Published in final edited form as:

J Am Chem Soc. 2011 November 16; 133(45): 18148–18160. doi:10.1021/ja202549q.

Activation of α -keto Acid Dependent Dioxygenases: Application of an $\{\text{FeNO}\}^7/\{\text{FeO}_2\}^8$ Methodology for Characterizing the Initial Steps of O_2 Activation

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Abstract

The α -keto acid dependent dioxygenases are a major subgroup within the O_2 -activating mononuclear non-heme iron enzymes. For these enzymes, the resting ferrous, the substrate plus cofactor-bound ferrous, and the $\text{Fe}^{\text{IV}}=\text{O}$ states of the reaction have been well studied. The initial O_2 -binding and activation steps are experimentally inaccessible and thus are not well understood. In this study, NO is used as an O_2 analog to probe the effects of α -keto acid binding in 4-hydroxyphenylpyruvate dioxygenase (HPPD). A combination of EPR, UV-vis absorption, magnetic circular dichroism (MCD), and variable-temperature, variable-field (VTVH) MCD spectroscopies in conjunction with computational models is used to explore the HPPD-NO and HPPD-HPP-NO complexes. New spectroscopic features are present in the α -keto acid bound $\{\text{FeNO}\}^7$ site that reflect the strong donor interaction of the α -keto acid with the Fe. This promotes the transfer of charge from the Fe to NO. The calculations are extended to the O_2 reaction coordinate where the strong donation associated with the bound α -keto acid promotes formation of a new, $S=1$ bridged Fe^{IV} -peroxy species. These studies provide insight into the effects of a strong donor ligand on O_2 binding and activation by Fe^{II} in the α -keto acid dependent dioxygenases and are likely relevant to other subgroups of the O_2 activating non-heme ferrous enzymes.

Introduction

The mononuclear non-heme iron enzymes catalyze a diverse range of chemical reactions that include dioxygenation, hydroxylation, ring closure, oxidative desaturation, carbon-carbon bond and aromatic ring cleavage, and halogenation.¹⁻⁸ Within this broad class, the

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Supporting Information Available

Full Reference 30; description of SOC mechanism; key geometric and electronic structure parameters for HPPD-HPP-NO and for E-NO and E- α KA-NO with different functionals; temperature-dependent EPR data with fits; Fe-EDTA-NO UV-vis abs and MCD spectra; VTVH MCD isotherms and fits for LF transitions; computational models and molecular orbital diagrams for E-NO, HPPD-HPP-NO, E- α KA-NO + salt bridge, and $S=2$ and $S=3$ E- α KA- O_2 ; comparison of energies of Fe d_{π} orbitals; TD-DFT comparisons for different models, to experimental data, for the $S=1$ peroxo-bridged structure; geometric and electronic structure comparison between B3LYP/LACVP and BP86+10%HF/Gen computational models; boundary surface plots for E-NO and $S=2$ E- α KA- O_2 ; spin crossover 2D-PES, molecular orbital diagrams and SOC; and Cartesian coordinates for stationary structures. This information is available free of charge via the Internet at <http://pubs.acs.org>.

oxygen-activating enzymes use an Fe^{II} center to activate triplet dioxygen for the formally spin forbidden reaction with organic substrates. These include the α -keto acid dependent dioxygenases. These enzymes use an α -keto acid group as a cosubstrate, providing electrons for dioxygen cleavage.^{1, 4, 5, 9} A general mechanistic strategy (Scheme 1) has been developed in which the binding of both the α -keto acid group and the substrate results in an open coordination position on the Fe^{II} for O_2 activation.¹ Reaction with O_2 leads to decarboxylation of the α -keto acid and generation of a highly reactive $\text{Fe}^{\text{IV}}=\text{O}$ species. This $\text{Fe}^{\text{IV}}=\text{O}$ species then undergoes an electrophilic reaction with the substrate.^{1, 4, 5, 8, 10-13} The binding of both the substrate and the α -keto acid and the reactive nature of the $\text{Fe}^{\text{IV}}=\text{O}$ species have been extensively studied.^{2-4, 8, 12, 14} The initial O_2 binding and activation steps, however, have not been definitively observed and therefore are not well understood.

As no O_2 intermediate prior to the $\text{Fe}^{\text{IV}}=\text{O}$ species has been experimentally trapped in the α -keto acid dependent dioxygenases, other approaches to probe these initial steps must be taken. NO binds to ferrous sites;¹⁵⁻²⁰ however, it is one electron deficient with respect to O_2 and does not react further. These complexes are described as $\{\text{FeNO}\}^7$ following the notation of Enemark and Feltham²¹ where the superscript refers to the total number of Fe d plus NO valence electrons (6 electrons from the ferrous site and one from NO). These $\{\text{FeNO}\}^7$ complexes are stable and chromophoric with half-integer spin (typically $S=3/2$),^{15, 18} making them good spectral probes and amenable to study by electron paramagnetic resonance (EPR), UV-visible absorption and magnetic circular dichroism (MCD) spectroscopies. Initial studies on the Fe-EDTA-NO complex showed an $S=3/2$ site (from EPR) with intense charge transfer transitions in the UV-vis absorption and MCD spectra (Supplementary Figures S2, S3A). XAS and resonance Raman studies assigned the electronic structure of the $\{\text{FeNO}\}^7$ unit as high spin Fe^{III} ($S=5/2$) antiferromagnetically coupled to NO^- ($S=1$).²² Computational modeling supported this description giving an $\text{Fe}^{\text{III}}-\text{NO}^-$ electronic structure characterized by 5 unoccupied β Fe d orbitals and 2 unoccupied α NO^- orbitals.²²⁻²⁴ The $\{\text{FeNO}\}^7$ computational description was then extended to provide experimentally calibrated models for $\{\text{FeO}\}^8$.²³ This $\{\text{FeNO}_2\}^7/\{\text{FeO}_2\}^8$ methodology was used to characterize the initial steps of the O_2 reaction of Isopenicillin N Synthase (IPNS).²⁵ In that study, the $\{\text{FeNO}\}^7$ complex provided an understanding of the effect of the native substrate (δ -(L- α -aminoadipoyl)-L-cysteiny-D-valine, ACV) coordination to the Fe through its thiolate ligand. Extension of the experimentally calibrated $\{\text{FeNO}\}^7$ description to an $\{\text{FeO}_2\}^8$ computational model led to a frontier molecular orbital for H-atom abstraction (the first O_2 -dependent step of the mechanism). The results for IPNS show the utility of the $\{\text{FeNO}\}^7/\{\text{FeO}_2\}^8$ methodology in evaluating the initial steps of the O_2 reaction.

The present study focuses on the α -keto acid dependent dioxygenase, (4-hydroxyphenyl)pyruvate dioxygenase (HPPD). HPPD catalyzes the reaction of (4-hydroxyphenyl)pyruvate (HPP) with dioxygen to release CO_2 and the product, homogentisate (Scheme 2).²⁶ HPPD has been chosen for this study because the resting ferrous and ES complexes have been studied previously.²⁷⁻²⁹ Additionally, in HPP the α -keto acid moiety is fused to the substrate facilitating formation of the catalytically relevant ES complex. From our previous study of the ferrous ES complex,²⁹ there is less MCD intensity from the ligand field transition of the Fe site at low energy (relative to other α -keto acid dependent dioxygenases) which facilitates a clear assignment of a new low energy transition present in the MCD spectrum of the HPPD-HPP-NO complex (*vide infra*).

