dependence (Figure 6, bottom left). In general, $+ZFS$ systems, which are split by the large axial ZFS (D), have much larger nesting behavior than $-ZFS$ systems.^{55,61}

The spin-Hamiltonian parameters can be obtained from VTVH MCD data by computationally fitting the field and temperature dependence of the normalized MCD intensity, taking into consideration linear B-terms, z-polarization, and thermal population of states (eq 1 in ref 55). Once δ and g_{\parallel} (and D and g_{\parallel} for $+ZFS$) are known, they can be used⁵⁸ to obtain Δ and $|V|$, the axial and rhombic splitting of the t_2 set of d orbitals. All the TH VTVH MCD data were fit with both $-ZFS$ and $+ZFS$ models, which can be distinguished from each other by comparing the spin-Hamiltonian values obtained. In particular, $+ZFS$ systems will give nonphysical, low g_{\parallel} values (vs $g_{\parallel} = 8.8\text{--}10$ cm⁻¹ for $-ZFS$ 6C), and a low-lying excited-state singlet when fit to a $-ZFS$ model.⁵⁸

From Figure 5E, the saturation data of the TH-[] bands exhibit a very small nesting and can be fit with a $-ZFS$ model giving spin-Hamiltonian parameters of $\delta = 3.1$ cm⁻¹ and $g_{\parallel} = 9.1$ cm⁻¹. These yield 5T_2 ground-state parameters of $\Delta = -200$ cm⁻¹ and $|V| = 60$ cm⁻¹. The doublet ground-state and small splitting of the t_{2g} d orbitals are typical of a 6C Fe^{II} site with moderate rhombic distortion ($|V/2\Delta| \approx 0.15$). The splitting of

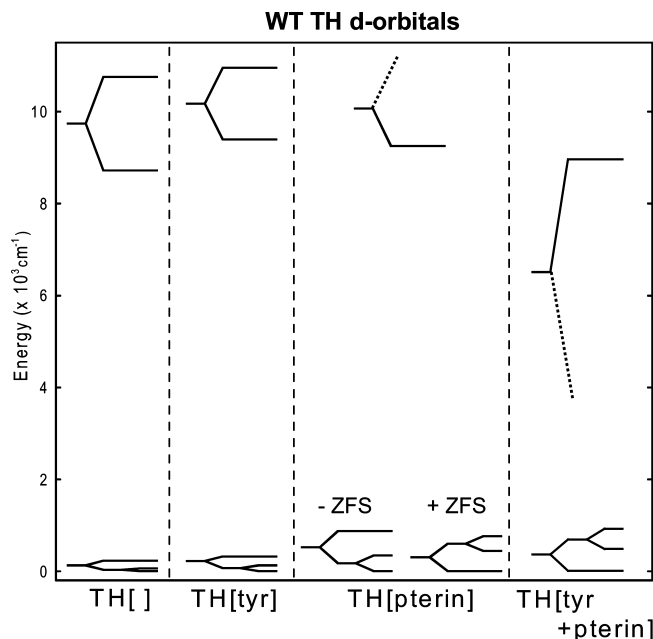


Figure 7. Experimental d-orbital energies of Fe^{II}TH complexes. The excited-state splittings are obtained from band positions in the NIR-MCD spectra, and ground-state splittings from VTVH MCD. Dotted lines represent uncertainty in energy positions of the orbitals.

the 5T_2 ground-state is combined with the excited-state splitting ($\Delta^5E = 2030$ cm⁻¹, from the MCD spectrum) to give the experimental d-orbital energy level diagram for TH-[] (Figure 7, left).

The VTVH MCD data for TH-[L-tyr] (Figure 5F) show increased nesting compared to resting TH, but are still described as a $-ZFS$ system with $\delta = 5.4$ cm⁻¹, $g_{\parallel} = 9.0$ cm⁻¹ to yield $\Delta = -250$ cm⁻¹ and $|V| = 130$ cm⁻¹. The large δ and relatively small Δ are consistent with an Fe^{II} site with a higher degree of rhombicity ($|V/2\Delta| = 0.26$), and thus a larger splitting of the t_2 orbitals is observed. The excited-state data presented earlier shows that tyrosine binding shifts the e orbitals to higher energy ($10 Dq = 10\,170$ cm⁻¹), and decreases their splitting to 1560 cm⁻¹. Overall, the experimental orbital energy levels for TH-[L-tyr] (Figure 7, second panel) are consistent with a distorted 6C iron site.

The VTVH data of TH-[6MPH₄] also show more nesting than that of resting TH and fit equally well to both $-ZFS$ and $+ZFS$ models (Supporting Information Figure S5). A larger total splitting of the orbitals of the ground-state is observed when pterin binds to the enzyme regardless of the sign of the ZFS (Figure 7, third panel). However, from the XAS data (vide supra) that TH-[6MPH₄] is 6C, which is consistent with a $-ZFS$. The parameters for the doublet ground state ($-ZFS$) in TH-[6MPH₄] are $\delta = 3.7$ cm⁻¹, $g_{\parallel} = 9.2$ cm⁻¹, $\Delta = -500$ and $|V| = 220$ cm⁻¹. The orbitals of the 5E excited-state of TH-[6MPH₄] are also shifted to higher energy, suggesting an overall stronger 6C LF at the Fe^{II} center relative to resting.

