*The facial triad in the α -ketoglutarate dependent
oxygenase FIH: a role for sterics in linking
substrate binding to O₂ activation*

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RECEIVED DATE

Running Title: Role of the facial triad in FIH

Abstract

The factor inhibiting hypoxia inducible factor-1 α (FIH) is a nonheme Fe(II)/ α KG oxygenase using a 2-His-1-Asp facial triad. FIH activates O₂ via oxidative decarboxylation of α -ketoglutarate (α KG) to generate an enzyme-based oxidant which hydroxylates the Asn⁸⁰³ residue within the C-terminal transactivation domain (CTAD) of HIF-1 α . Tight coupling of these two sequential reactions requires a structural linkage between the Fe(II) and the substrate binding site to ensure that O₂ activation occurs after substrate binds. We tested the hypothesis that the facial triad carboxylate (Asp²⁰¹) of FIH linked substrate binding and O₂ binding sites. Asp²⁰¹ variants of FIH were constructed and thoroughly characterized *in vitro* using steady-state kinetics, crystallography, autohydroxylation, and coupling measurements. Our studies revealed each variant activated O₂ with a catalytic efficiency similar to that of wild-type (WT) FIH ($k_{cat}/K_{M(O_2)} = 0.17 \mu\text{M}^{-1} \text{min}^{-1}$), but led to defects in the coupling of O₂ activation to substrate hydroxylation. Steady-state kinetics showed similar catalytic efficiencies for hydroxylation by WT-FIH ($k_{cat}/K_{M(CTAD)} = 0.42 \mu\text{M}^{-1} \text{min}^{-1}$) and D201G ($k_{cat}/K_{M(CTAD)} = 0.34 \mu\text{M}^{-1} \text{min}^{-1}$); hydroxylation by D201E was greatly impaired, while hydroxylation by D201A was undetectable. Analysis of the crystal structure of the D201E variant revealed steric crowding near the diffusible ligand site supporting a role for sterics from the facial triad carboxylate in the O₂ binding order. Our data support a model in which the facial triad carboxylate Asp²⁰¹ provides both steric and polar contacts to favor O₂ access to the Fe(II) only after substrate binds, leading to coupled turnover in FIH and other α KG oxygenases.

Introduction

The Fe(II)/ α KG dioxygenases catalyze a wide range of oxidations including hydroxylation of proteins, demethylation of RNA and DNA, and desaturations of antibiotic precursors [1, 2]. Enzymes in this superfamily bind the nonheme Fe(II) cofactor with a 2-His-1-Carboxylate facial triad, completing the coordination environment with a bidentate α -ketoglutarate (α KG) and an exchangeable ligand site that is occupied by H₂O in resting enzyme (Scheme 1) [1, 3]. Binding primary substrate near the cofactor increases the reactivity of Fe toward O₂ [4, 5], leading to a sequential mechanism with ordered O₂ activation, resulting in tight coupling between the two half reactions. The linkage between substrate binding and O₂ reactivity is thought to involve changes in contacts within the second coordination sphere of the Fe cofactor [6-12]. FIH, an Fe(II)/ α KG oxygenase responsible for O₂ sensing in humans, is one of the few α KG oxygenases that is active with a substituted facial triad carboxylate residue [13]. This affords a unique opportunity to understand how local contacts to the facial triad carboxylate ligand affect the coupling between O₂ reactivity and substrate binding.

The consensus mechanism for FIH and other Fe(II)/ α KG oxygenases (Scheme 1) involves the sequential half reactions of O₂ activation followed by substrate hydroxylation [1, 14-18]. Substrate binding leads to release of the aquo ligand in FIH and other Fe(II)/ α KG oxygenases as shown by variable temperature and variable field magneto circular dichroism (VTVH-MCD) spectroscopy [19-21], as part of a process that has been termed triggering [22] or priming [23]. O₂ is proposed to bind as a ferric superoxide at the open coordination site, then attack the C-2 carbonyl of α KG, passing through a transition state resembling a peroxohemiketal [17, 24, 25] to form succinate and a ferryl as the active oxidant. The ferryl oxidant abstracts an H-atom to form a substrate radical and ferric hydroxide, which then rebounds to form the ferrous

cofactor and hydroxylated product [22, 26-31]. As primary substrate does not directly coordinate the Fe(II), the structural linkage between substrate binding and increased O₂ reactivity are crucial to tight coupling in this family of enzymes.

