Direct coordination of pterin to Fe$^{II}$ enables neurotransmitter biosynthesis in the pterin-dependent hydroxylases

Shyam R. Iyer$^a$, Kasper D. Tidemand$^b$, Jeffrey T. Babicz Jr$^a$, Ariel B. Jacobs$^a$, Leland B. Gee$^a$, Laerke T. Haahr$^b$, Yoshitaka Yoda$^c$, Masayuki Kurokuzu$^d$, Shinji Kitao$^d$, Makina Saito$^d$, Makoto Seto$^d$, Hans E. M. Christensen$^{b,e}$, Günther H. J. Peters$^{b}$, and Edward I. Solomon$^{a,1}

$^a$Department of Chemistry, Stanford University, Stanford, CA 94305; $^b$Department of Chemistry, Technical University of Denmark, 2800 Kongens Lyngby, Denmark; $^c$Japan Synchrotron Radiation Research Institute, Hyogo 679-5198, Japan; $^d$Institute for Integrated Radiation and Nuclear Science, Kyoto University, Osaka 590-0494, Japan; and $^e$Bioneer A/S, 2970 Hørsholm, Denmark

Edited by JoAnne Stubbe, Massachusetts Institute of Technology, Cambridge, MA, and approved January 26, 2021 (received for review October 26, 2020)

The pterin-dependent nonheme iron enzymes hydroxylate aromatic amino acids to perform the biosynthesis of neurotransmitters to maintain proper brain function. These enzymes activate oxygen using a pterin cofactor and an aromatic amino acid substrate bound to the Fe$^{II}$ active site to form a highly reactive Fe$^{IV}$ = O species that initiates substrate oxidation. In this study, using tryptophan hydroxylase, we have kinetically generated a pre-Fe$^{IV}$ cis sites available for cofactor, substrate, and oxygen binding served 2-His/1-carboxylate facial triad motif, which leaves three circular dichroism spectroscopy), these studies both experimen-

tal amino acids to perform the biosynthesis of neurotransmit-

ers and demonstrates a unified mechanism for oxygen activation by the cofactor-dependent nonheme iron enzymes.

Edited by JoAnne Stubbe, Massachusetts Institute of Technology, Cambridge, MA, and approved January 26, 2021 (received for review October 26, 2020)

The pterin-dependent nonheme iron enzymes hydroxylate aromatic amino acids to perform the biosynthesis of neurotransmitters to maintain proper brain function. These enzymes activate oxygen using a pterin cofactor and an aromatic amino acid substrate bound to the Fe$^{II}$ active site to form a highly reactive Fe$^{IV}$ = O species that initiates substrate oxidation. In this study, using tryptophan hydroxylase, we have kinetically generated a pre-Fe$^{IV}$ cis sites available for cofactor, substrate, and oxygen binding served 2-His/1-carboxylate facial triad motif, which leaves three circular dichroism spectroscopy), these studies both experimen-
tal amino acids to perform the biosynthesis of neurotransmit-
ers and demonstrates a unified mechanism for oxygen activation by the cofactor-dependent nonheme iron enzymes.

Edited by JoAnne Stubbe, Massachusetts Institute of Technology, Cambridge, MA, and approved January 26, 2021 (received for review October 26, 2020)

The pterin-dependent nonheme iron enzymes hydroxylate aromatic amino acids to perform the biosynthesis of neurotransmitters to maintain proper brain function. These enzymes activate oxygen using a pterin cofactor and an aromatic amino acid substrate bound to the Fe$^{II}$ active site to form a highly reactive Fe$^{IV}$ = O species that initiates substrate oxidation. In this study, using tryptophan hydroxylase, we have kinetically generated a pre-Fe$^{IV}$ cis sites available for cofactor, substrate, and oxygen binding served 2-His/1-carboxylate facial triad motif, which leaves three circular dichroism spectroscopy), these studies both experimen-
tial amino acids to perform the biosynthesis of neurotransmit-
ers and demonstrates a unified mechanism for oxygen activation by the cofactor-dependent nonheme iron enzymes.

The mononuclear pterin-dependent nonheme iron enzymes catalyze the rate-limiting step in neurotransmitter biosynthesis and are essential in maintaining proper brain function. These enzymes utilize molecular oxygen, a redox active pterin cofactor, and a ferrous active site to generate an Fe$^{IV}$-oxo intermediate that catalyzes substrate oxidation. This study demonstrates that the pterin cofactor directly coordinates to the iron center before oxygen activation and also coordinates to a kinetically generated peroxy-Fe$^{III}$ intermediate that is transiently observed in Fe$^{IV}$-oxo formation. The direct coordination of the pterin cofactor to the iron center enables facile electron transfer to promote rapid oxygen reduction that facilitates the biological function of this family of enzymes and thus defines a unified oxygen activation mechanism for the cofactor-dependent nonheme iron enzymes.