The saturation behavior of TH-[L-tyr, 6MPH₄] is dramatically different from TH-[]. The saturation curves are significantly more nested, and must be fit with a $+ZFS$ system. The ground-state of TH-[L-tyr, 6MPH₄] is now $M_S = 0$ and gives $D = 11.2$ cm⁻¹, $|E| = 2.6$ cm⁻¹, $\Delta = 700$ cm⁻¹ and $|V| = 420$ cm⁻¹. The large t_2 orbital splittings and $+ZFS$ for TH-[L-tyr, 6MPH₄] are consistent with a 5C site that is closer to square pyramidal in geometry. The ground-state analysis yields $|V/2\Delta| = 0.3$, indicating a high degree of rhombic distortion. In addition, the

(61) Campochiaro, C.; Pavel, E. G.; Solomon, E. I. *Inorg. Chem.* **1995**, *34*, 4669–4675.

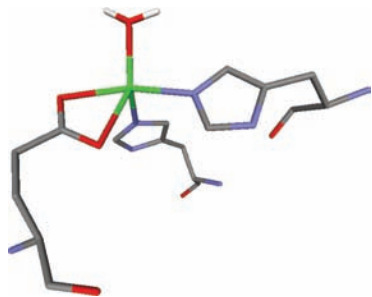


Figure 8. Geometry of Fe^{II} site in PAH with substrate analogue and cofactor bound (PDB 1MMT),¹⁶ showing a rhombically distorted equatorial ligand field and a short Fe–OH₂ bond.

excited-state transitions for TH-[L-tyr, 6MPH₄] occur at 8960 and <5000 cm⁻¹, a range consistent with a 5C site with a strong axial distortion. Although there is no crystal structure of this ternary complex of TH, the MCD data of PAH are very similar and examination of the structure of PAH with cofactor and substrate analog bound (PDB ID 1MMT¹⁶) shows a 5C Fe^{II} site that has one short axial bond and rhombically distorted equatorial ligand field (Figure 8). Excited and ground-state parameters obtained from MCD spectra and VTVH analyses of the four TH complexes are summarized in Table 3.

3.2.3. TH Mutants: E332A and S395A. The S395A-[] spectrum, shown in Figure 9A (brown) has a shape very similar to wild-type TH-[], but is almost 2-fold more intense. The spectrum is fit well with two Gaussian bands at 8850 and 10 870 cm⁻¹, and the saturation behavior of the LF bands shows small nesting (Figure 9B) and fits to a -ZFS system. Together, these indicate that the iron site in S395A-[] is distorted 6C. As in wild-type TH, the substrate + cofactor bound form of S395A has only one resolvable transition (9330 cm⁻¹) in its MCD spectrum (Figure 9C tan). Also, as with the WT enzyme, the VTVH data of S395A-[L-tyr, 6MPH₄] (Figure 9D) show a large increase in nesting and yield a +ZFS system, showing that the active site has become 5C square pyramidal.

The MCD spectrum of E332A-[] (Figure 10A, purple) shows two bands centered around 10 000 cm⁻¹ but looks qualitatively quite different from that of wild-type TH. The intensity ratios of the two bands are reversed—the higher energy band now has more intensity than the lower energy band. The Gaussian-resolved band positions are 8880 and 10 610 cm⁻¹ and give Δ⁵E of 1830 cm⁻¹. The smaller excited-state splitting suggests less distortion of this 6C site than in resting wild-type TH. The saturation data (Figure 10B) of this mutant show small nesting and are fit to a -ZFS system, confirming that the ferrous site is 6C. When both substrate and cofactor are bound to E332A, the MCD spectrum changes to show only one band (9150 cm⁻¹ Figure 10C, light blue). The VTVH data of this band (Figure 10D) again exhibit very large nesting and yield a +ZFS system, confirming that the iron site in E332A-[L-tyr, 6MPH₄] is 5C distorted square pyramidal.

The ligand field, spin-Hamiltonian, and ground-state parameters for TH S395A and E332A are listed in Tables S3 and S4 in the Supporting Information.

3.3. Kinetics. Stopped-flow single-turnover kinetic experiments were performed on WT and E332A TH to further probe the mechanism of the reaction of TH with pterin and O₂. The oxidation of Fe^{II}TH to Fe^{III}TH results in a broad increase in the absorbance spectrum between 200 and 450 nm (Figure 11A), corresponding to a ligand to Fe^{III} charge transfer transitions.³⁷ In addition, the reduced and oxidized pterin species have

distinctive absorbance spectra over the same wavelength region (Figure 11B); these can be used to identify pterin-based intermediates formed during the reaction. In the rapid kinetic experiments, the oxidation of Fe^{II} was followed as absorbance increases at 246 and 350 nm (Figure 11A, black to orange). The oxidation of 6MPH₄ to quinonoid 6MPH₂ (q-6MPH₂) is uniquely characterized by increased absorbance at 350–450 nm (solid red to dashed green in Figure 11B). The formation of 4a-HO-6MPH₃ from 6MPH₄ was detected by absorbance changes at two diagnostic wavelengths: a maximal initial absorbance increase at 246 nm and an accompanying decrease at 300–340 nm (solid red to dotted blue in Figure 11B). The slow dehydration of 4a-HO-6MPH₃ to q-6MPH₂ is accompanied by a decrease at 246 nm and an increase at 300–450 nm (blue to green in Figure 11B).