Scheme 1. Consensus Mechanism for FIH

As the primary substrate for FIH is the C-terminal transactivation domain (CTAD) of the hypoxia inducible factor, the Fe active site is exposed to solvent until the primary substrate is bound as shown by crystal structures of (Fe+ α KG)FIH and (Fe+ α KG)FIH/CTAD [32, 33]. MCD spectroscopy indicates that (Fe+ α KG)FIH is six coordinate[20] and crystal structures show the facial triad carboxylate, Asp²⁰¹, is well positioned to hydrogen bond to the distal aquo ligand [32-34]. In structures where the aquo ligand is resolved, the distal O-atom of Asp²⁰¹ is within hydrogen bonding distance to the aquo ligand (O_{distal}---OH₂ = 2.52Å - 2.85Å) [35, 36]. Although O₂ activation occurs slowly in the absence of CTAD [37, 38], CTAD binding alters the local contacts to Asp²⁰¹ and stimulates O₂ reactivity [10, 13, 33, 34], suggesting that changes in the chemical environment near the diffusible ligand site connects CTAD binding to O₂ reactivity.

We test the facial triad carboxylate as the nexus connecting substrate binding to O₂ reactivity in FIH. Previously, variants of Asp²⁰¹ were shown to hydroxylate CTAD with varying yields, but the role of these variants in linking O₂ activation with substrate binding and hydroxylation was not explored[13]. In this work, three facial-triad variants (Glu, Gly, Ala) were prepared and purified to perturb the chemical environment near the diffusible ligand. Steady-state kinetics, crystallography, and assays to measure the coupling between O₂ consumption and CTAD hydroxylation indicated that the identity of the facial triad residue had little effect on O₂ activation, but played a deciding role in coupling the O₂ activation to substrate

hydroxylation. Overall, these data indicate that the facial triad carboxylate is not essential to activate O₂, but rather serves to ensure that O₂ activation is coupled with substrate hydroxylation.

Methods

Materials

All reagents were purchased from commercial vendors and were used as received, with the exception of the 39-residue CTAD peptide. The sequence of the 39-residue CTAD peptide (theoretical molecular weight = 42544.6 Da; pI=3.95) followed that of the C-terminal activation domain of human HIF-1 α , (HIF-1 α ^{788–826}), with the exception of a Cys⁸⁰⁰→Ala substitution to prevent peptide oxidation (Asn⁸⁰³), DESGLPQLTSYDAEVNAPIQGSRNLLQGEELLRALDQVN. CTAD was purchased as a desalted peptide from EZBiolab (Carmel, IN, USA) with free N and C-termini, then purified as previously described using reversed-phase HPLC to obtain peptide of >95% purity [4].

Protein Expression and Purification

The QuikChange mutagenesis kit (Stratagene) was used to make mutations in the pET28a-FIH construct [38]. The DNA sequence of each mutated plasmid was sequenced (Genewiz, NJ, USA) to confirm the desired point mutation. WT-FIH and each variant was overexpressed in BL21-DE3 *E. coli* with an N-terminal His₆ tag and purified as previously described [4]. Three non-native residues (NH₂-GlySerHis-) remained on the N-terminus of FIH following thrombin cleavage. Purified FIH was buffer exchanged into 50 mM HEPES pH 7.00. The purity (>95%) of each variant was assessed by SDS-PAGE.

Steady-State Kinetics

The standard reaction conditions used 50 mM HEPES pH 7.00 (37.0 °C) containing 100 mM NaCl, ascorbic acid (2 mM), α KG (100 μ M), FeSO₄ (25 μ M). Assays in which CTAD was the varied substrate (15-275 μ M) used saturating α KG (100 μ M). Assays in which α KG was the varied substrate (0-200 μ M) contained ascorbate (2 mM), FeSO₄ (25 μ M) and a fixed CTAD concentration (80 μ M). Initial rate measurements were made as previously described using a Bruker MicroFlexII MALDI-TOF-MS and fit to the Michaelis-Menten equation [4].