This study examines the effect of the α -keto acid binding to the $\{\text{FeNO}\}^7$ unit. The NO-bound complexes of both resting HPPD and HPPD-HPP are studied, where the HPPD-NO complex provides a reference to define the new features which arise from the bound α -keto acid in the HPPD-HPP-NO complex. The experimental study of the HPPD-NO and HPPD-HPP-NO complexes is then extended to computational models. In order to obtain a general

picture of α -keto acid non-heme Fe reactivity and to compare to the published literature, the computational model presented in this study is an Fe^{II} α -ketoglutarate-bound facial triad. However, the key computational steps have also been evaluated with HPP as the substrate and are presented in the Supporting Information. Analysis of the {FeNO}⁷ computational models in conjunction with the experimental HPPD-(HPP)-NO data leads to an understanding of the effects of the α -keto acid on the {FeNO}⁷ bond. Insight gained from the {FeNO}⁷ complex is extended to the mechanistically relevant {FeO₂}⁸ complex. Evaluation of the {FeO₂}⁸ complex and the subsequent reaction coordinate allows an evaluation of proposed mechanisms of reactivity and provides insight into the initial steps of the O₂ reaction in the α -keto acid dependent dioxygenases leading to the formation of the Fe^{IV}=O intermediate for subsequent reaction with substrate.

2. Materials and Methods

HPPD was purified according to previously published procedures.²⁶ Apo-HPPD was exchanged into 50 mM 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES) buffer in D₂O at a pD of 7.1 using an Ultrafree-4 filter with a 10kDa cutoff membrane (Millipore) to a concentration of 2-4 mM. All other reagents were used as received without further purification. Buffer, HPP, ferrous ammonium sulfate, methyl-[6-[methyl-[nitroso(oxido)amino]amino]hexyl]azanium (NONOate) and glycerol were made anaerobic by purging with Ar on a Schlenk line. HPPD was made anaerobic by alternating cycles of vacuum and purging with Ar at 273K. All samples for spectroscopy were prepared in an inert atmosphere, N₂-purged “wet box” to maintain an O₂ free environment. Ferrous ammonium sulfate was dissolved in buffer and added to HPPD in microliter quantities to a concentration of 90% of the enzyme (monomer) concentration to avoid free iron in the sample. HPP was added to buffer to form a saturated solution. Residual solid HPP was filtered out of the solution and the concentration of the HPP solution was determined from the intensity of the 270 nm maximum in the absorption spectrum. For the substrate-bound samples, HPP was added in microliter quantities to a concentration of 5-10 times the concentration of the protein. Solid NONOate was dissolved in 220 mM NaOH for a final concentration of 90 mM and added to the HPPD-Fe^{II} and HPPD-Fe^{II}-HPP solutions in microliter quantities to a concentration of ~2 times the concentration of the protein. Glycerol (~50-60% v/v) was added as a glassing agent to samples for MCD. CD control experiments showed no effect of the glycerol on the Fe site.

NIR (600-2000 nm) MCD spectra were taken on either a Jasco J-200D or Jasco J-730 spectropolarimeter with a liquid N₂ cooled InSb detector (Teledyne Judson Technologies) and an Oxford Instruments SM-4000-7T superconducting magnet. UV-vis (300-900 nm) MCD spectra were taken on a Jasco J-810D spectropolarimeter equipped with an extended S20 photomultiplier tube and a SM-4000-7T superconducting magnet. UV-vis Abs spectra were taken on an Agilent 8453 diode array spectrometer. Abs spectra were taken at 278K in an anaerobic cuvette and were corrected for buffer and protein baseline effects by subtraction. Natural CD features and baseline effects were eliminated from the MCD spectra by taking half the difference of positive and negative field data. For VTVH MCD, a calibrated Cernox resistor (Lakeshore Cryogenics, calibrated 1.5 – 300 K), inserted into the sample cell, was used for accurate temperature measurement. The VTVH MCD data obtained were normalized to the intensity maximum. X-band EPR spectra were taken on a Bruker EMX spectrometer with a Bruker ER 041XG/ER microwave bridge and ER 4102ST/ER 5106QT cavity. Spectra were taken at temperatures between 3.8 and 50K using an Oxford ITC503 temperature controller with an ESR 900 continuous flow cryostat.

The computational models for the resting enzyme plus NO (E-NO) and the α -keto acid bound enzyme plus NO (E- α KA-NO) were taken from the CS2- α -ketoglutarate-NO crystal

structure (PDB entry: 1gvg). Histidine residues were truncated to methyl imidazole and glutamate was truncated to propionate for the models. Constraints imposed by the protein backbone were simulated in both computational models by fixing the relative positions of the β -carbons of the backbone.

Density functional theory (DFT) calculations were performed with the Gaussian 03 program^{30, 31} with the unrestricted BP86 functional³² with 10% Hartree-Fock Exchange under tight convergence criteria. The Pople triple- ζ basis set, 6-311G*, was used to describe Fe, NO/O₂, and the α -keto acid moiety (OCCO₂). The double- ζ basis set, 6-31G* was used for all other atoms. This method has been calibrated previously for {FeNO}⁷ complexes.²³ For comparison with literature results, calculations were also performed with the unrestricted B3LYP functional under tight convergence criteria. The LANL2DZ effective core potential basis set was used in these to describe the Fe and the Pople double- ζ basis set, 6-31G, was used to describe all other atoms. All structures were optimized and found to be stable with no imaginary frequencies $< 15 \text{ cm}^{-1}$ (which are associated with the constraints placed on the β -carbons). Effects of the protein environment were included by applying the polarized continuum model (PCM)³³ with a dielectric constant, $\epsilon = 4.0$, to calculate solvated energies for the complexes. These solvated single point calculations were done with the 6-311+G(2d,p) basis set. (For the B3LYP models, the 6-311G** basis set was used, consistent with literature.^{34, 35} The energies given include thermal and zero-point corrections. As the Gaussian package does not allow for Δ SCF calculations (convergence of a wavefunction in a user-defined excited state), the Amsterdam Density Function (ADF 2010.02) package³⁶⁻³⁸ was used to calculate the Δ SCF excitations. Using the Gaussian 03 optimized geometries, excited states for the ligand field transitions were calculated with the unrestricted BP86 functional with the uncontracted triple- ζ basis set (TZP) with a single polarization function without a frozen core approximation. To allow for differences between the pure functional and hybrid calculations, the Z of Fe was set to 25.6 (single point calculation) as was previously calibrated for similar calculations on {FeNO}⁷ model complexes.²³ All excitations were calculated as Slater transition states. ZFS parameters were calculated in ORCA (version 2.8) for both models using the B1P functional with 10% H-F exchange and with the CP(PPP) basis set on Fe, TZVP on N and O, and SVP on all other atoms.³⁹ Structures were visualized with GaussView 3.09;⁴⁰ orbital compositions were determined with QMForge;⁴¹ and molecular orbitals were visualized with Molden version 4.1.⁴² Time-dependent DFT (TD-DFT) calculations were performed (with Gaussian 03 using BP86 with 10% HF exchange and the split 6-311G*/6-31G* basis set as described above) to compare to the experimental spectra and SWizard was used to parse the results.^{43, 44}