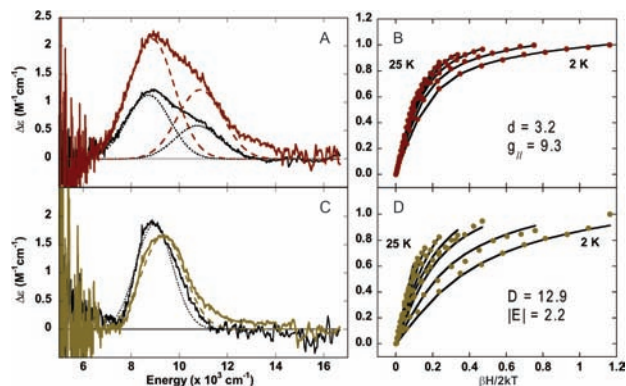
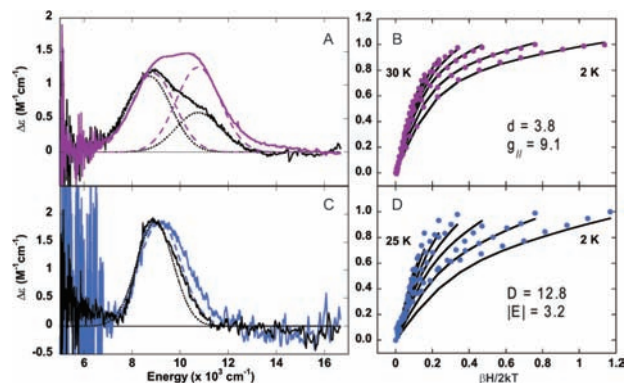
Representative stopped-flow absorbance traces from the reactions of different TH complexes (~100 μM final concentration) with varied concentrations of O₂ (95–950 μM) at 5 °C are shown in Figures 12 and 13, and S7–S10. All the reactions could be successfully simulated using one of the mechanisms in Scheme 3. The rate constants used in the simulations are summarized in Table 4. Addition of O₂ to resting Fe^{II}TH-[] results in monophasic absorbance increases at 246 and 350 nm (Figure 12, dashed red trace) with a single step second-order mechanism with a rate constant of 0.037 mM⁻¹ s⁻¹ (Scheme 3A, Table 4 and Figure S7), in agreement with previous studies at 20 °C.³⁷ Similarly, the oxidation of 6MPH₄ to q-6MPH₂ in the presence of apo-TH is well-described as a single second-order reaction with a rate constant of 1.7 × 10⁻⁴ mM⁻¹ s⁻¹ (Table 4), comparable to the rate of tetrahydropterin auto-oxidation in solution under the same conditions (1.3 × 10⁻⁴ mM⁻¹ s⁻¹, results not shown).

The absorbance changes accompanying the reaction with oxygen of TH complexes containing substrates or analogs are more complex. All are better described by an initial reversible oxygen binding step to form Enz:O₂ complexes followed by chemical transformations (Scheme 3B,C and Figures S8–S10). The presence of tyrosine or 6MPH₂ has little effect on the kinetics of Fe^{II} oxidation (Figure 12). The Fe^{II}TH-[5-deaza-6MPH₄] complex is an exception; in this case, the rate constants are the same as for Fe^{II}TH-[6MPH₂], but the magnitudes of the absorbance changes are much smaller and are consistent with the amount of the free enzyme in these samples (Figure 12, gray dotted and black solid lines, respectively), suggesting that binding of 5-deaza-6MPH₄ to TH significantly decreases the reactivity with oxygen.

The rate constant for Fe^{II} oxidation in the Fe^{II}TH-[6MPH₄] complex is about 5-fold faster than that for the other binary complexes containing the redox-inactive pterins. This suggests that a change in the oxidation state of the pterin accompanies oxidation of the Fe^{II}. Scheme 3B, in which the reaction of O₂ with Fe^{II}TH-[6MPH₄] results in concomitant oxidation of Fe^{II} to Fe^{III} and 6MPH₄ to 6MPH₃[•] in the first step, followed by disproportionation of 6MPH₃[•] to give q-6MPH₂ and 6MPH₄[•], accounts well for the absorbance traces at 246 and 350 nm (Figures 12 and S9). The rate constant for 6MPH₄ oxidation in the Fe^{II}TH-[6MPH₄] complex shows a 3 orders of magnitude acceleration over the auto-oxidation reaction in apo-TH-[6MPH₄] (0.16 vs 1.7 × 10⁻⁴ s⁻¹, respectively). The mechanism of Scheme 3B is consistent with the redox potentials of the individual species. Using an E⁰ of + 210 mV for the Fe^{II} in

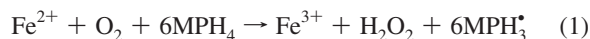
Table 3. Excited State Transition Energies, Spin-Hamiltonian, and Ground State Parameters for Fe^{II}TH Complexes^a

sample	band 1	band 2	10 Dq	Δ ⁵ E	d	g	D	E	Δ	V	V/2Δ
TH-[]	8720	10 750	9735	2030	3.1	9.1	—	—	-200	60	0.15
TH-[L-tyr]	9390	10 950	10 170	1560	5.4	9	—	—	-250	130	0.26
TH[6MPH ₄]	9250	~10 900	~10 100	1610	3.7	9.2	—	—	-500	220	0.22
TH[L-tyr, 6MPH ₄]	8960	—	—	>4000	5.6	8.0	11.4	2.4	600	324	0.27
					5.2	8.0	11.2	2.6	700	420	0.30