Steady-State Kinetics varying O₂

Assays in which the O₂ concentration was varied were performed as previously described [25]. Briefly, HEPES buffer (50 mM, pH 7.00) was stirred to equilibrate the solution under an atmosphere of N₂ and O₂ at 37.0°C within an AtmosBag (Sigma-Aldrich). The concentration of dissolved O₂ was measured in buffer using a Clarke electrode (YSI) prior to each reaction. The reaction mixture (45 μ L) containing ascorbic acid (2mM), α KG (100 μ M), FeSO₄ (25 μ M), NaCl (100 mM) and CTAD (80 μ M), was incubated at 37.0 °C for two minutes prior to addition of FIH to initiate the reaction. The FIH stock was gently pipetted down the side of the microcentrifuge tube to equilibrate the solution with the N₂/O₂ mixture before initiating turnover. Reaction aliquots were quenched in 75% acetonitrile/0.2% TFA saturated with 3,5-dimethoxy-4-hydroxycinnamic acid prior to analysis on a Bruker microFlex MALDI-TOF-MS to quantify product formation. Initial rates were determined as previously described[4] and fit to the Michaelis-Menten equation.

Succinate Quantification

Reactions to determine the production of succinate and CTAD^{OH} concentrations were run at 37.0° C in 50 mM Tris, pH 7.00 and analyzed as previously reported [11]. Briefly, aliquots (20

μL) from a common reaction containing αKG (100 μM), FeSO_4 (100 μM), CTAD (100 μM) and FIH (100 μM) were quenched in 500 mM H_2SO_4 containing 500 μM succinate (5 μL). From each quenched aliquot, HPLC-UV/Vis was used to quantitate the succinate concentration produced from the reaction and MALDI-TOF-MS was used to quantitate the CTAD^{OH} concentration. The coupling ratio (C) was determined by taking the ratio of the concentration of succinate and the concentration of CTAD^{OH} from matching quenched reactions.

UV-Vis absorption spectroscopy

All reagents, except the buffer, were made anaerobic by argon sparging. The buffer was made anaerobic by multiple cycles of vacuum/ N_2 flush. Samples of $(\text{Fe}+\alpha\text{KG})\text{FIH}$, where the active-site occupancy is denoted within parenthesis, were prepared in an anaerobic glove box as solutions containing FIH (400 μM), FeSO_4 (375 μM) and αKG (400 μM) in 50 mM HEPES pH 7.00. An Avantes AvaSpec-2048 Fiber Optic spectrometer with a 2048 pixel CCD Detector Array was used to record all spectra in a 1 cm path-length cuvette. An $(\text{Fe})\text{FIH}$ spectrum was subtracted from each $(\text{Fe}+\alpha\text{KG})\text{FIH}$ spectrum to record the changes in spectral features due to formation of the $\text{Fe}+\alpha\text{KG}$ chromophore.

Autohydroxylation

Autohydroxylation assays were performed as previously described [37, 38]. All reagents except buffer were degassed individually by argon flush before being handled in a glove box. The buffer was made anaerobic using multiple cycles of vacuum and N_2 flush. Samples containing FIH (100 μM), FeSO_4 (100 μM) and αKG (500 μM) in 50 mM HEPES pH 7.00 were prepared in a masked cuvette and sealed. Removal of the cap exposed the anaerobic samples to air, allowing for slow oxidation of the samples. Experiments were performed at room

temperature and all spectra were collected using an Agilent HP 8453 diode-array UV-Vis spectrophotometer.