3. Results

3.1 Spectroscopic

Addition of NO to the resting, Fe^{II}-bound form of HPPD results in the EPR spectrum shown in Figure 1A. The HPPD-NO complex exhibits effective g' values of 3.96 and 2.00 indicating that it is a close to axial $S=3/2$ species with an $E/D = 0.008$. A plot of the temperature dependence of the EPR signal intensity under non-saturating conditions is shown as an insert in Figure 1a and in Supplementary Figure S1 (red). Addition of NO to HPP bound Fe-HPPD results in the EPR spectrum shown in Figure 1B. The EPR signal of the HPPD-HPP-NO complex indicates that the spin of the ground state is still $S=3/2$ but with $g_x' = 4.14$ and $g_y' = 3.90$ giving an E/D of 0.02. The inverse dependence of the EPR intensity with increasing temperature for the HPPD-HPP-NO spectrum is shown as an insert in Figure 1b and in Supplementary Figure S1 (blue).

These EPR spectra are described by the spin Hamiltonian given in Equation 1, where $g_0=2.0$ and D and E are the axial and rhombic ZFS parameters, respectively.⁴⁵

$$\hat{H}_{spin}=D\left[S_z^2-5/4+E/D(S_x^2-S_y^2)\right]+g_0\beta\vec{S}\cdot\vec{H} \quad (1)$$

This splits the $S=3/2$, $M_s = \pm 1/2, \pm 3/2$ by an amount $2D$. The inverse temperature dependence of the EPR signal intensity indicates that D is positive with $M_s = \pm 1/2$ lowest and is fit to a Boltzmann population of the Curie law dependence (Equation 2 where C is the Curie constant, T is the temperature in Kelvin and k is the Boltzmann constant).

$$Intensity=\frac{C}{T}\left[\frac{1}{1+\exp(-2D/kT)}\right] \quad (2)$$

For the HPPD-NO EPR sample, this gives $D = 16 \pm 2 \text{ cm}^{-1}$ while for the HPPD-HPP-NO sample, the value of the axial ZFS parameter has decreased to $D = 8.4 \pm 0.7 \text{ cm}^{-1}$.

The room temperature UV-vis absorption spectra of the HPPD-NO and HPPD-HPP-NO complexes are shown in Figure 2 (with the Gaussian resolutions of the bands from a simultaneous fit with the MCD spectra presented below). The two spectra are very similar up to $25,000 \text{ cm}^{-1}$. However, a new intense transition resulting from HPP binding is observed above $25,000 \text{ cm}^{-1}$. The Fe-EDTA-NO complex has been extensively studied and provides a point of reference for this study.²² The HPPD-NO UV-vis absorption spectrum is very similar to that of Fe-EDTA-NO (Supplementary Figure S2). For the Fe-EDTA-NO complex, the two sets of bands observed are assigned as ligand-to-metal charge transfer transitions from NO^- to Fe^{III} d (at $20,000 - 25,000 \text{ cm}^{-1}$) and Fe^{III} ligand field (LF) transitions (at $\sim 15,000 \text{ cm}^{-1}$).

The 5K, 7T MCD spectra of the HPPD-NO and HPPD-HPP-NO complexes are shown in Figure 3. The MCD spectra of these two complexes are generally similar with only small differences in energy and intensity of the major features. (The starred feature in the HPPD-NO MCD spectrum is due to unreacted resting HPPD.) The major differences between the MCD spectra of the resting and substrate-bound NO complexes are the new bands at low energy ($< 5000 \text{ cm}^{-1}$) and at high energy ($\sim 29,000 \text{ cm}^{-1}$, negative MCD intensity) in the HPPD-HPP-NO complex in Figure 3B. The Gaussian resolutions of the bands in the two MCD spectra are also included in Figure 3. The MCD spectrum of HPPD-NO is very similar to that observed for the Fe-EDTA-NO complex (compare Gaussian resolutions in Supplementary Figure S3 including tabulated transition energies). From the strong spectral correlation of Fe-EDTA-NO and HPPD-NO in absorption, MCD and VTVH MCD (*vide infra* - the transition at $\sim 22,000 \text{ cm}^{-1}$ in Fe-EDTA-NO is also z polarized, data not shown), the three transitions at $\sim 18,000\text{--}24,000 \text{ cm}^{-1}$ of the HPPD-NO complex are assigned as NO^- $2\pi^*$ to Fe^{III} d $_{\pi}$ charge transfer transitions, while the two lower energy transitions are assigned as two of the Fe^{III} ligand field transitions (which are formally spin forbidden, but gain intensity through spin-orbit coupling with the nearby charge transfer transitions, *vide infra*). For the MCD spectrum of the HPPD-HPP-NO complex, the three transitions at $\sim 17,000\text{--}25,000 \text{ cm}^{-1}$ are also assigned as NO^- to Fe^{III} d charge transfer excitations and the two lower energy transitions at $11,500 \text{ cm}^{-1}$ and $15,000 \text{ cm}^{-1}$ are assigned as Fe^{III} ligand field transitions. This assignment is consistent with spin-forbidden ΔSCF calculations and TD-DFT calculations (see section 4.1). The transition at $\sim 29,000 \text{ cm}^{-1}$ corresponds to the new feature in the absorption spectrum of HPPD-HPP-NO. Assignment of the $< 5000 \text{ cm}^{-1}$ and $29,000 \text{ cm}^{-1}$ transitions in the HPPD-HPP-NO complex will be considered in the analysis. VTVH MCD data give the polarizations of the transitions observed in Abs/MCD

spectra.⁴⁶ The arrows in Figure 3 indicate the energies at which the VTVH MCD isotherms were taken to evaluate the polarizations of the corresponding transitions (energy positions where the associated Gaussian shows little overlap with other transitions). The VTVH MCD isotherms for the 21,700 cm⁻¹ transition in HPPD-NO are shown in Figure 3C. The VTVH MCD for the ligand field transitions taken at 15,100 cm⁻¹ is shown in Supplementary Figure 4A. These isotherms were taken on the less intense ligand field transition to avoid any contribution to the VTVH MCD isotherms from the residual resting HPPD (which has negative MCD intensity in this region). For the HPPD-HPP-NO complex, VTVH MCD isotherms were taken at 21,400 cm⁻¹ (Figure 3D), at 15,000 cm⁻¹ for the ligand field transition (Supplementary Figure S4B), and at 5,650 cm⁻¹ for the low energy transition (Figure 3E, the large error bars reflect the noise at this limit of our accessible spectral region). These VTVH MCD data were modeled by Equation 3.⁴⁶

$$\frac{\Delta\epsilon}{E} = \frac{\gamma}{4\pi S} \int_0^\pi \int_0^{2\pi} \sum_i N_i \left(l_z \langle S_z \rangle_i M_{xy}^{eff} + l_y \langle S_y \rangle_i M_{xz}^{eff} + l_x \langle S_x \rangle_i M_{yz}^{eff} \right) \sin \theta d\theta d\phi \quad (3)$$