^a Values are in cm⁻¹.**Figure 9.** MCD spectra at 5 K and 7 T of S395A mutant. Gaussian-resolved bandshapes for each spectrum are shown in dashed lines. (A) S395A-[] (brown) compared to WT TH-[] (black), (B) VTVH data (•) and fit (—) of S395A-[] collected at 7830 cm⁻¹, (C) S395A-[L-tyr, 6MPH₄] (tan) compared to WT TH-[L-tyr, 6MPH₄] (black), (D) VTVH data (•) and fit (—) of S395A-[L-tyr, 6MPH₄] collected at 8333 cm⁻¹.**Figure 10.** MCD spectra at 5 K and 7 T of E332A mutant. Gaussian-resolved bandshapes for each spectrum are shown in dashed lines. (A) E332A-[] (purple) compared to WT TH-[] (black), (B) VTVH data (•) and fit (—) of E332A-[] collected at 8650 cm⁻¹, (C) E332A-[L-tyr, 6MPH₄] (light blue) compared to WT TH-[L-tyr, 6MPH₄] (black), (D) VTVH data (•) and fit (—) of E332A-[L-tyr, 6MPH₄] collected at 8306 cm⁻¹.

TH,⁶² the one-electron oxidation of Fe²⁺ + O₂ → Fe³⁺ + O₂^{-•} (O₂ + e⁻ → O₂^{-•}, E⁰ = -330 mV)⁶³ has E⁰ = -537 mV and is uphill by 12.5 kcal/mol. Similarly, the reaction of 6MPH₄ to 6MPH₃·H⁺ (E⁰ = -270 mV)⁶⁴ with O₂ has E⁰ = -600 mV and is energetically uphill by 14 kcal/mol. However, the two-electron reaction between Fe²⁺, O₂ and 6MPH₄, (O₂ + 2e⁻ → H₂O₂, E⁰ = +280 mV)⁶³ has E⁰ = +83 mV and is downhill at -1.9 kcal/mol. Taken together, all these results indicate that the concerted two-electron transfer to O₂ from 6MPH₄ and Fe^{II}

(eq 1) is more favorable than the individual one-electron oxidation processes.



When O₂ is mixed with Fe^{II}TH-[L-tyr,6MPH₄] to form a catalytically competent complex, a reversible oxygen complex is initially formed with no observable absorbance change. This is followed by biphasic absorbance changes at 246, 318, and 330 nm over ~4 s (Figure 13, dashed green trace). This rapid initial first phase corresponds to the formation of 4a-OH-6MPH₃ with a rate constant of 24 s⁻¹ (Scheme 3C and Figure S10). The 4a-OH-6MPH₃ is formed in the same step as the Fe^{IV}=O intermediate (Schemes 2 and 3C). Rapid-quench Mössbauer spectroscopy has previously shown that the Fe^{IV}=O intermediate is also formed with a rate constant of 24 s⁻¹ under the same conditions used here.²⁶ The slower second phase was successfully modeled as the release of 4a-OH-6MPH₃ from the enzyme active site into the solution with a rate constant of 0.42 s⁻¹ (Figure S10), a value which matches *k*_{cat} under these conditions. The 4a-OH-6MPH₃ subsequently dehydrates in solution to produce q-6MPH₂, resulting in a gradual decrease at 246 nm and increases at 318 and 330 nm, with a rate constant of ~0.02 s⁻¹. The reactions of O₂ with Fe^{II}TH-[L-tyr,5-deaza-6MPH₄] and Fe^{II}TH-[L-tyr,6MPH₂], which presumably have similar 5C sites,⁶⁵ are similar to Fe^{II} oxidation in Fe^{II}TH with either 6MPH₂ or tyrosine bound (Figure 13A and Table 4). This indicates that both a 5C Fe^{II} and a redox active pterin are needed for the 2 (and greater) orders of magnitude rate enhancement in the reactivity with O₂ seen with Fe^{II}TH-[L-tyr,6MPH₄] compared to Fe^{II}TH-[], Fe^{II}TH-[L-tyr], and Fe^{II}TH-[6MPH₄].

The absorbance changes upon addition of O₂ to E332A-Fe^{II}TH-[L-tyr,6MPH₄] are shown in Figure 13 (solid blue traces). The enzyme clearly catalyzes a reaction between Fe^{II}, O₂, and pterin, but the absorbance changes are distinct from those seen with WT Fe^{II}TH-[L-tyr,6MPH₄]. In the case of the E332A mutant, the observed absorbance changes can be accounted for by initial reversible oxygen addition followed by formation of an intermediate in a single slow step with a rate constant of 0.3 s⁻¹; this value matches *k*_{cat} for this mutant enzyme. The intermediate decays nonenzymatically with almost the same rate constant (~0.02 s⁻¹) as the dehydration of 4a-HO-6MPH₃ to q-6MPH₂. The reaction catalyzed by the E332A mutant is clearly slower than that of the WT enzyme. The rate constant for the second step is similar to the rate constant for release of the 4a-HO-6MPH₃ from the WT enzyme. This raises the possibility that the observed rate constant is a net rate constant which includes formation of the intermediate and its release from the enzyme; however, these two rate constants are too similar to resolve. Compared to WT TH, the magnitudes of the absorbance

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(65) While there is no structural data on Fe^{II}TH-[L-tyr,5-deaza-6MPH₄], parallel MCD and XAS data on Fe^{II}PAH-[L-phe,5-deaza-6MPH₄] indicate that the Fe^{II} site is 5C (refs 14 and 15). Similarly, the crystal structure of Fe^{II}PAH-[thietylalanine,6MPH₂], the PAH analogue of Fe^{II}TH-[L-tyr,6MPH₂], show that the Fe^{II} site is 5C (refs 16 and 17).