Crystallography and data refinement

Crystals of (Fe+ α KG)D201E, (Zn+ α KG)D201E and (Zn+ α KG)D201E/CTAD were grown anaerobically using conditions similar to those used in the literature[13]. Crystals were grown anaerobically for (Fe+ α KG)FIH via hanging drop vapor diffusion method by mixing 2 μ L of a 20 mg/ml protein solution containing 0.55 mM FeSO₄, and 2mM α KG in 50 mM HEPES pH 7.00 with 1 μ L of the reservoir buffer containing 0.1 M HEPES, 1.2 M (NH₄)₂SO₄, and 3 % PEG-400, pH 7.50. Crystals grew within 5 days at 20 °C inside the glovebox, then were harvested quickly outside the glovebox to minimize O₂ diffusion and flash frozen in reservoir solution containing 24% glycerol. Crystals of the (Zn+ α KG)D201E and (Zn+ α KG)D201E/CTAD were grown aerobically in a similar manner using 0.55 mM ZnCl₂ in the place of FeSO₄. A 19 residue CTAD peptide corresponding to HIF-1 α ⁷⁸⁸⁻⁸⁰⁷ was used for co-crystallization studies. This 19-mer peptide was purchased from EZBiolabs as a >95% pure peptide with free N and C termini with sequence DESGLPQLTSYDAEVNAPI (Asn⁸⁰³ underlined).

Data were collected at the Brookhaven National Synchrotron Radiation facility at 100 K using radiation of $\lambda = 1.08$ Å. 240 frames were collected for (Fe+ α KG)D201E and (Zn+ α KG)D201E at a crystal to detector distance of 225 mm with 0.5° oscillation and 10 sec exposure per image. 136 frames were collected for (Zn+ α KG)D201E/CTAD at a crystal to detector distance of 250 with 0.5° oscillation and 15 second exposure per image. The data sets were processed using HKL2000 [39]. Coordinates from PDBID 3D8C [13] were employed for molecular replacement using Phaser [40] then refined using COOT [41] and Refmac5 [42] in the

CCP4 software package [43]. During refinement, 5% of the data was withheld and used to obtain an R_{free} value.

Results and discussion

UV-Vis Absorption Spectroscopy

The electronic environment for the Fe(II) cofactor in the D201X variants was assessed by UV-Vis absorption spectroscopy. The expected metal-to-ligand charge transfer (MLCT) electronic transition for each of the (Fe+ α KG)FIH D201X variants was observed as a broad absorption peak centered near 500 nm (Figure 1) indicating that each variant bound Fe(II) along with α KG. Both variants with a carboxylate residue (X = Asp, Glu) exhibited a similar peak wavelength ($\lambda_{\text{max}} = 500$ nm); however, the variants lacking a facial triad carboxylate (X = Gly, Ala) exhibited a blue-shifted peak wavelength ($\lambda_{\text{max}} = 485$ nm). The blue shift in the MLCT transition for the D201X (X = Gly, Ala) variants was consistent with a stronger Lewis acidity for the Fe(II) in these variants than for X = Asp or Glu variants, which we attribute to stabilization of the Fe $t_{2g}(\pi)$ orbitals due to removal of the π -donor carboxylate ligand for D201G and D201A. Increased Lewis acidity of the cofactor might have been expected to increase the intrinsic reactivity for the D201(G/A) variants, however the kinetics studies (below) did not bear this out.

Figure 1. Anaerobic UV-Vis spectra of WT-FIH and the D201X variants for the (Fe+ α KG) form of WT-FIH (line), D201E (dashed), D201A (dotted) and D201G (dot/dash); FIH (400 μ M), FeSO₄, (375 μ M), α KG (400 μ M).

Steady-state kinetics with varied CTAD and α KG

The relative activity levels for CTAD hydroxylation by the Asp²⁰¹ variants was previously shown to follow the trend WT > D201G >> D201E = D201A = 0, with a large amount of succinate formed due to uncoupling by the Glu and Ala variants [13]. We observed a similar trend, with the notable exception that the D201E variant was competent to hydroxylate CTAD before rapidly inactivating. This led us to suspect that the facial triad carboxylate residue strongly impacted the coupling between CTAD binding and O₂ activation, and that the D201E variant might autohydroxylate as seen for WT-FIH after prolonged incubation [37, 38].