A fit of the MCD intensity to Equation 3 using the spin Hamiltonian parameters g_0 , D and E allowed for the M_{ij}^{eff} transition moments to be obtained for the transition being probed. These transition moments were used to obtain the % polarization in the x, y and z directions using Equation 4, shown for % x polarization. The % polarizations in the y and z directions are obtained from cyclic permutations of the indices of Equation 4.⁴⁶

$$\%x = 100 \times \left[\frac{(M_{xy}^{eff} M_{xz}^{eff})^2}{(M_{xy}^{eff} M_{xz}^{eff})^2 + (M_{xy}^{eff} M_{yz}^{eff})^2 + (M_{yz}^{eff} M_{xz}^{eff})^2} \right] \quad (4)$$

From this analysis, the VTVH MCD data for HPPD-NO taken at 21,700 cm⁻¹ are 85% z-polarized (fit to the isotherms is included in Figure 3C). This is consistent with the assignment of this transition as NO⁻ to Fe^{III} d CT. The z polarization refers to the axial tensor of the ZFS which is collinear with the Fe-N^{NO} bond, and thus the NO⁻ to Fe^{III} d CT is polarized along this bond. For the HPPD-HPP-NO complex, the fit of the VTVH MCD data at 21,400 cm⁻¹ (fit shown in Figure 3D) is 49% z-polarized and 51% x,y-polarized reflecting a new interaction resulting from the binding of the α -keto acid substrate. Fits of the VTVH isotherms of the ligand field transitions give the same polarizations as their respective NO⁻ to Fe^{III} d charge transfer transitions (predominantly z polarized for HPPD-NO and mixed polarization for HPPD-HPP-NO, Supplementary Figure S4). The < 5000 cm⁻¹ transition in HPPD-HPP-NO can be fit with a range of polarizations between 88% and 47% z-polarized (Supplementary Figure S5). A more definitive fit cannot be obtained due to the noise in the data; however, the lowest energy transition is no more x,y polarized than the NO⁻ to Fe^{III} d CT transition at 21,400 cm⁻¹.

3.2 Computational – {FeNO}⁷

In order to further evaluate the geometric and electronic structures of the {FeNO}⁷ complexes, computational models have been studied. A previous study evaluated a number of different functional and basis set combinations to provide an experimentally validated rationale for the choice of computational parameters.²³ From this study, the BP86 functional with 10% Hartree-Fock exchange included, in combination with a split basis set consisting of triple- ζ on the Fe-N-O unit and double- ζ on the remaining atoms (for computational efficiency, hereafter referred to as Gen) proved to be the most consistent with experiment. Previous computational studies on α KA by other groups have used a combination of B3LYP and the LACVP basis set.^{12, 34, 35} Further rationale for the current choice of functional and

basis set will be presented below. There are a number of structural choices for the computational model of α -keto acid reactivity. To understand dioxygen reactivity for the broader family of α -keto acid dependent dioxygenase mononuclear non-heme iron enzymes and for comparison to the computational studies in the literature, the results for α -ketoglutarate bound to a facial triad (abbreviated as E- α KA) are shown in the text. As indicated in the text, comparable results were obtained for HPPD-HPP-NO in the orientation used in previous studies^{27, 28} and are presented in the Supporting Information. To develop a mechanism general to this broad family of enzymes, the model has been truncated to include only first sphere residue interactions. A survey of available crystal structures does not reveal any additional conserved residues that could interact with the NO or O₂ binding steps of the mechanism.⁴⁷

The HPPD-NO complex was modeled using the three protein derived residues making up the facial triad (2His/1 carboxylate), 2 water ligands and NO coordinated to Fe. The geometry optimized computational model (E-NO) is shown in Supplementary Figure S6A and key geometric parameters are given in Table 1. The geometric structure shows a strong Fe-N^{NO} bond at 1.76Å (and an Fe-N-O angle of 147°) which defines the z-axis of the molecule. The geometric structure of the FeNO unit in E-NO is similar to both the experimental EXAFS and geometry optimized structure of Fe-EDTA-NO (Table 1). Examination of the molecular orbital diagram (Supplementary Figure S6B, left) and boundary surface plots (Supplementary Figure S7) shows two unoccupied α NO π^* and 5 unoccupied β Fe d orbitals. This electronic structure is consistent with the Fe^{III}-NO⁻ description determined for Fe-EDTA-NO.¹ To model the HPPD-HPP-NO complex, the two water ligands of the E-NO complex were replaced by α -ketoglutarate (α KA) as described above.⁴⁷ This geometry optimized computational model (E- α KA-NO) also shows a strong Fe-N^{NO} bond (1.76Å, Fe-N-O angle of 149°) which again defines the z-axis of the molecule (Figure 4A and Table 1).⁴⁸ The electronic structure of the Fe-NO unit of E- α KA-NO is very similar to that of E-NO (molecular orbital diagram in Figure 4B, right and boundary surface plots in Figure 5). The α KA moiety has two molecular orbitals (the HOMO and LUMO) that interact with the Fe center (Figure 6). The occupied α KA n orbital (the α KA HOMO) is primarily a lone pair on the carboxylate with some in-plane character on the carbonyl and will have a σ bonding interaction with the Fe d_{xy} and $d_{x^2-y^2}$ orbitals. (Note that in the coordinate system in Figure 4A, y is in the Fe-N-O plane that bisects equatorial ligand-metal bonds therefore $d_{x^2-y^2}$ is a d_π orbital with lobes bisecting the ligand bonds and d_{xy} is the d_σ orbital with its lobes along the ligand-metal bonds.) The unoccupied α KA π^* LUMO is mostly on the carbonyl with conjugation onto the carboxylate and will have a π interaction with the Fe d_{yz} and d_{xz} orbitals. In the E- α KA-NO complex, these α KA based MOs are β 117 (n) and β 122 (π^*) in Figure 4B, right and Figure 5.

4. Analysis

4.1 Correlation of calculations to experiment

Using the geometry optimized computational models E-NO and E- α KA-NO presented above, the effects of α KA binding on the experimental spectra were evaluated.

Time-dependent density functional theory (TD-DFT) was used to generate a predicted absorption spectrum for each computational model. The TD-DFT predicted absorption spectrum for the E-NO complex is shown in Figure 7A where the transitions with significant NO⁻ to Fe d character are modeled with Gaussian bandshapes with a width at half height of 3000 cm⁻¹. The primary transitions and their corresponding transition dipole moment directions are given in Table 2. The TD-DFT predicted absorption spectrum for the E- α KA-NO complex is shown in Figure 7B.⁴⁹ Binding of the α KA ligand introduces new bands in the predicted spectrum with significant contributions from the α KA n to Fe $d_{x^2-y^2}$ (d_π)

transition. These are included in Figure 7B (teal). The TD-DFT calculation predicts these transitions to be higher in energy than the NO^- to Fe d transitions (Table 2). From this, the highest energy transition in the UV-vis absorption (and MCD) spectrum of HPPD-HPP-NO is assigned as an αKA n to Fe $d_{x^2-y^2}$ CT transition. Overlays of the experimental absorption and calculated TD-DFT spectra for HPPD-NO and HPPD-HPP-NO are in reasonable agreement and are shown in Supplementary Figure S11.