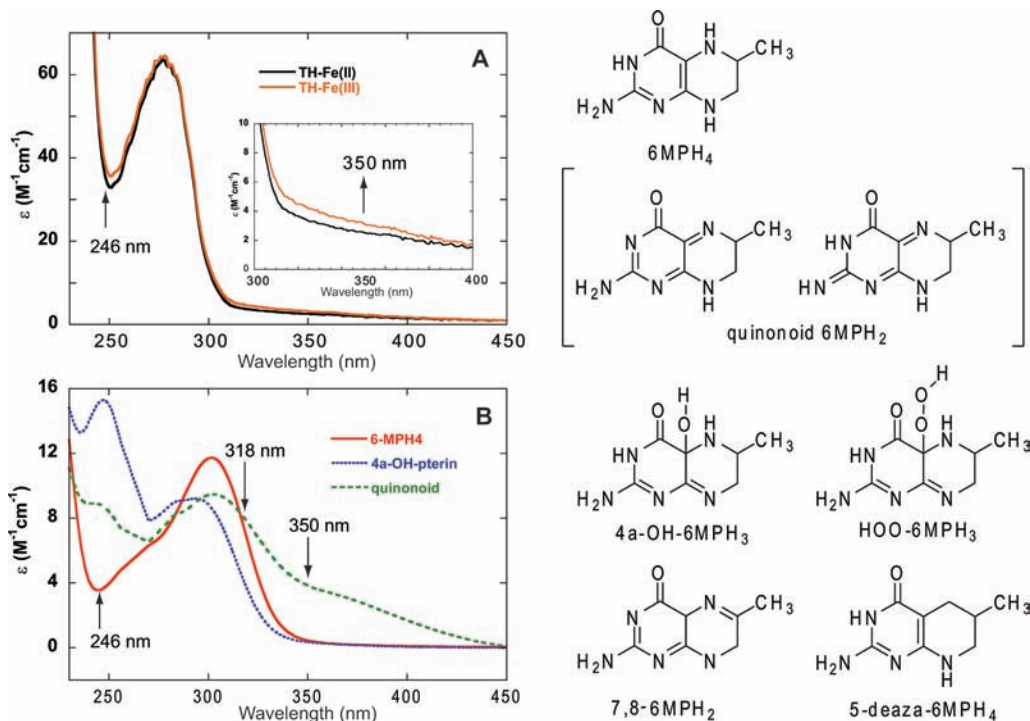


Figure 11. (Left) Absorbance spectra of $Fe^{II}TH$ (A) and pterin species (B) at neutral pH. (Right) Structures of pterin species.

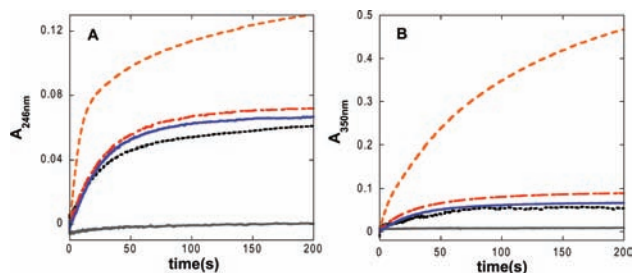


Figure 12. Kinetic traces at 246 (A) and 350 nm (B) upon mixing a final concentration of 950 μM O_2 with the following $Fe^{II}TH$ complexes (90–140 μM): $Fe^{II}TH-[]$ (dashed red line), $Fe^{II}TH-[6MPH_4]$ (dashed orange line), $Fe^{II}TH-[5-deaza-6MPH_4]$ (solid gray line), $Fe^{II}TH-[L-tyr]$ (solid blue line), and $Fe^{II}TH-[6MPH_2]$ (dotted black line).