The initial steady-state characterization of the Asp²⁰¹ variants with CTAD as the varied substrate was performed under standard assay conditions using saturating concentrations of FeSO₄ (25 μ M), α KG (100 μ M), and ascorbate (2 mM) in 50 mM HEPES, 100 mM NaCl, pH 7.00 (Figure 2). These assays revealed WT-like hydroxylation activity for D201G ($k_{cat} = 23 \pm 2$ min⁻¹, $k_{cat}/K_{M(CTAD)} = 0.34 \pm 0.05$ μ M⁻¹ min⁻¹) and diminished activity for D201E ($k_{cat} = 1.6 \pm 0.2$ min⁻¹, $k_{cat}/K_{M(CTAD)} = 0.022 \pm 0.002$ μ M⁻¹ min⁻¹), with D201A activity below our detection level (Table 1). These significant decreases in these kinetic parameters for D201E indicated that CTAD hydroxylation was impeded in this variant.

Comparison of the Michaelis constant for α KG revealed that $K_{M(\alpha KG)}$ was insensitive to the identity of the residue at position 201 (Table 1). This was consistent with the limited second-sphere contacts between α KG and the residues of the facial triad, and suggested minimal impact of the facial triad carboxylate upon the α KG chemical environment.

Figure 2. Steady-state kinetics of FIH variants with CTAD as the varied substrate. WT (■) D201G (●) Inset: D201E (▼) D201A (◆). All assays contained saturating concentrations

of FeSO₄ (25 μM), αKG (100 μM), and ascorbate (2 mM), with an ambient O₂ concentration (217 μM) and were performed in 50 mM HEPES containing 100 mM NaCl, pH 7.00, 37°C.

Steady-State Kinetics Varying O₂

While O₂ activation was quite slow for WT-FIH ($k_{cat}/K_{M(O_2)}^{app}=0.17 \mu M^{-1} min^{-1}$) the kinetic parameters for the O₂-reaction of these facial triad variants has not been previously reported. To test for changes in O₂ activation due to the D201X substitutions, steady-state kinetics with O₂ as the varied substrate were measured using fixed concentrations of CTAD (80 μM), saturating concentrations of FeSO₄ (25 μM), αKG (100 μM), and ascorbate (2 mM) in 50 mM HEPES, 100 mM NaCl, pH 7.00 (Figure 3). The conservative substitution in the D201E variant led to depressed kinetic parameters ($k_{cat}/K_{M(O_2)} < 0.02 \mu M^{-1} min^{-1}$), whereas the D201G variant exhibited kinetics that were similar to those of WT-FIH ($k_{cat}/K_{M(O_2)} \sim 0.02 \mu M^{-1} min^{-1}$); the D201A variant failed to hydroxylate CTAD at both high and low O₂ concentrations (Table 2). Although these results suggested a very complex relationship between the identity of residue 201 and O₂-activation, it is important to consider that reaction rates were obtained by measuring CTAD hydroxylation. As defects in the coupling of the active oxidant to CTAD hydroxylation could have led to low yields of hydroxylated CTAD, we measured the succinate formation in these variants to account for all consumed αKG.

Figure 3. Steady-state kinetics varying O₂ for WT (■) and D201G (●) (Insert) Steady-state kinetics varying O₂ for D201E (▼). All assays contained ascorbic acid (2mM), αKG (100 μM), FeSO₄ (25 μM) and CTAD (80 μM).

Succinate forms stoichiometrically with the active oxidant (Eq. 1a), making succinate quantitation a direct measurement of O₂ activation regardless of the fate of the putative [Fe(IV)=O]²⁺ oxidant identified in related enzymes [5, 14, 44] (Eq. 1b, c). As the putative ferryl oxidant can hydroxylate CTAD to form the normal product (Eq. 1b, R = CTAD-Asn⁸⁰³), inactivate by autohydroxylating (Eq. 1b, R = FIH-Trp²⁹⁶) [37, 38], or potentially react with an exogenous reagent as seen for related enzymes AtsK and CS2 (Eq. 1c) [45, 46], there is the very real potential for the D201X substitutions to lead to normal O₂ activation chemistry that is simply poorly coupled to CTAD hydroxylation.