Significance

The mononuclear pterin-dependent nonheme iron enzymes catalyze the rate-limiting step in neurotransmitter biosynthesis and are essential in maintaining proper brain function. These enzymes utilize molecular oxygen, a redox active pterin cofactor, and a ferrous active site to generate an Fe$^{IV}$-oxo intermediate that catalyzes substrate oxidation. This study demonstrates that the pterin cofactor directly coordinates to the iron center before oxygen activation and also coordinates to a kinetically generated peroxy-Fe$^{III}$ intermediate that is transiently observed in Fe$^{IV}$-oxo formation. The direct coordination of the pterin cofactor to the iron center enables facile electron transfer to promote rapid oxygen reduction that facilitates the biological function of this family of enzymes and thus defines a unified oxygen activation mechanism for the cofactor-dependent nonheme iron enzymes.

PNAS 2021 Vol. 118 No. 15 e2022379118

Published online March 5, 2021.


The authors declare no competing interest.

This article is a PNAS Direct Submission.

Published under the PNAS license.

1To whom correspondence may be addressed. Email: solomone@stanford.edu.

This article contains supporting information online at https://www.pnas.org/lookup/suppl/doi:10.1073/pnas.2022379118/-/DCSupplemental.

Published April 5, 2021.

https://doi.org/10.1073/pnas.2022379118

1 of 8
which the pterin cofactor donates its two electrons for O₂ activation with the low barrier required for its biological function.

**Results**

**Spectroscopic Definition of the Ternary Complex.** From absorption spectroscopy (Fig. 1A, Top), addition of tryptophan (Trp) to the pterin (BH₄) bound Fe²⁺-TPH site (forming the ternary complex) produces a new absorption feature at 330 nm (30,300 cm⁻¹, ε = 3,000 M⁻¹ · cm⁻¹) that was previously observed in PAH (22). From low temperature magnetic circular dichroism (MCD) data (Fig. 1A, Bottom Inset), an excited state spectroscopy sensitive to the paramagnetic nature of the ground state at low temperature, substrate binding to the (Fe²⁺/BH₄)-TPH site also results in a large spectral change in the ligand field region with the appearance of a transition at ~5,000 cm⁻¹, indicating that the Fe²⁺ has converted to a 5C site in this ternary complex (analysis in SI Appendix, Defining Coordination Geometry Using Magnetic Circular Dichroism (MCD) Spectroscopy). Extending the MCD data into the charge transfer region (Fig. 1A) shows that the ~30,000 cm⁻¹ absorption band in the ternary complex has temperature-dependent intensity at 5 K (SI Appendix, Fig. S1), which requires that the paramagnetic Fe²⁺ participate in this transition. Thus, this 330 nm absorption feature must involve a charge transfer between the Fe²⁺ and either the pterin or tryptophan, as transitions involving the other first sphere ligands to the Fe²⁺ do not contribute in this energy region (SI Appendix, Fig. S2). Although this absorption feature forms upon tryptophan addition to the (Fe²⁺/BH₄)-TPH site, tryptophan cannot coordinate to the Fe²⁺ and perform productive chemistry because of the lack of a heteroatom oriented toward the Fe²⁺ center. On the other hand, the carbonyl functional group of the reduced pterin cofactor can directly bind to the Fe²⁺ center to produce the high absorption intensity of this charge transfer.

From time-dependent (TD) density functional theory (DFT) calculations (SI Appendix, Figs. S3–S6), the 30,000 cm⁻¹ absorption feature is assigned as a ligand-to-metal charge transfer (LMCT), which is a transition between the π HOMO (highest occupied molecular orbital) of the reduced pterin and a dn* orbital on the Fe²⁺. DFT calculations on the one electron oxidized pterin cofactor result in structural distortions along the carbonyl (C=O) and C₄α-N₅ bonds (SI Appendix, Figs. S7 and S8) that would be...
present in resonance Raman spectroscopy, which is a technique that probes the vibrations associated with metal–ligand distortions of the charge transfer transition leading to resonance enhancement. Several resonance enhanced features are observed in the resonance Raman data collected on the ternary complex ($\lambda_{ex} = 334.5$ nm, Fig. 1B and SI Appendix, Fig. S9), where the highest energy vibration at $1,601$ cm$^{-1}$ is lower than the corresponding vibration observed for reduced pterin cofactor, which has its carbonyl stretch at $1,695$ cm$^{-1}$ (23). From frequency calculations on the pterin-bound ternary complex, the direct coordination of the pterin through its carbonyl functional group elongates the C$–$O bond by 0.037 Å, which results in a reduction of the C$–$O stretching frequency by 63 cm$^{-1}$ (SI Appendix, Table S1) that reproduces the experimental Raman data. While there were early reports that suggested a metal–pterin interaction from spectroscopic studies on CuII-PAH (24, 25) and the ternary complex in PAH (26), the subsequent crystal structures of the ternary complex of PAH in 2002 showed no pterin coordination (18, 19, 27), and since then, it has been generally thought that the pterin cofactor does not bind directly to the iron in the pterin-dependent hydroxylases. Here, our spectroscopic data have defined the direct coordination of the pterin cofactor through its carbonyl functional group to the FeII ternary active site before O$_2$ reactivity.