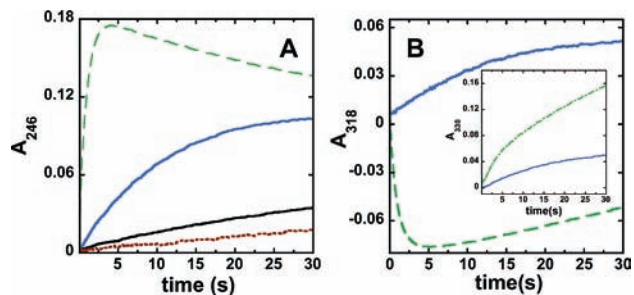
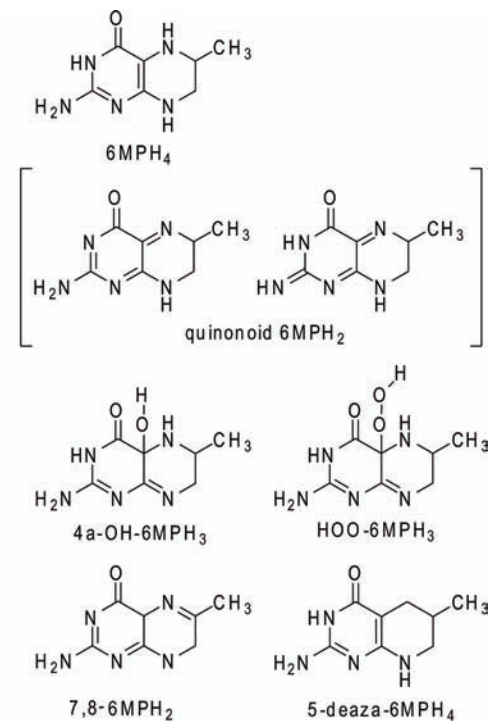
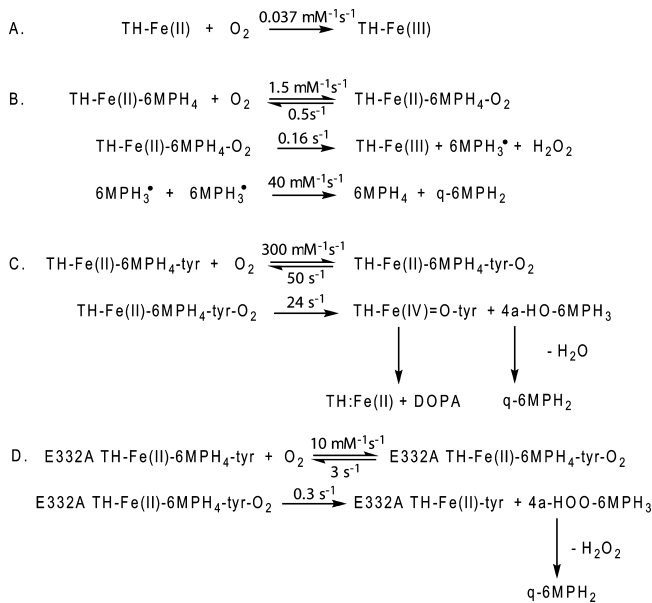


Figure 13. Kinetic traces at 246 (A) and at 318 and 330 nm (B) upon mixing a final concentration of 95 μM O_2 with the following $Fe^{II}TH$ complexes: 150 μM WT $TH-[L-tyr,6MPH_4]$ (dashed green line), 150 μM E332A $TH-[L-tyr,6MPH_4]$ (light blue line), 100 μM WT $TH-[L-tyr,5-deaza-6MPH_4]$ (dotted brown line), and 120 μM WT $TH-[L-tyr,6MPH_2]$ (solid black line).

changes at 246 and 330 nm and the sign of the absorbance change at 318 nm after O_2 binding are different for E332A TH (Figure 13, solid blue traces). The spectrum of the intermediate could be obtained by global analysis of the absorbance changes between 240 and 450 nm and fitting the data to a three-step



Scheme 3. Reactions of Various TH Complexes with O_2



kinetic model (Figure S11). The starting spectrum and those of the final two species match those for 6MPH₄, q-6MPH₂, and 7,8-6MPH₂. The spectrum of the unknown intermediate exhibits a number of differences from that of 4a-HO-6MPH₃, including a much lower absorbance at 246 nm and a red-shifted absorbance maximum. These differences establish that, in contrast to WT TH, a 4a-HO-pterin is not formed in the reaction of E332A- $Fe^{II}TH$ [L-tyr, 6MPH₄] with O_2 . However, the decay of the intermediate in the E332A TH reaction to q-6MPH₂ with a rate constant nearly identical to that for 4a-HO-6MPH₃ suggests that this intermediate is similar to it. Spectral differences similar to those between this intermediate and that of 4a-HO-6MPH₃ have been observed previously for 4a-HO- and 4a-HOO-flavins, intermediates in the reaction of the flavoprotein phenol

Table 4. Rate Constants for the Reactions of WT and E332A TH Complexes with O₂ at 5 °C

Fe ^{II} TH + O ₂ → Fe ^{III} TH (mM ⁻¹ s ⁻¹)				
Fe ^{II} TH-[]	0.037			
	Fe ^{II} TH + O ₂ → Fe ^{II} TH:O ₂			
	<i>k</i> _{on} (mM ⁻¹ s ⁻¹)	<i>k</i> _{off} (s ⁻¹)	Fe ^{II} TH:O ₂ → Fe ^{III} TH (s ⁻¹)	6MPH ₄ → q-6MPH ₂ (mM ⁻¹ s ⁻¹)
Fe ^{II} TH-[6MPH ₄]	1.5	0.5	0.16	40
apoTH-[6MPH ₄]				0.00017
Fe ^{II} TH-[5-deaza-6MPH ₄]			no reaction	
Fe ^{II} TH-[6MPH ₂]	2.0	0.5	0.032	
Fe ^{II} TH-[L-tyr]	2.0	0.5	0.040	
Fe ^{II} TH-[5-deaza-6MPH ₄ ,L-tyr]	2.0	0.4	0.026	
Fe ^{II} TH-[6MPH ₂ , L-tyr]	6.0	0.5	0.050	
Fe ^{II} TH:O ₂ → Fe ^{IV} =O + 4a-HO-6MPH ₃				
Fe ^{II} TH-[L-tyr, 6MPH ₄]	300		50	24 s ⁻¹
Fe ^{II} TH:O ₂ → Fe ^{II} + HOO-6MPH ₃				
E332A-[L-tyr, 6MPH ₄]	10		3	0.3 s ⁻¹

hydroxylases.^{66,67} Thus, a possible candidate for this intermediate species in the E332A TH reaction is a hydroperoxy-pterin, HOO-6MPH₃.⁶⁷

The rapid single-turnover kinetics experiments presented above allow the dissection of individual steps of TH reaction, and show that the presence of a 5C Fe^{II} site and a redox-active pterin greatly accelerates the enzyme reaction with O₂. The data on the WT vs E332A reactions also provide insights into the nature of the intermediates formed in the coupled vs uncoupled reaction.