The assignment of the new high energy transition in the absorption/MCD spectra to an αKA n to Fe d CT transition provides insight into the mixed polarization observed in the VTVH MCD data of the HPPD-HPP-NO. From the calculated transition dipole moment directions given in Table 2, the NO^- to Fe d CT transitions in E-NO are predominantly polarized in the z direction. This polarization direction refers to the axis of the ZFS and is consistent with the strong Fe- N^{NO} bond which defines the z-axis of the system. For the predicted E- αKA -NO complex NO^- to Fe d CT transitions, however, there is an x,y component of the transition dipole moment leading to an off axis transition moment direction. This reflects mixing with the αKA n to Fe d transition that has a transition dipole moment dominantly in the x,y direction (Table 2) consistent with αKA binding in the equatorial plane. The two charge transfer transitions are close in energy and mix through configuration interaction either through orbital overlap to generate the off axis transition dipole moment calculated or through SOC to give the mixed polarization observed in the VTVH MCD data.

The lower energy transitions in the MCD spectra of $\{\text{FeNO}\}^7$ complexes are assigned as the formally spin forbidden Fe^{III} , ligand field transitions. As the TD-DFT calculations only give the spin-allowed transitions, ΔSCF calculations were undertaken to probe the energies of the specific Fe^{III} ligand field transitions. As promotion of a full electron resulted in wavefunctions which would not converge, these transitions were calculated as Slater transition states.⁵⁰ Table 3 gives the calculated transition energies for the Fe^{III} αd_{σ} to βd_{π} ligand field transitions.⁵¹ From Table 3, for most of the ligand field transitions, the energies change only slightly between the E-NO and E- αKA -NO complexes. The most notable exception lies in the lowest energy, Fe^{III} αd_{z^2} to $\beta d_{x^2-y^2}$, transition which increases by $\sim 3800\text{ cm}^{-1}$ upon binding of αKA . This change is consistent with the appearance of a new band in the low energy region of the HPPD-HPP-NO MCD spectrum in Figure 3B; this transition would then be at still lower energy in the HPPD-NO MCD spectrum (below our 5000 cm^{-1} cutoff), and moved up in energy with αKA binding. As mentioned previously, the Fe^{III} ligand field transitions are formally spin-forbidden. These transitions become allowed through metal-based spin-orbit coupling (SOC) with the nearby spin allowed charge transfer transitions. (For a more in-depth description of the SOC mechanism, see the supporting information.)

The increase in energy of this Fe^{III} d_{z^2} to $d_{x^2-y^2}$ transition (axes in Figure 4A and Supplementary Figure S6A) upon binding of αKA reflects either a decrease in the energy of Fe d_{z^2} or an increase in the energy of the $d_{x^2-y^2}$ orbital. Alignment of the MO energy levels to the highest occupied NO^- orbital (Supplementary Figure S12) shows that d_{z^2} does not significantly change in energy between the E-NO and E- αKA -NO complexes (in fact, it increases in energy). This is consistent with the similar Fe- N^{NO} bond lengths in the geometry optimized structures, and reflects the strong σ interaction of the NO with Fe d_{z^2} . However, $d_{x^2-y^2}$ increases in energy with addition of αKA which reflects the addition of a much stronger donor ligand in the equatorial plane than the two water ligands it replaces from the E-NO complex. Note that even this BP86 + 10% HF exchange calculation does not fully account for the charge donation of the α -keto acid. While the calculation correctly predicts the shift up in energy of the lowest energy Fe^{III} ligand field transition due to donation by the α -keto acid, it does not predict the observed shift down in energy of the $\sim 12,000\text{--}13,000\text{ cm}^{-1}$ ligand field transition. Additionally, the αKA to Fe^{III} transition at

$\sim 29,000\text{ cm}^{-1}$ is significantly more intense in the experimental absorption spectrum than in the corresponding TD-DFT predicted spectrum, supporting the assignment of α KA as a strong donor ligand, but again suggesting that the strength of the α KA donation is underestimated in these calculations. Quantitatively, the amount of α KA present in the unoccupied valence orbitals of the NO complex (relative to the amount of O from the waters it replaces) gives the relative donor strength. These values are 23% for α KA relative to 10% for the water ligands (from Mulliken population analyses). Assignment of the α KA moiety as a strong donor ligand has important implications for O_2 reactivity.

The axial ZFS parameter, D , obtained from fitting the temperature dependence of the $S=3/2$ EPR signal (Supplementary Figure S1) decreases by half in the α KA bound FeNO complex (from 16.0 cm^{-1} to 8.4 cm^{-1}). This is reproduced in the DFT calculations where the ZFS parameters calculated using the optimized geometries for E-NO and E- α KA-NO (both calculated to be axial with z along Fe-N^{NO}) decrease from 10 cm^{-1} to 6.6 cm^{-1} . Rodriquez, et. al. have evaluated the origin of the large ZFS in $S=3/2$ {FeNO}⁷ complexes (for Fe-[Me₃TACN]-(N₃)₂-NO where $D=22\text{ cm}^{-1}$).⁵² They attribute the large value of the ZFS to delocalization of electron density from the NO⁻ into the Fe d_{yz} and d_{xz} orbitals which strongly overlap in the {FeNO}⁷ unit. From Table 4, the amount of NO⁻ character in the β Fe d_{yz} and d_{xz} molecular orbitals does in fact decrease from 72% to 54% upon binding of α KA. This reflects the competition from the strong donation of the α KA into the d_π orbitals. Thus the high energy CT feature in absorption/MCD, the mixed polarization from the VTVH MCD isotherms, the new low energy LF band in MCD and the decreased value of D all reflect the strong donation of the α KA ligand to the Fe^{III}.

4.2 Comparison to B3LYP/LACVP

Most calculations published on the reactivity of α -keto acid dependent mononuclear non-heme Fe complexes used the B3LYP/LACVP combination of functional and basis set.^{12, 34, 35} Therefore, it was useful to evaluate its performance with respect to these experimental data. Geometry optimization of the E-NO and E- α KA-NO complexes with B3LYP/LACVP results in structures with Fe-N^{NO} bond lengths $\sim 0.3\text{ \AA}$ longer than the structures optimized with BP86+10%HF/Gen (Supplementary Figure S13 and Table S2). This long Fe-N^{NO} bond results in less electron density transferred to NO giving electronic structures which are best described as Fe^{II}-NO^{*} (4 unoccupied β Fe d orbitals, 2 unoccupied α NO orbitals and 1 unoccupied β NO orbital shown in the energy level diagram in Supplementary Figure S14). The TD-DFT predicted absorption spectra of these complexes show a large change upon binding α KA (Supplementary Figure S15), and the TD-DFT calculations also predict transitions which are predominantly Fe d to NO in character, consistent with the initial electronic structure description as Fe^{II}-NO^{*}. This calculated large change in the TD-DFT predicted absorption spectrum upon binding α KA is not consistent with the observed experimental data (compare Figure 2 to Figure S15). Using the B3LYP/LACVP optimized structures and the “spectroscopic parameters” basis set (CP(PPP) on Fe) with the B3LYP functional to calculate the ZFS gives values of D that are negative and E/D values that are significantly rhombic (Supplementary Table S2). These are also not consistent with the experimentally observed strongly axial EPR signals with positive values of D (in Figure 1).