**O$_2$ Reaction with Ternary Complex: Formation and Decay of a Pre-Fe$^{IV} = O$ Intermediate.** The single turnover reaction of O$_2$ with (Fe$^{II}$/BH$_4$/Trp)-TPH (with stoichiometric amounts of BH$_4$ and Trp) was monitored by stopped-flow absorption spectroscopy. The results from this reaction in D$_2$O/sucrose buffer are presented below as it enables the maximal accumulation of an intermediate species (reactions were performed in both H$_2$O and D$_2$O and with and without 20 weight/volume% sucrose added to the reaction buffer). From Fig. 2A, the decay of the 333 nm ternary complex LMCT leads to the formation of a new absorption feature at $442$ nm within the first 175 ms of the reaction, which decays within 2 s (Fig. 2B). The decay of this species is concomitant with the growth of an absorption feature at $248$ nm (Fig. 2C, green), which corresponds to the formation of hydroxybiopterin (hydrolyzed to quinonoid dihydrobiopterin at long times as shown in SI Appendix, Fig. S10) (28). The formation of hydroxybiopterin is the marker for the corresponding formation of an Fe$^{IV} = O$ species, indicating that the O$–$O bond of O$_2$ is cleaved in this stage of the reaction. Thus, the 442 nm absorption feature is associated with a pre-Fe$^{IV} = O$ intermediate and is similar to a previously observed transient signal in another isozyme of TPH (29).

To probe the mechanism of formation and decay of the 442 nm intermediate, its O$_2$ dependence and H/D solvent kinetic isotope effect (KIE) were evaluated. In Fig. 2D, the formation of the intermediate is dependent on the concentration of O$_2$ and is approaching saturation at the highest oxygen concentration (1 mM), requiring an O$_2$ equilibrium binding step. Furthermore, in deuterated buffer, the absorption maximum is higher and occurs at longer time (Fig. 2E), reflecting a normal KIE on the decay of the intermediate but no KIE on its formation. Additionally, the decay of this species has a fast phase and a slow phase (SI Appendix, Fig. S11, purple and green lines), which cannot be simply fitted as two decay pathways for the intermediate since it does not capture the biphasic behavior of the absorption time.
trace between 0.6 and 2 s (SI Appendix, Fig. S11, green line). To capture this biphasic decay, the formation of the intermediate requires two parallel reactions with two O₂ binding equilibria (with the same ternary-O₂ decay rate, k₂ in Scheme 2). This kinetic behavior is interpreted to reflect the differential O₂ reactivity of the two ternary 5C sites observed in the MCD data (Fig. 1A) for the ternary Fe³⁺ complex (as described in SI Appendix, Fig. S12 and associated text). Fitting the kinetic data in Fig. 2D and SI Appendix, Fig. S13 to the model in Scheme 2 that includes two O₂ binding equilibria (with the 60/40 speciation of the ternary Fe³⁺ site from Mössbauer spectroscopy (Fig. 3A)), the absorption feature of the 442 nm intermediate has a molar extinction coefficient of 5,500 M⁻¹ cm⁻¹, the fast decay phase has a normal KIE of 4.2 (k₁ in Scheme 2, Top), and the slow decay phase (k₃p) also has a normal KIE but is too small to accurately estimate. Importantly, the fast rate of formation (k₁) and slow decay (especially in D₂O, k₃) enables accumulation of this intermediate for spectroscopic characterization.

**Spectroscopic Definition of the Pre-Fe⁴⁺ = O Intermediate.** The oxidation state of the pre-Fe⁴⁺ = O intermediate was characterized using Mössbauer spectroscopy, which is a nuclear absorption technique sensitive to the electron density at and around an ^⁵⁷Fe nucleus. Compared to the ternary Fe³⁺ site (Fig. 3A), a new quadrupole doublet (Fig. 3B, purple) appears with a similar isomer shift (δ = 1.25 mm/s) but a smaller quadrupole splitting (∆E_Q = 2.80 mm/s), defining it as an Fe³⁺ species. The formation of the 442 nm absorption feature occurs with the decay of the 330 nm perin-to-Fe³⁺ charge transfer in the ternary complex, which is indicative of perin oxidation upon reaction with O₂. Since, from Mössbauer, the intermediate is Fe³⁺ and its decay leads to the formation of an Fe⁴⁺ = O, this intermediate can be formulated as either an Fe³⁺-superoxide/BH₄⁺⁺ (or BH₄⁻) or an Fe³⁺-peroxo/oxidized perin species. The absorption spectra of BH₄⁺⁺ and BH₄⁻ do not reproduce the absorption spectrum in Fig. 2A (30, 31) and thus indicate that this species is an Fe³⁺-peroxo/oxidized perin intermediate.