4. Discussion

In this study, we present new spectroscopic insight and information on the catalytically relevant ferrous active site of TH and its interaction with tyrosine and 6-methyltetrahydropterin (6MPH₄). XAS and MCD studies show that the Fe^{II} active site is 6C in resting TH. The geometric and electronic structure of the Fe^{II} site is slightly perturbed when either tyrosine or pterin is bound to TH, but the site remains 6C in both cases. The active site goes 5C when both tyrosine and pterin are bound, opening up a coordination position for the O₂ reaction. The EXAFS data for TH-[L-tyr,6MPH₄] are also consistent with a 5C Fe^{II} site with two His, one bidentate Glu, and one water as ligands, comparable to the crystal structure determined for Fe^{II}PAH with thienylalanine and BH₄ bound (PDB 1KWO¹⁷). The 6C → 5C change in the TH active site is consistent with the general mechanistic strategy observed in other nonheme iron enzymes that use redox-active cofactors to activate O₂ for reaction with substrates.^{28,60,68}

Earlier steady-state kinetic studies on WT TH were consistent with an ordered binding sequence of pterin followed by oxygen in rapid equilibrium and then tyrosine before the DOPA product is released.¹⁸ This result suggested that the Fe^{II} site in TH could

undergo different structural changes upon pterin binding which allow the Fe^{II}, pterin, and O₂ to react before tyrosine binds. The protein itself does undergo conformational changes upon pterin binding.⁶⁹ However, our spectroscopic data show only a slight perturbation to the 6C Fe^{II} site upon pterin binding. While steady-state experiments give information on the overall reaction, they do not provide information on individual mechanistic steps.⁷⁰ In addition, these experiments were complicated by the use of a large excess of 6MPH₄, required to ensure steady-state conditions, because the excess 6MPH₄ also reduces the Fe. Here, we have used single-turnover kinetics experiments with tyrosine and 6MPH₄ (added in stoichiometric amounts to minimize side reactions) to evaluate the individual steps and identify short-lived intermediates in the TH reaction.

The single turnover kinetic results indicate that the isolated one-electron oxidation reactions of Fe^{II} to Fe^{III} in Fe^{II}TH-[] and tetrahydropterin to quinonoid dihydropterin (q-6MPH₂) in apoTH-[6MPH₄] are slow due to unfavorable thermodynamics. However, the combination of Fe^{II} and redox-active pterin in Fe^{II}TH-[6MPH₄] accelerates both the Fe^{II} and uncoupled pterin oxidation reactions, even though the Fe^{II} site remains 6C. The faster reaction rates in Fe^{II}TH-[6MPH₄] can be ascribed to the favorable thermodynamics of the two-electron reduction of O₂ to peroxide by the combined reaction of Fe^{II} → Fe^{III} and 6MPH₄ → 6MPH₃[•]. When both pterin and tyrosine are bound to give a 5C Fe^{II}TH site, the first observable step in the O₂ reaction, corresponding to the breakdown of the putative Fe^{II}-OO-pterin intermediate, is 2–3 orders of magnitude faster than the Fe^{II}, 6MPH₄, and Fe^{II} + 6MPH₄ oxidation reactions in the 6C and noncatalytic 5C forms of TH (Table 4). The measured rate constant for this step necessarily sets a lower limit for the rate constant for the initial reaction with oxygen to form this intermediate. Thus the two electron transfer from the 5C Fe^{II} and reduced pterin to O₂ is far more favorable than in the 6C Fe^{II}TH-[6MPH₄] site and than in the 5C Fe^{II}TH-[L-tyr,5-deaza-6MPH₄] and Fe^{II}TH-[L-tyr,6MPH₂] sites, confirming that *the open coordination position on the Fe^{II} and the presence of a redox active pterin are both critical for catalytic TH reactivity.*

(66) The absorbance spectrum of 4a-OH-flavin and HOO-flavin are compared in ref 67. The λ_{max} of H-OO-flavin is shifted to lower energy and has lower ε compared to 4a-OH-flavin. A similar type of perturbation can be expected for the HOO-pterin vs 4a-OH-pterin spectra. In fact, a 2 nm red-shift is observed in the peroxy vs hydroxy adduct of 5-deaza-6MPH₄ by Moad et. al.³⁹

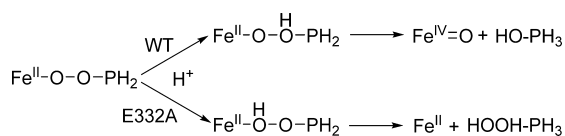
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Scheme 4. Proposed Protonation Sites in WT and E332A TH