Finally, the free energy of NO binding to a resting, facial triad enzyme complex can be extracted from the known dissociation constant for NO binding to metapyrochatecase⁵³ and compared to the energies for the E-NO computational models. (In metapyrocatechase, the Fe center is also ligated by the facial triad, 2 water ligands, and NO and the resulting complex has an $S=3/2$ ground state.) The experimentally derived free energy of NO binding is ~ -4.4 kcal/mol while the calculated values for the two model systems are -4.9 kcal/mol for BP86+10%/Gen and $+9.4$ kcal/mol for B3LYP/LACVP ($T = 298\text{ K}$, solvation $-\epsilon = 4.0$).

Thus, the calculated NO binding energy for B3LYP/LACVP predicts that the E-NO complex is not stable which is inconsistent with experiment.

Thus two different computational methods produce different electronic structures. The spectroscopic data support the choice of BP86+10%/Gen as an experimentally calibrated computational model to best evaluate the NO complex and by extension the O₂ complex. The difference between these two electronic structures reflects the extent of charge donation from Fe to NO which will have implications on the interactions of O₂ with the Fe center in the evaluation of the O₂ reaction coordinate to form the Fe^{IV}=O species.

4.3 Extension to {FeO₂}⁸

Based on the above analysis, the BP86+10%HF/Gen computational approach was used to evaluate the {FeO₂}⁸ complex and the reaction coordinate of α KA dependent dioxygenases. The free energy of O₂ binding to the E computational model in this study is 17 kcal/mol (T = 298K, solvation – ϵ = 4.0), consistent with the previous computational study of $\Delta G^\ddagger$ of 22 kcal/mol for the resting form of metapyrocatechase and the fact that the holoenzyme does not react with O₂ in the absence of α KA (or substrate in the case of metapyrocatechase).⁵⁴ To evaluate the {FeO₂}⁸ complex, the NO of the E- α KA-NO complex was replaced with O₂ and the structures for the different possible spin states were reoptimized.

The complex of O₂ (S=1) bound to Fe^{II} (S=2) can have a total spin of S=3, S=2, S=1, or S=0. Dioxygen binding in each of these spin states was evaluated.⁵⁵ The geometry optimized structures and MO diagrams for the E- α KA-O₂ complexes are shown in Figure 8 and Supplementary Figures S16-17, with key geometric parameters given in Table 5. The S=3 E- α KA-O₂ optimized structure (end-on) is best described as a Fe^{III} ferromagnetically coupled to O₂^{•-} (5 unoccupied β Fe d orbitals and 1 unoccupied β O₂ π^* orbitals, Supplementary Figure S17, left). The optimized E- α KA-O₂ structure in the S=2 spin state is again end on and best described as a high spin Fe^{III} antiferromagnetically coupled to O₂^{•-} (5 unoccupied β Fe d orbitals and 1 unoccupied α O₂ π^* orbital, Supplementary Figure S17, right, and Supplementary Figure S18 for boundary surface plots). For the S=1 E- α KA-O₂ complex, the lowest energy structure is a new bridged binding mode to the α KA ligand (Figure 8, right – bottom left corner). This structure is best described as a low spin Fe^{IV}-O₂²⁻ (4 unoccupied β Fe d orbitals and 2 unoccupied α Fe d orbitals, Figure 8, left, with boundary surface plots in Figure 8, right). Comparison of the free energy of O₂ binding for each spin state (Table 5) reveals the S=1 bridged binding mode is significantly lower in energy than the S=2 and S=3 spin states.⁵⁶ Consistent with the endergonic nature of the O₂ binding, no O₂ bound species is observed prior to the formation of the Fe^{IV}=O intermediate.¹⁴

A linear transit calculation was used to evaluate O₂ binding to form the new peroxide bridged binding mode (Figure 9 and Table 6). At long Fe—O₂ distances, an S=1 description of the E- α KA-O₂ complex starts as an antiferromagnetic coupled E- α KA (Fe^{II}, S=2) and O₂ (S=1) system (Figure 9, left). As the O₂ approaches the Fe^{II} E- α KA complex, charge is transferred from Fe to O₂, resulting in more ferric character. This increases the interaction with the α KA resulting in shorter Fe-O ^{α KA} bonds that increases the ligand field at the iron center resulting in an E- α KA-O₂ complex that is a low spin, ferromagnetically coupled Fe^{III}-O₂^{•-} species (Figure 9, step 1). Further interaction of the superoxide with the Fe center leads to transfer of a second electron to form an Fe^{IV}-O₂²⁻ species (Figure 9, step 2). To form the bridge, the occupied O₂²⁻ π^* _(ip) orbital has a nucleophilic interaction with the unoccupied α KA π^* orbital (Scheme 3) forming a σ bond between the distal O of the peroxide and the carbonyl carbon of the α KA.

This structure is of interest as a potential intermediate for study experimentally. Although based on the equilibrium between the unbound O₂ and peroxy-bridged structures the

population of peroxy-bridged species will be small, there is still a barrier after formation of the peroxy-bridged structure (*vide infra*). The TD-DFT predicted absorption spectrum was calculated to evaluate where the peroxy to Fe charge transfer transitions are expected. This calculated spectrum with Gaussian broadening of the peroxy to Fe CT transitions is shown in Supplementary Figure S19. An Fe^{IV}-alkylperoxy model complex has been reported by Que and coworkers.⁵⁷ The TD-DFT calculated absorption spectrum in figure S19 is in good agreement with the experimental absorption spectrum of this complex and strengthens their assignment of the two lowest energy transitions as peroxy to Fe^{IV} CT.

4.4 Completion of α -keto acid dependent non-heme iron reaction coordinate

The first experimentally observed intermediate for the α -keto acid dependent dioxygenases is an Fe^{IV}=O which has an S=2.^{14, 58, 59} From the low spin Fe^{IV}-O₂²⁻ S=1 structure in Figure 8, a spin intersystem crossing must occur to allow the reaction coordinate to proceed on the S=2 surface. Cleavage of both the C-C (to release CO₂) and O-O bonds occurs before formation of the Fe^{IV}=O species. Changing the O-O coordinate increases the energy of both spin states (Supplementary Figure S20). However, on the C-C coordinate, the two spin states cross in energy, and this coordinate was pursued. Intersystem crossing on the C-C coordinate requires that the two spin surfaces have a similar energy and geometry (minimum energy crossing point, MECP), and their electronic structures should differ by one electron on the same center, such that the two states can spin orbit couple. The optimizations of the bridged O₂ structure in both the S=1 and S=2 spin states show that the geometries are very similar with the exception of the Fe-O^{CO2} distance (which is 1.93Å in S=1 and 2.30Å in S=2). Thus a 2-D potential energy scan (elongation of the C-C bond while decreasing the difference between the Fe-O^{CO2} bond lengths) was evaluated to find the spin surface crossing point (Figure 10). A crossing point in the energies was found at a C-C bond length of ~2.1Å. The electronic structures of the S=1 and S=2 models (Figure 11, structures 2 and 3) are both Fe^{III}-O₂²⁻ where as the C-C was elongated, one e⁻ transferred from the α KA n orbital to the Fe (Supplementary Figure S21). The S=1 and S=2 states at this MECP differ by one e⁻: occupied β Fe d_{xz} and α Fe d_{z2}⁶⁰ (Figure 11, structures 2 and 3). Thus, these two states SOC via L_y (Supplementary Figure S22).^{60, 61}

Once on the S=2 surface, the C-C bond cleaves without any additional barrier and transfers the second α KA n electron to Fe forming a high spin Fe^{II}-peracid (Figure 11, structure 4). The transfer of 2 e⁻s from Fe^{II} into the peracid σ^* orbital to break the O-O bond is accomplished with a minimal additional barrier (calculated <1kcal/mol) to form the experimentally observed S=2 Fe^{IV}=O. The free energies for these steps of the O₂ reaction are given in Figure 12. The reaction of the Fe^{IV}-oxo with substrate in HPPD has been evaluated in Ref 28.