The Fe³⁺-peroxo/oxidized perin intermediate can either maintain or lose the bond between the Fe³⁺ and the perin carbonyl present in the ternary Fe³⁺ site (SI Appendix, Figs. S14 and S15 for structural comparison). TD-DFT calculations show that, for both cases, the lowest energy absorption transition is a metal-to-ligand charge transfer (MLCT) into the oxidized perin π orbital, with the more intense transition predicted for the carbonyl bound structure because of direct orbital overlap (SI Appendix, Figs. S16 and S17). This assignment is confirmed by resonance Raman spectroscopy (Fig. 3C and SI Appendix, Figs. S18 and S19 for resonance enhancement) in the metal-ligand region between 400 and 800 cm⁻¹, as the resonance enhanced vibrations at 451, 489, and 792 cm⁻¹ only have modest isotope shifts upon ^¹⁸O₂ substitution. DFT calculations for the one electron reduction of a peroxy oxidized perin (resulting from the MLCT) predict structural distortions that contribute to the resonance enhancement along the carbonyl C = O and the pyrazine ring C₆-N₅ bonds (SI Appendix, Fig. S20). From the resonance Raman data in the intraperin region above 1,000 cm⁻¹ (Fig. 3C, Inset and SI Appendix, Figs. S18 and S19 for resonance enhancement), the highest energy vibration at 1,570 cm⁻¹ is 110 cm⁻¹ lower relative to the carbonyl stretch (1,680 cm⁻¹) of an oxidized perin (23). Frequency calculations on computational models with and without the perin carbonyl bound to the peroxy-Fe³⁺ intermediate demonstrate that, as in the ternary complex, carbonyl binding leads to a ~100 cm⁻¹ decrease in the C = O stretching frequency (SI Appendix, text below SI Appendix, Fig. S20 and Table S2 for detailed peak assignment), reproducing the experimental data. Additionally, these calculations also assign the vibrations in the metal-ligand region, where the most intense vibration at 489 cm⁻¹ is the Fe-O-carbonyl stretch, while the 451 cm⁻¹ feature is the Fe-O-peroxy stretch (SI Appendix, Table S2 and Fig. S21). The modest ^¹⁸O₂ isotope shift of the Fe-O-peroxy stretch (∆ = 5 cm⁻¹) is due to its distribution into a number of stretching and bending modes associated with the formation of a six-membered ring. This derives from the simultaneous coordination of the pterin carbonyl and peroxide, thus defining the geometric structure of the peroxy-Fe³⁺ intermediate (Fig. 3F).

The electronic and geometric structures of the Fe³⁺ ternary complex and the peroxy intermediate were further investigated using nuclear resonance vibrational spectroscopy (NRVS). NRVS measures the vibrational sidebands of the ^⁵⁷Fe nuclear Mössbauer transition where the spectral intensity gives the Fe motion in a normal mode at the observed energy. Thus, this technique is sensitive to changes in ligation around an ^⁵⁷Fe center. In Fig. 3D, Top, the NRVS spectrum of the ternary complex (black) has been overlaid with the spectrum of the intermediate (red). The NRVS data on the ternary complex (black) and peroxy intermediate (red) show a redistribution of intensity from 230 and 260 cm⁻¹ (black arrow) to 200 to 220 cm⁻¹, 240 cm⁻¹, and 325 cm⁻¹ (red arrows). In order to interpret the changes in the NRVS data, we employed an NRVS/DFT methodology to reproduce the vibrational changes in the experimental data that has been previously used to characterize Fe⁴⁺ = O, Fe⁵⁺-peroxy and Fe⁵⁺-superoxide intermediates (32-34). The simulations (Fig. 3D, Bottom) for the ternary complex, with

---

**Scheme 2.** Kinetic model for the formation and decay of the O₂-dependent intermediate observed at 442 nm in stopped-flow absorption spectroscopy. The details of how this model was developed are presented in the SI Appendix. From spectroscopy, 60% of the reaction occurs through the top pathway, and 40% occurs through the bottom pathway. The kinetic rate constants and equilibrium constants that fit the data are given for each pathway.
Fig. 3. Mössbauer spectra of the (A) ternary complex and (B) the 442 nm pre-Fe⁴⁺ = O intermediate generated by reacting 0.5 mM (Fe⁴⁺BH₄/Trp)-TPH with 1 mM O₂ in Hepes/(NH₄)₂SO₄/sucrose buffer (pD 7). The parameters for the ternary complex and the intermediate are listed in their respective tables. (C) Resonance Raman spectra using the 457.9 nm laser line of the 442 nm intermediate (0.5 mM (Fe⁴⁺BH₄/Trp)-TPH + 1 mM O₂) rapid-freeze quenched at 150 ms. (Inset) Raman shifts measured in the 1,480 to 1,610 cm⁻¹ region showing resonance enhanced features at 1,514 and 1,570 cm⁻¹. The change in the energies of the vibrations from the ¹⁸O₂/¹⁸O₂ isotope perturbation are indicated. Note that the 1,514 and 1,570 cm⁻¹ features do not show an O₂ isotope effect. (D, Top) NRVS spectra of the ternary Fe¹ complex (black) and the rapid-freeze quenched intermediate (red, 1 mM (Fe⁴⁺BH₄/Trp)-TPH + 1 mM O₂). The error bars in the processed spectra are represented by vertical lines. The contribution of the ternary complex to the intermediate spectrum was removed and the spectrum renormalized as described in SI Appendix, Fig. S22. The black arrow depicts loss of intensity, while the red arrows show gain in intensity going from the ternary Fe¹ complex to the intermediate spectrum. (Bottom) Simulation of NRVS spectra of the ternary complex (black) and the peroxy intermediate (red) with the pterin carbonyl bound to the Fe²⁺ center. Optimized structures of (E) the ternary complex and the (F) peroxy intermediate with the pterin carbonyl bound. The metal–ligand distances are indicated.