Although each of the facial triad variants was reported to produce significant levels of succinate, the level of coupling between O₂ activation and CTAD hydroxylation was not reported [13]. We measured the ratio of succinate to hydroxylated CTAD, in order to identify the coupling ratio ($C = [\text{succinate}]/[\text{CTAD}^{\text{OH}}]$) for each variant. As seen previously [10], WT-FIH produced 1.05 equivalents of succinate for every equivalent of CTAD^{OH} formed ($C = 1.05$) and exhibited tight coupling between O₂ activation and the subsequent hydroxylation step. In contrast, the other variants produced excess succinate ($C \gg 1$), with appreciable uncoupling of O₂ activation to CTAD hydroxylation (Table 3). The conservative D201E variant exhibited $C = 20$ and D201G exhibited $C = 2.0$; the D201A variant exhibited $C \gg 80$, however the CTAD^{OH} level was undetectable such that C could be significantly larger than reported.

The product of $C \times k_{\text{cat}}/K_{\text{M}(\text{O}_2)}^{\text{app}}$ yields the rate constant for total O₂ activation independent of whether or not the active oxidant succeeds in hydroxylating CTAD. Intriguingly,

this product for both D201G and D201E ($0.36 \pm 0.05 \mu\text{M}^{-1} \text{min}^{-1}$ and $0.28 \pm 0.06 \mu\text{M}^{-1} \text{min}^{-1}$, respectively) is *greater than* that of WT-FIH ($0.18 \pm 0.03 \mu\text{M}^{-1} \text{min}^{-1}$), indicating that O₂ activation to was not impaired by either of these variants (Table 3). Furthermore, although the hydroxylated CTAD levels could only be estimated for the D201A variant, even this variant produced succinate at nearly the same rate as WT-FIH. As the trend in Lewis acidity for the Fe(II) cofactor (D201G ~ D201A > WT-FIH ~ D201E) did not track with the O₂ activation trend (D201G > D201E > WT-FIH > D201A), it is unlikely that the increased O₂ activation rates for the D201G and D201E variants arose from an intrinsically more reactive Fe(II). Further, it is apparent that the Asp²⁰¹ in WT-FIH was not essential to support O₂ activation but rather played a decisive role in coupling O₂ activation to the subsequent hydroxylation step.

Autohydroxylation

The rapid kinetic parameters for O₂ activation suggested some of these variants were constitutively triggered, which would allow for increased O₂ activation even in the absence of CTAD. As WT-FIH was previously shown to autohydroxylate slowly in the absence of CTAD [37, 38], the D201X variants were tested for increased autohydroxylation kinetics. Samples containing a D201X variant, FeSO₄ and α KG were prepared anaerobically and then exposed to air allowing for the slow auto-hydroxylation of the D201X variant. UV-Vis spectroscopy was used to monitor the changes in the (Fe+ α KG)FIH absorption spectra as a function of time.

The slow oxidation of (Fe+ α KG)WT-FIH resulted in the formation of the previously identified indigo-coloured species with a characteristic visible absorption spectrum ($\lambda_{\text{max}} = 583 \text{ nm}$) at a rate of $0.35 \mu\text{M min}^{-1}$. This species results from the auto-hydroxylation of FIH at Trp²⁹⁶, forming the Fe(III)-O-Trp²⁹⁶ chromophore [37, 38] which can be thought of as the basal rate for O₂ activation in when WT-FIH is not triggered. Autohydroxylation of the D201E variant formed

the same chromophore as observed for WT-FIH ($\lambda_{\text{max}} = 583 \text{ nm}$) but at significantly faster initial rate ($2.2 \mu\text{M s}^{-1}$) (Figure 4). This faster auto-hydroxylation by D201E was consistent with an enzyme that was constitutively triggered to react with O_2 .