5. Discussion

The comparison of the NO complexes of HPPD and HPPD-HPP provides a probe of the new spectral features which arise from the α -keto acid bonding to the ferric site. Four new spectral features are observed in the HPP bound HPPD-NO complex that reflect a new electronic structure: a new α KA to Fe^{III} charge transfer transition above 25,000 cm⁻¹ in the UV-vis absorption and MCD spectra, mixed polarization of the NO⁻ to Fe^{III} charge transfer bands, a new low energy, ligand field MCD transition at < 5000 cm⁻¹, and a decrease by half in the ZFS. The new charge transfer transition reflects the donor interaction of the α -keto acid ligand to the Fe^{III}. The < 5000 cm⁻¹ ligand field band in the HPPD-HPP-NO complex also reflects the strong equatorial donor interaction of the α -keto acid with the {FeNO}⁷ unit which further leads to the decrease in the ZFS. Taken together, these spectroscopic changes indicate that the α -keto acid ligand is a strong donor that promotes increased charge transfer from Fe^{III} to NO⁻.

Replacement of NO with O₂ in calculations that reproduce the α -keto acid-{FeNO}⁷ spectral features results in a new, low energy S=1 peroxy-bridged structure. Charge donation from iron to O₂ results in increased ferric character that increases the bonding and donation of the α -keto acid ligand⁶² which promotes formation of this low spin Fe^{IV}-peroxide bridge (Figure 9). As the experimentally observed Fe^{IV}=O product of O-O cleavage has an S=2 ground state, the S=1 bridged peroxide must crossover to the S=2 surface to form the Fe^{IV}=O species. A minimum energy crossing point from the S=1 to S=2 surface along the C-C coordinate (C-C = 2.10 Å) was found. The free energy to this step is ~ 17 kcal/mol (Figure 12, starting from the reaction of E- α KA with dioxygen, T = 298K, solvation - ϵ = 4.0) which is in reasonable agreement with experiment ($\Delta G^\ddagger$ ~ 14 kcal/mol).^{14, 58, 59} Thus a bridged Fe^{IV}-peroxide binding mode is a viable step along the α -keto acid dependent mononuclear non-heme Fe-O₂ reaction coordinate. From this S=2 peroxy species, decarboxylation occurs and the resultant Fe^{II}-peracid undergoes O-O cleavage to give the Fe^{IV}=O species with a minimal (< 1 kcal/mol) barrier.

Previous studies, using the B3LYP/LACVP approach, suggested that an S=2 Fe^{III}-O₂^{*} species initiates the reaction with α -keto acids.^{12, 34, 35} However, this functional/basis set combination generates structures with long Fe-O₂ bonds and less charge transfer from Fe to O₂ (i.e. less Fe^{III} character) which in turn results in less donor bonding by the α KA and prevents the formation of a low energy peroxy bridged structure. In this study, the experimentally validated computational methods lead to α KA binding as a strong donor ligand (from experiment) that drives the formation of the S=1, low spin bridged Fe^{IV}-peroxide intermediate.

As triplet O₂ approaches the Fe^{II}, orbital overlap would lead to antiferromagnetic coupling to give the S=1 surface. Continuing along the S=1 surface leads to the S=1 Fe^{IV}-peroxide intermediate that can nucleophilically attack the α -keto acid π^* LUMO on the carbonyl. This intermediate (not present in B3LYP/LACVP calculations^{12, 34, 35}) has been postulated since early studies of α -keto acid dependent mononuclear non-heme iron enzymes,¹⁰ but had not been validated until now. Finally, the effect, elucidated in this study, associated with the binding of a strong donor ligand (i.e. α KA) on the initial dioxygen activation steps likely has implications for the mechanisms of other non-heme Fe^{II} oxygen activating enzymes in which the substrate or cofactor binds directly to the metal center.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This research was supported by NIH Grant GM40392 (EIS) and NSF Grant MCB0843619 (GRM). We would like to thank Dr. Martin Srnec for assistance in performing calculations.

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60. Movement towards the crossing point results in a weaker interaction along N^{eq}-Fe-O^{CO2} and a tetragonal elongation of the site which rotates the coordinate system such that the z-axis is along N^{eq}-Fe-O^{CO2}.
61. Further elongation of the C-C bond on the S=1 surface continues to be energetically unfavorable as there is no transfer of the second electron from the α KA n orbital (Supplementary Figure S21). The S=3 surface was also considered. However, at the crossing point, the S=3 structure is 10kcal/mol higher in energy than the S=1 and S=2 structures, making its involvement unlikely in this reaction.
62. The α KA donation relative to the O_{water} it replaces can again be estimated from their relative character in the unoccupied valence orbitals in the FeO₂ complexes which are 38% α KA versus 11% O of the 2 water molecules (from Mulliken population analyses).

Scheme 1.

Proposed mechanism for α -keto acid dependent dioxygenases. (Adapted from Ref 1)

Scheme 2.
Reaction catalyzed by HPPD

Scheme 3.

Nucleophilic interaction between the occupied $O_2^{2-} \pi^*_{(ip)}$ orbital and the unoccupied $\alpha KA \pi^*$ orbital.

Figure 1. EPR of $\{\text{FeNO}\}^7$ enzyme complexes

EPR of S=3/2 HPPD-NO (A) and HPPD-HPP-NO (B) showing the inverse temperature dependence of the signal intensity. The inlays show the best fit to the data.

Figure 2. Room temperature UV-vis absorption spectra of HPPD-NO (red) and HPPD-HPP-NO (blue). Gaussian resolution of the bands is shown (dashed for HPPD-NO and dot for HPPD-HPP-NO).

Figure 3. VTVH MCD of $\{FeNO\}^7$

(A, B) The 5K, 7T MCD spectra of HPPD-NO (A) and HPPD-HPP-NO (B). The Gaussian fits to the spectra are shown. For HPPD-NO, the star indicated unreacted resting HPPD. VTVH MCD isotherms were taken at the points indicated with arrows on the spectra of HPPD-NO and HPPD-HPP-NO. (C-E) The VTVH MCD isotherms and best fits to the data. (Data were collected at 1.8K, 3K, 5K, 7.5K, 10K, 15K, and 25K for C & D and at 3K, 5K, 7.5K, 12K, and 15K for E.) VTVH MCD taken for HPPD-NO at 21,700 cm^{-1} (C) and HPPD-HPP-NO at 21,400 cm^{-1} (D) and 5650 cm^{-1} (E).