The pterin carbonyl bound to the iron center (black) show two Fe–His modes at 228 and 241 cm⁻¹ as well as two Fe–carboxylate modes at 275 and 364 cm⁻¹. Going from the ternary complex to the peroxo intermediate with the carbonyl bound to the Fe²⁺ (red), the Fe–His modes shift to 160 and 195 cm⁻¹ (lower in energy by 68 and 46 cm⁻¹, respectively) and the Fe–carboxylate modes shift to 260 and 322 cm⁻¹ (lower in energy by 15 and 42 cm⁻¹, respectively). In the calculations, these energy decreases reflect elongation of the Fe–His and Fe–carboxylate bonds in going from the 5C ternary site to the 6-coordinate (6C) intermediate due to the coordination of an anionic peroxide ligand (as shown in Fig. 3 E and F). These simulations reproduce the experimental data as the gain in intensity in the peroxo NRVS spectrum at 200 to 220 cm⁻¹ is due to the Fe–His modes shifting down in energy and at 240 and 325 cm⁻¹ are due to the Fe–carboxylate modes shifting to lower energy. Additionally, the loss of intensity at 260 cm⁻¹ going from the ternary complex to the peroxo intermediate is captured by the decrease in calculated energy of the Fe–carboxylate mode from 275 to 260 cm⁻¹. Note that while there is a discrepancy in the absolute energy values of the calculated spectra relative to the experimental data, the trends in intensity going from the ternary to the peroxo complex are internally consistent. On the other hand, the active site models without the pterin carbonyl bound do not reproduce the experimental changes observed in the NRVS data (SI Appendix, see description with SI Appendix, Fig. S23). Thus, the NRVS data complement the resonance Raman data on both the ternary site and peroxo intermediate and demonstrate the direct coordination of the pterin carbonyl with the Fe²⁺ in both structures.

O₂ Reaction Coordinate: Role of Direct Coordination of Pterin to Fe⁴⁺.
To evaluate the impact of direct pterin coordination on oxygen activation, we have calculated the reaction coordinates of two 5C ternary complexes (SI Appendix, Fig. S4), one with and one without the pterin carbonyl bound to the metal center (Fig. 4). When the pterin carbonyl is bound, O₂ binds to the open coordination site on the Fe²⁺ to form a superoxide species that is only uphill by 1.4 kcal/mol (Fig. 4A, green). In contrast, when the pterin is not bound to Fe⁴⁺, O₂ binding is uphill by 11.7 kcal/mol (Fig. 4A, red), comparable to other nonheme iron enzyme calculations (34, 35). The superoxide then forms the peroxide species through the attack of its distal O (relative to Fe) on the C4α position of the pterin cofactor along with a proton transfer from the N₃ amine on the pterin to a nearby carboxylate residue (Fig. 4B, right circled). When the pterin carboxyl is bound, the formation of the peroxo intermediate from the superoxo has a calculated reaction barrier of 5.6 kcal/mol (Fig. 4A and SI Appendix, Fig. S24), compared to the ~10 kcal/mol barrier estimated (based on the two-dimensional potential energy surface, SI Appendix, Fig. S25) when the pterin carbonyl is not bound. Thus, comparing the reaction coordinates with and without pterin carbonyl bound to Fe⁴⁺, the direct binding of the pterin to the
Fe\textsuperscript{II} center reduces the barrier for O\textsubscript{2} activation by 14.7 kcal/mol (Fig. 4A), which corresponds to an \(\sim 10^{10}\) increase in kinetic rate.