When analogous samples containing D201G or D201A mixed with FeSO_4 and αKG were exposed to air, pink chromophores formed with $\lambda_{\text{max}} = 515 \text{ nm}$ ($\epsilon_{515} = 2500 \text{ M}^{-1} \text{ cm}^{-1}$) and $\lambda_{\text{max}} = 510 \text{ nm}$ ($\epsilon_{510} = 2000 \text{ M}^{-1} \text{ cm}^{-1}$) respectively (Figure 4B and S3). The formation of these chromophores was O_2 -dependent, as they did not form in the absence of oxygen. The rate of formation was significantly higher ($1.4, 2.2 \mu\text{M min}^{-1}$) when compared to that of WT FIH, and within a factor of two of the rate observed for the D201E variant, suggesting that $\sim 2 \mu\text{M}^{-1} \text{ s}^{-1}$ may be the upper limit for O_2 activation by FIH variants. The new chromophores observed for D201G and D201A indicated an altered electronic environment of the Fe site, which could include coordination by a catecholate formed from autohydroxylation of a nearby Tyr residue instead of the Trp^{296} , or the binding of an exogenous anion.

Figure 4. Autohydroxylation following exposure to air of FIH variants ($100 \mu\text{M}$) mixed anaerobically with FeSO_4 ($100 \mu\text{M}$) and αKG ($500 \mu\text{M}$) in 50 mM HEPES pH 7.00 containing 100 mM NaCl. (A) Timecourse for WT-FIH and the D201E variant; inset, D201E spectra. (B) Timecourse for the D201G variant; inset, D201G spectra.

When TauD autohydroxylates to form an Fe(III)-catecholate species, that chromophore absorbs visible light ($\lambda_{\text{max}} = 720 \text{ nm}$) that can blue-shift to 550 nm upon the addition of excess bicarbonate [47]. Inspection of the structure of FIH shows that the aromatic residue closest to the Fe center is Trp^{296} ($4 \text{ \AA}$), with Tyr^{102} ($8 \text{ \AA}$) significantly further away, consistent with Trp^{296}

as the site of autohydroxylation in WT-FIH and suggesting that Tyr¹⁰² would be an unlikely site for modification. Similarly, comparing the electronic spectra of the autohydroxylation products from the FIH D201(G/A) variants ($\lambda_{\text{max}} = 515 \text{ nm} / 510 \text{ nm}$) with that of TauD ($\lambda_{\text{max}} = 720 \text{ nm}$) suggests that catecholate formation is unlikely in these D201(G/A) variants. As the crystal structure of the D201A variant exhibited electron density near the Fe that was refined as CO_3^{2-} , it is more likely that both D201G and D201A autohydroxylate at Trp²⁹⁶ but bind a bicarbonate or carbonate anion, leading to the blue –shifted UV-Vis absorption for autohydroxylated D201(G/A).

Structure of Asp²⁰¹ Variants

In the consensus mechanism, substrate binding leads to aquo release from the resting enzyme, thereby creating the site to bind O_2 (Scheme 1). While there is strong evidence that the aquo ligand is released from FIH upon binding CTAD, structural information explaining why this happens is limited. In part this is because the initially reported X-ray crystal structures of (Fe+NOG)FIH and (Fe+ α KG)FIH did not contain a resolved aquo ligand to the metal center [32, 33], although a hydrogen bond between the backbone amide of CTAD-Asn⁸⁰³ and Asp²⁰¹ was noted to link the Fe(II) binding site and the CTAD binding site [33]; a more recent structure for (Fe+ α KG)FIH included a resolved aquo ligand with an Fe-OH₂ bond length of 2.00 Å [34], however it is worth noting that precise identification of non-protein ligands near metal centers via crystallography is quite challenging. MCD spectroscopy showed that the Fe(II) converts from a 6-coordinate geometry in the (Fe+ α KG)FIH state to a mixed 5- and 6-coordinate geometry in the (Fe+ α KG)FIH/CTAD structure [20], consistent with aquo release to ensure that O_2 activation only occurs once CTAD is present. Asp²⁰¹ of the His₂Asp facial triad is oriented to

permit a polar contact between the distal O-atom and the Fe(II)-bound water molecule or to the CTAD-Asn⁸⁰³ backbone, suggesting that shifted polar contacts are central to this coupling – but sterics with residues near this aquo ligand site have been implicated to play a significant role in O₂ binding/reactivity with WT-FIH [34]. A comparison of the structures and reactivity of the D201X variants suggests that both polar contacts and sterics involving the D201X residue impact communication between the O₂ and CTAD binding sites.