Figure 4. Computational model for $S=3/2$ $\{FeNO\}^7$ E- α KA-NO
 (A) Geometry optimized structure and (B) Molecular orbital diagram.

Figure 5.
Select β boundary surface plots for the geometry optimized structure of $S=3/2$ E- α KA-NO.

Figure 6.
Boundary surface plots of αKA available for bonding to Fe.

Figure 7. TD-DFT predicted absorption spectra

Plots of E-NO (A) and E- α KA-NO (B) predicted absorption spectra with Gaussian bandshapes with a width at half height of 3000 cm⁻¹ shown for transitions with NO⁻ to Fe d (red) and α KA to Fe d (teal) charge transfer character.

Figure 8. Molecular orbital diagram (left) and select β boundary surface plots (right) for geometry optimized structure of S=1 E- α KA-O₂. Optimized structure shown in lower left corner. Selected bond lengths given in Table 5.

Figure 9. Formation of S=1 $Fe^{IV}-O_2^{2-}$ bridge

Four points along formation of S=1 $Fe^{IV}-O_2^{2-}$ bridge structure: initial $Fe^{II}-O_2$ (left, Fe-O = 2.50 Å), step 1 (middle left, Fe-O = 2.10 Å), step 2 (middle right, Fe-O = 1.90 Å) and bridged $Fe^{IV}-O_2^{2-}$ (right).

Figure 10. 2-D spin crossover surface

Electronic energies for elongation of C-C while decreasing the difference in Fe-O^{CO₂} bond length. The energies of the two spin states cross at an Fe-O^{CO₂} distance of 2.15 Å and a C-C bond length of 2.10 Å. For clarity, only the minimum energy points are shown; however, points were taken at C-O bond distances on either side of the minimum energy points. The energies are relative to the optimized S=1 bridged structure.

Figure 11. Electron flow for steps of E- α KA- O_2 reaction to form $Fe^{IV}=O$, S=2

Dioxygen binding occurs on the S=1 surface with formation of a bridge to the α -keto acid moiety. Two electrons are transferred to the O_2 π^* orbital generating a low spin Fe^{IV} -peroxide (1). Elongation of the C-C bond results in transfer of $1e^-$ from the α KA n orbital to form an Fe^{III} -peroxide which crosses over to the S=2 surface (2-3). Once on the S=2 surface, the second electron of the α KA n orbital is transferred to Fe, generating an Fe^{II} -peracid and free CO_2 (4). Finally, cleavage of the O-O bond leads to formation of an Fe^{IV} -oxo S=2 (5).

Figure 12. Free energy diagram for initial steps of E- α KA-O₂ reaction (T = 298K; solvation – $\epsilon = 4.0$)

Table 1Key geometric parameters for E-NO, E- α KA-NO, and Fe-EDTA-NO

	E-NO	E-αKA-NO	Fe-EDTA-NO
Fe-N ^{NO}	1.76Å	1.76Å	1.78Å ^a /1.76Å ^b
N-O	1.17Å	1.17Å	1.10Å/1.18Å
Fe-N-O	147°	149°	156°/149°

^aEXAFS parameters^bcomputational parameters

Table 2

TD-DFT transition energies and directions of transition dipole moments

Transition	Experiment HPPD-NO	HPPD-HPP-NO	Calculated E-NO	E- α KA-NO
NO ⁻ $\rightarrow$ Fe d	19,500cm ⁻¹	17,600cm ⁻¹	13,900cm ⁻¹ ; z	13,500cm ⁻¹ ; z
	20,800cm ⁻¹	19,800cm ⁻¹	16,600cm ⁻¹ ; x,y/z ^a	17,500cm ⁻¹ ; x,y/z
	22,100cm ⁻¹ ^b	22,000cm ⁻¹ ^b	23,700cm ⁻¹ ; z ^b	22,000cm ⁻¹ ; x,y/z ^b
α KA n $\rightarrow$ Fe d		~30,000cm ⁻¹		25,500cm ⁻¹ ; x,y

^aTransition is mixed with a His $\rightarrow$ Fe CT transition. These His CT transitions are calculated to be significantly lower in energy than would be seen experimentally.

^bThe polarization of this transition can be measured experimentally to compare to the direction of the transition dipole moment. HPPD-NO 85% z (exp), E-NO 89% z (calc); HPPD-HPP-NO 51% x,y and 49% z (exp), E- α KA-NO 70% x,y and 30% z (calc).

Table 3 $\Delta\text{SCF Fe}^{\text{III}}$ spin forbidden ligand field transition energies

Transition	E-NO	E- α KA-NO
$d_{z^2} - d_{x^2-y^2}$	3600 cm^{-1}	7400 cm^{-1}
$d_{xy} - d_{x^2-y^2}$	8000 cm^{-1}	8700 cm^{-1}
$d_{xy} - d_{yz}$	12,500 cm^{-1}	12,500 cm^{-1}
$d_{z^2} - d_{xz}$	14,100 cm^{-1}	14,800 cm^{-1}
$d_{xy} - d_{xz}$	16,100 cm^{-1}	19,100 cm^{-1}

Table 4NO⁻ 2 π^* character in Fe d_{yz} and d_{xz}

Orbital	E-NO	E- σ KA-NO
d _{yz}	24%	22%
d _{xz}	48%	32%
Total	72%	54%

Table 5Key geometric and electronic structure parameters for E- α KA-O₂ complexes

	S=3	S=2	S=1
Fe-O	2.18 Å	2.06 Å	1.80 Å
O-O	1.28 Å	1.28 Å	1.41 Å
Fe-O-O	118.0°	118.5°	110.3°
electronic structure	Fe ^{III} -O ₂ ⁻	Fe ^{III} -O ₂ ⁻	Fe ^{IV} -O ₂ ²⁻
ΔG O ₂ binding ^a	12.0 kcal/mol	12.5 kcal/mol	5.8 kcal/mol

^a(T = 298K; solvation - ϵ = 4.0)

Table 6

Steps to form the S=1 peroxo-bridged structure

	approach	step 1	step 2	bridged structure
Fe-O	2.50Å	2.10Å	1.90Å	1.80Å
O-O	1.23Å	1.30Å	1.34Å	1.41Å
O-C	3.15Å	1.90Å	1.70Å	1.47Å
Fe-O ^{CO}	2.31Å	1.91Å	1.88Å	1.84Å
Fe-O ^{CO2}	2.02Å	1.90Å	1.90Å	1.89Å
Fe-O-O	120.0°	111.7°	112.2°	110.3°
Fe-O ^{CO} -C-C	5.2°	39.4°	48.5°	55.8°
relative electronic energy	0.0kcal/mol	-9.5kcal/mol	-15.2kcal/mol	-19.4kcal/mol
electronic structure	Fe ^{II} -O ₂	Fe ^{III} -O ₂ ⁻ (low spin)	Fe ^{IV} -O ₂ ²⁻	Fe ^{IV} -O ₂ ²⁻