To understand the origin of this reduction in barrier, the changes in electronic structures along each reaction coordinate were assessed and compared. Before the O\textsubscript{2} reaction, the pterin carbonyl–bound ternary complex donates electron density to the Fe center stabilizing the ternary complex by \(\sim 8\) kcal/mol (SI Appendix, Figs. S26 and S27). This electron donation corresponds to the LMCT observed in the absorption spectrum for the ternary complex in Fig. 1 at \(\sim 30,000 \text{ cm}^{-1}\) and plays a key role in O\textsubscript{2} activation. The electronic structures of the superoxide species in Fig. 4 C and D (formally high-spin Fe(III) [\(\alpha\)-spins] antiferromagnetically coupled to O\textsuperscript{2-} [\(\beta\) spin] to give \(S_{\text{tot}} = 2\); see SI Appendix, Figs. S28 and S29 for septet versus quintet) with and without pterin bound demonstrate that there is \(\beta\) electron transfer from the Fe to O\textsubscript{2} (red arrow) and \(\alpha\) electron transfer from the pterin to the O\textsubscript{2} moiety (blue arrow in Fig. 4 C and D and SI Appendix, Figs. S30 and S31). However, when the pterin carbonyl is bound to the Fe\textsuperscript{II}, there is additional direct donation of electron density from the pterin cofactor to the iron center (green arrow) that results in the \(\sim 10\) kcal/mol stabilization of O\textsubscript{2} binding relative to superoxo formation without bound pterin. From the bound superoxo species, formation of the peroxo intermediate completes the three-electron transfer processes shown in Fig. 4 C and D (red, blue, and green arrows). When the pterin carbonyl is bound, the electronic structure of the transition state for peroxo formation (Fig. 4 C and SI Appendix, Figs. S32 and S33) reveals that the electron transfer processes between the Fe and O\textsubscript{2} (red arrow) and the pterin and Fe (green arrow) are far along. However, when the pterin carbonyl is not bound, the lack of direct overlap between the pterin and iron requires that this electron transfer be mediated by O\textsubscript{2}. From Fig. 4D TS, this process involves the concerted electron transfer from the O\textsubscript{2} \(\pi^*\) to Fe \(d_{xz}\) and from the pterin \(\pi\) to O\textsubscript{2} \(\pi^*\) (SI Appendix, Figs. S34 and S35). In a transition state–like structure for the reaction coordinate without pterin carbonyl bound (PES in SI Appendix, Fig. S25), the electron transfer from the iron to the O\textsubscript{2} is far along, but there is only partial electron transfer from the pterin (\(\beta\)) to the iron (Fig. 4D TS, green arrows). Thus, relative to the electronic structure of the transition state with the pterin carbonyl bound, the lack of carbonyl binding results in less total electron transfer and increases the barrier for superoxo to peroxo formation by 5 kcal/mol (Fig. 4D). Thus, the direct donation from the pterin cofactor to the Fe\textsuperscript{II} through its carbonyl leads to more favorable O\textsubscript{2} binding and efficient electron transfer, which collectively result in the 14 kcal/mol lower reaction barrier.

**Discussion**

In this study, we have defined the geometric and electronic structures of the pterin- and tryptophan-bound Fe\textsuperscript{II}-TPH active site and the Fe\textsuperscript{II}-peroxy-pterin intermediate and the reaction mechanism through which the pterin cofactor provides its two electrons for O\textsubscript{2} activation. When both pterin and tryptophan are bound to Fe\textsuperscript{II}-TPH, we observe a new absorption feature at 330 nm, which is not present when only pterin is bound to the Fe\textsuperscript{II} site. The 330 nm absorption feature is paramagnetic from MCD spectroscopy, which establishes that the charge transfer involves pterin dependent hydroxylases.

*Fig. 4.* (A) O\textsubscript{2} activation reaction coordinate of the ternary complex with pterin carbonyl bound to Fe\textsuperscript{II} (green) and without the pterin carbonyl bound to Fe\textsuperscript{II} (red). (B) The geometries for the ternary, superoxo, and peroxo complexes in the reaction coordinate with the pterin carbonyl bound. The red dashed ovals demonstrate the proton transfer from the pterin cofactor to a nearby carboxylate residue. (C and D) Qualitative electronic structures and electron transfer pathways for the superoxo and TS structures for (C) the reaction coordinate with the pterin carbonyl bound and (D) the reaction coordinate without the pterin carbonyl bound to Fe\textsuperscript{II}.

*Fig. 5.* Comparison of the Fe-superoxide structures without (Left) and with (Right) the pterin carbonyl bound. The red arrows represent electron transfer from the iron to O\textsubscript{2}, the blue arrows represent electron transfer from the pterin to O\textsubscript{2}, and the green arrows represent electron transfer from the pterin to the iron center.
Additionally, the resonance Raman spectrum associated with the 330 nm band reveals a direct interaction between the pterin carbonyl and the metal center based on the presence of a carbonyl stretch, which decreases from 1,695 cm\(^{-1}\) in a reduced pterin cofactor to 1,601 cm\(^{-1}\) in the pterin and tryptophan-bound ternary complex. Reaction of this ternary complex with O\(_2\) generates a 442 nm absorption feature associated with an Fe\(^{II}\)-peroxy-pterin intermediate. The 442 nm band is a metal-to-pterin charge transfer transition based on the lack of a resonance-enhanced O–O stretching vibration and the presence of intrapterin stretches in the resonance Raman spectrum associated with this band. Furthermore, the highest energy resonance–enhanced features are more than 100 cm\(^{-1}\) lower than the carbonyl stretch for an oxidized pterin cofactor (1,680 cm\(^{-1}\)), which demonstrates that the pterin carbonyl is also directly bound to the iron center in the peroxo-bridged intermediate.