In an earlier study on PAH mutants related to PKU, structural differences in the active site were observed that suggested that the pterin will react with O_2 to make peroxypterin which decays in the uncoupled reaction to give off H_2O_2 .³² Here, we are able to observe individual reaction steps in the coupled WT and uncoupled E332A reactions when both tyrosine and 6MPH₄ are bound. The first step with observable kinetics in WT TH is the decay of this putative intermediate, which occurs at a rate of 24 s^{-1} . The 4a-OH-pterin that forms in WT TH (and in the S395A mutant) is not observed in the E332A mutant, which also does not hydroxylate tyrosine, indicating that the $\text{Fe}^{\text{IV}}=\text{O}$ intermediate also does not form. The first observable step of the E332A TH reaction is the formation of an oxidized pterin species, possibly the HOO-pterin, with a rate constant of 0.3 s^{-1} . This intermediate may form either by direct reaction of O_2 with 6MPH₄ (however, this reaction is much slower at $1.7 \times 10^{-4} \text{ mM}^{-1} \text{ s}^{-1}$), or, more likely, through a $\text{Fe}^{\text{II}}-\text{OO}$ -pterin intermediate as in WT TH. In the latter case, the E332A mutation may change the reactivity of the $\text{Fe}^{\text{II}}-\text{OO}$ -pterin intermediate such that it decomposes with a slower rate and through a different mechanism than WT. In both, decay of the intermediate likely involves protonation of the $\text{Fe}^{\text{II}}-\text{OO}$ -pterin. In WT TH, the nature of the cleavage products, $\text{Fe}^{\text{IV}}=\text{O}$ and 4a-OH-pterin indicate that the O distal (O_{dis}) to the Fe^{II} is protonated to heterolytically cleave (Scheme 4, top). In the E332A mutant, an oxidized pterin species, possibly the HOO-pterin, is formed. This would involve the protonation of the O proximal to Fe (O_{prox}) in the $\text{Fe}^{\text{II}}-\text{OO}$ -pterin, followed by the cleavage of the $\text{Fe}-\text{O}_{\text{prox}}$ bond (Scheme 4, bottom). These results suggest that the E332 residue participates in the reactivity of TH by directing the distal protonation of the $\text{Fe}^{\text{II}}-\text{OO}$ -pterin intermediate.

The crystal structure of Fe^{II} -PAH with thienylalanine and tetrahydrobiopterin bound (PDB 1KW0, Figure 14)¹⁷ indicates that the E332 carboxylate group is oriented toward the pterin,

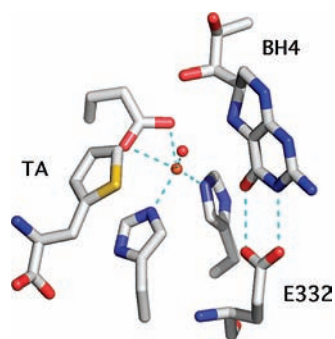


Figure 14. Crystal structure of the Fe^{II} active site of PAH showing the binding positions of the amino acid substrate (TA, thienylalanine, cofactor (BH₄, tetrahydrobiopterin), metal ligands and the E332 residue (using the TH numbering). The figure was created from the PDB file 1KW0.¹⁷

and could hydrogen bond to it, either through direct interaction with the proton on the N3 or through solvent molecules. The E332 residue could either directly transfer the proton from N3 to O_{dis} (N3 is deprotonated in the final quinonoid pterin product), or direct a solvent water via an H-bond to the O_{dis} . The water ligand is not likely to be the proton source for heterolytic O–O cleavage because the MCD data of the TH-[L-tyr, 6MPH₄] complexes in WT and E332A indicate that the geometric and electronic structures of the 5C sites are very similar (Figure 10C). Apparently, when E332 is mutated to an alanine, the enzyme can no longer direct a proton to O_{dis} , and the O_{prox} is protonated instead, leading to the cleavage of the $\text{Fe}-\text{O}$ bond to regenerate Fe^{II} and HOO-pterin.

In summary, we have shown through XAS and MCD spectroscopy that the Fe^{II} active site in TH is 6C until both tyrosine and pterin are bound. This 6C \rightarrow 5C conversion is consistent with the general mechanistic strategy observed in other mononuclear nonheme iron enzymes. Single turnover kinetic data on WT TH indicate that at a 6C active site, the two electron reduction of O_2 to peroxide by Fe^{II} and pterin is favored over individual one electron reactions. However, this reaction is still slow. Importantly, this reaction is greatly accelerated in the Fe^{II} TH-[L-tyr, 6MPH₄] complex, indicating that both 5C Fe^{II} and pterin are required for the O_2 reaction. Comparison of the kinetics of WT and E332A TH-[L-tyr, 6MPH₄] reactions with O_2 indicate that the E332 residue plays a role in directing the protonation of the bridged $\text{Fe}^{\text{II}}-\text{OO}$ -pterin complex to produce the $\text{Fe}^{\text{IV}}=\text{O}$ hydroxylating intermediate and hence is important for tuning the TH active site for productive coupled turnover. Application of the spectroscopic and mechanistic analyses described in this study to variants of TH related to neurological disorders would provide molecular-level insight into enzyme dysfunction and define potential therapeutic approaches.

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Supporting Information Available: Details of EXAFS fits, the MCD spectrum and VTVH data of WT Fe^{II} TH-[5-deaza-6MPH₄], ligand field and spin Hamiltonian parameters for E332A and S395A, kinetic traces at different wavelengths for single-turnover experiments and proposed mechanisms based on the kinetics. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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