By computationally evaluating the O\(_2\) reaction coordinate with and without carbonyl bound to the iron center, we calculate a 14 kcal/mol lower reaction barrier with the peroxo addition, which corresponds to a 10\(^{15}\)-fold increase in reaction rate. When the pterin is not bound to the Fe\(^{II}\) center, O\(_2\) first binds, the iron is partially oxidized to Fe\(^{III}\), and the O\(_2\) is partially reduced to superoxide, which is 10 kcal/mol uphill. In going from the superoxy to the peroxy species, the pterin cofactor needs to transfer one electron to the O\(_2\) and one electron to the iron center to form the Fe\(^{III}\)-peroxy oxidized pterin (Fig. 5, Left). While the electron transfer from the pterin to the O\(_2\) is straightforward (Fig. 5, blue arrow), the second electron transfer from the pterin to the d\(^*\) orbital on the iron center needs to proceed through one of the superoxide \(\pi^*\) molecular orbitals (green arrow). The lack of direct orbital overlap between the pterin and iron makes this electron transfer less efficient, which gives rise to the high reaction barrier. On the other hand, when the pterin carbonyl is bound to the metal center, O\(_2\) binding is thermoneutral due to donation from the pterin to the metal center that compensates the electron transfer from iron to O\(_2\) as there is direct orbital overlap between the pterin \(\pi\) HOMO and d\(^*\) orbital (Fig. 5, Right, green arrow), which results in a much lower reaction barrier. In the pterin-dependent hydroxylases, under turnover conditions, product dissociation is the rate-limiting step and has a barrier of \(\sim 16\) kcal/mol (based on a rate constant of 0.1 to 1 s\(^{-1}\)) (22, 29, 36, 37). When carbonyl is not bound to the iron center, the overall calculated barrier of >21 kcal/mol would result in \(\sim 10^{14}\)-fold slower turnover. This barrier would be similar to that of uncoupled turnover (oxidation of pterin cofactor, but not substrate) (24, 38), which would result in enzymatic dysregulation and impact key metabolic pathways necessary for proper brain function. As the pterin-dependent hydroxylases catalyze the rate-limiting steps in dopamine and serotonin biosynthesis as well as aromatic amino acid degradation, impairment of their reactivity has been linked to neurological diseases such as phenylketonuria (PAH), Parkinson’s, schizophrenia (TH) and depressive disorders (TPH), highlighting the importance of their coupled reactivity.

Finally, this study defines a unifying mechanism employed by the cofactor-dependent iron enzymes to enable rapid O\(_2\) activation (Scheme 3). For both the pterin and \(\alpha\)-KG–dependent nonheme iron enzymes, the cofactor and substrate must simultaneously bind to open a coordination position on the Fe\(^{III}\), which enables O\(_2\) activation. In the \(\alpha\)-KG–dependent nonheme iron enzymes, substrate coordination to the Fe\(^{III}\)/\(\alpha\)KG site induces formation of the 5C site needed for O\(_2\) activation. For the pterin-dependent hydroxylases, binding of the substrate causes both the pterin to bind to the iron and also opens a coordination position for O\(_2\) activation. Both enzyme classes thus prevent uncoupled reactivity (i.e., O\(_2\) activation without substrate oxidation) and autooxidation when only the cofactor is bound to the enzyme—the \(\alpha\)-KG–dependent enzymes by keeping the site 6C and the pterin-dependent enzymes by cofactor binding to the Fe\(^{III}\) only in the presence of substrate. O\(_2\) binding results in some electron transfer from the iron to the O\(_2\) (i.e., Fe\(^{III}\)-superoxy character), which then requires the cofactor to donate two electrons (one to O\(_2\) and one to Fe) to generate the peroxo-bound Fe\(^{III}\) intermediate observed here and proposed to form in the \(\alpha\)-KG–dependent enzymes (35, 39, 40). The peroxo-Fe\(^{IV}\) species then undergoes a two-electron reductive cleavage of the O–O bond to form the Fe\(^{IV}\) = O intermediate, primed to initiate substrate chemistry. We have demonstrated that for both subclasses, electron transfer from the cofactor to the iron requires orbital overlap and thus the direct coordination of the cofactor to the Fe\(^{III}\). Without the cofactor binding to the iron, the oxygen reaction would be too slow for metabolic function.

**Materials and Methods**

Details about protein expression and purification, sample preparation, spectroscopic methods, stopped-flow absorption experiments, and computational methods are provided in SI Appendix, Materials and Methods.

**Data Availability.** All study data are included in the article and/or supporting information.

**ACKNOWLEDGMENTS.** This research was supported by US NIH Grants GM40392 (to E.I.S., S.R.I., J.T.B., and A.B.J.) and F32GM122194 (to L.B.G.) and Independent Research Fund Denmark Grant DFF-6108-00247 (to H.E.M.C. and E.I.S.) and Independent Research Fund Denmark Grant DFF-6108-00247 (to H.E.M.C. and E.I.S.). K.D.T. acknowledges financial support via an Academic Excellence Independent Research Fund Denmark Grant DFF-6108-00247 (to H.E.M.C. and E.I.S.) and Independent Research Fund Denmark Grant DFF-6108-00247 (to H.E.M.C. and E.I.S.).

**Authors contributions.** J.E.B. and E.A. conceived the project. J.E.B., E.A., and K.D.T. designed experiments. J.E.B. performed experiments. J.E.B., E.A., and K.D.T. analyzed data. J.E.B. and E.A. wrote the article. All authors contributed to the discussion and writing of the manuscript. J.E.B. and E.A. contributed equally. J.E.B. and E.A. contributed equally.

**Conflict of interest.** The authors declare no competing interests.

**Funding information.** Funding information for this article has been deposited with the article.
5. D. Lando et al., FHII-1 is an asparaginyl hydroxylase enzyme that regulates the tran-

6. T. Gerken et al., The obesity-associated FTO gene encodes a 2-oxoglutarate-dependent

7. F. H. Vailancourt et al., Characterization of extradiol dioxygenases from a poly-
chlorinated biphenyl-degrading strain that possess higher specificities for chlorinated

8. B. G. Keenan, T. K. Wood, Orthic Rieske dioxygenases for degrading mixtures of 2,4-
dinitrotoluene/naphthalene and 2-amino-4,6-dinitrotoluene/4-amino-2,6-dinitrotoluene.

DNA as probed through chemical cross-linking studies. Nucleic Acids Res. 32,

10. S. Kai, L. Que, Dioxyn activation by nonheme iron enzymes with the 2-His-1-car-

11. E. I. Solomon, S. Goudarzi, K. D. Sutherlin, O2 activation by non-heme iron enzymes.

12. E. G. Pavel et al., Circular dichroism and magnetic circular dichroism spectroscopic
studies of the non-heme ferrous active site in clavaminase synthase and its interaction


to the α-ketoglutarate-dependent non-heme iron enzyme clavaminase synthase 2:
Coupling mechanism of oxidative decarboxylation and hydroxylation. J. Am. Chem.
Soc. 120, 13539–13540 (1998).

and magnetic circular dichroism spectroscopy of the catalytically competent ferrous
active site of phenylalanine hydroxylase and its interaction with pterin cofactor.

16. M. S. Chow et al., Spectroscopy and kinetics of wild-type and mutant tyrosine hy-
donase: Mechanistic insight into O2 activation. J. Am. Chem. Soc. 131, 7685–7698
(2009).

17. M. D. Krzyaniak, B. E. Eser, H. R. Ellis, P. F. Fitzpatrick, J. McCracken, Pulsed EPR study
of amino acid and tetrahydropterin binding in a tyrosine hydroxylase nitric oxide
complex: Evidence for substrate rearrangements in the formation of the oxygen-

18. O. A. Andersen, T. Flatmark, E. Hough, High resolution crystal structures of the cata-
ytic domain of human phenylalanine hydroxylase in its catalytically active FeIII form

19. O. A. Andersen, A. I. Stokka, T. Flatmark, E. Hough, 2.0Å resolution crystal structures of
the ternary complexes of human phenylalanine hydroxylase catalytic domain with
tetrahydrobiopterin and 3,2-thienyl-L-alanine or L-norleucine: Substrate specificity

20. X. Zhang, J.-M. Beaulieu, T. D. Sotnikova, R. R. Gainetdinov, M. G. Caron, Tryptophan

21. M. S. Windahl, J. Boesen, P. E. Karlins, H. E. M. Christensen, Expression, purification
and enzymatic characterization of the catalytic domain of human tryptophan hy-

22. K. M. Roberts, J. A. Pavon, P. F. Fitzpatrick, Kinetic mechanism of phenylalanine hy-
donase: Intrinsic binding and rate constants from single-turnover experiments.

drobioppterin: Fourier transform Raman investigations provide mechanistic implica-
tions for the enzymatic utilization and recycling of this essential cofactor. J. Raman
Spectrosc. 33, 610–617 (2002).

24. G. Eberlein, T. C. Bruce, R. A. Lazarus, R. Henrie, S. J. Benkovic, The interconversion of
the 5,6,7,8-tetrahydro-, 6,7,8-dihydro-, and radical forms of 6,6,7,7-tetramethylhy-
dropterin. A model for the biotinperin center of aromatic amino acid mixed function

formation between the cupric site of phenylalanine hydroxylase from Chromobacterium

26. K. Teigen, N. Å. Frøystein, A. Martínez, The structural basis of the recognition of
phenylalanine and pterin cofactors by phenylalanine hydroxylase: Implications for the

27. C. F. Morasch et al., The obesity-associated FTO gene encodes a 2-oxoglutarate-dependent


29. K. M. Roberts, J. A. Pavon, P. F. Fitzpatrick, Kinetic mechanism of phenylalanine hy-
donase: Intrinsic binding and rate constants from single-turnover experiments.