Although the structures of the D201A and D201G variants have been reported [13], there were very few structural differences from WT-FIH even near the Fe(II) site. The most notable difference was the loss of the facial triad carboxylate and its accompanying polar contacts, although a large planar molecule in the D201A structure, refined as CO₃²⁻, occupying the place of the missing carboxylate sidechain from the D201A variant. As both the D201G and D201A variants uncoupled, the authors proposed that the loss of polar contacts from Asp²⁰¹ weakened the interaction with the aquo ligand and led to a more facile O₂ reaction in the absence of CTAD [13]. Our results agree with this prior report, supporting a strong role for polar contacts near the diffusible ligand site in defining the O₂ access.

The great mystery was that the conservative substitution in the D201E variant retained the polar contacts from the facial triad, and yet this variant uncoupled to a large extent. This observation pointed to some factor in addition to polar contacts as determining the ordered addition of O₂ to the enzyme. We hoped that structural data would help to resolve this puzzling observation and provide insight into how this variant could react so poorly with CTAD but so efficiently with O₂. Crystals of the D201E variant containing Fe(II) were grown anaerobically while crystals containing Zn(II) were grown aerobically using the hanging drop method. The structure of (Fe+αKG)D201E was refined to 2.3 Å ($R_{\text{work}} = 18.6\%$ and $R_{\text{free}} = 22.7\%$ and the

(Zn+ α KG)D201E structure was refined to 2.4 Å ($R_{\text{work}} = 18.0\%$ and $R_{\text{free}} = 22.7\%$). The protein backbone of (Fe+ α KG)D201E and (Zn+ α KG)D201E were essentially identical to each other, and very similar to that of wild-type (Fe+ α KG)FIH (PDB:4Z2W 2.4 Å)[34] (Figure 5), indicating that the D201E substitution did not significantly alter the structure relative to WT-FIH (rmsd = 0.178 Å over residues 9-349).

Each of the four facial triad variants exhibited nearly identical protein structures [13, 32-34], indicating that the reactivity changes did not arise from gross conformational changes. The active site metal retained pseudo-octahedral geometry in the D201X variants [13], and was coordinated by His¹⁹⁹, His²⁷⁹, α KG with the C-1 carboxylate bound *trans* to His¹⁹⁹, and a variable ligand for each variant: Glu²⁰¹ (D201E), Asp²⁰¹ (WT), H₂O (D201G), CO₃²⁻ (D201A). That loss of polar contacts to the diffusible ligand site with the Gly and Ala substitutions led to uncoupling, and rapid autohydroxylation, pointed to an active site that easily releases bound H₂O from the Fe(II), allowing O₂ to access to the metal center without regard to the presence of CTAD as previously proposed [13].

The D201E variant structure showed a slight expansion of the beta-barrel near residues 199 - 202 to accommodate the longer Glu²⁰¹ sidechain in a suitable bonding configuration with the metal ion. WT-FIH exhibited fairly typical M-O distances for a monodentate carboxylate from Asp²⁰¹: Fe-O1 (1.9 Å); Fe-O2 (3.1 Å). Both Fe-O distances were changed by less than 0.1 Å in the D201E variant (Table 5), showing that D201E retained the potential to form adequate polar contacts to the putative aquo ligand. Although bonded contacts involving the facial triad carboxylate were only slightly altered by the D201E substitution, the slight rotation of the carboxylate in this variant led to the distal O-atom impinging on the sterics of the diffusible ligand site (Figure 5A). The O2 – O_{keto} distance was decreased by 0.3 Å for the D201E variant

from that of WT-FIH (Table 5), which narrowed the available space for the exchangeable ligand site. This is well demonstrated as the exchangeable site angle ($O_{\text{keto}} - \text{Fe} - \text{O2}$) decreased from 151° in WT-FIH to 134° by the D201E substitution, indicating an increased steric ‘push’ in the D201E variant (Figure 6). Although polar contacts from Glu²⁰¹ to a bound H₂O ligand would be possible, sterics appear to be sufficient to disfavor this ligand based on the rapid inactivation of the D201E variant.

Figure 5. (A) Active site overlays of (Fe+ α KG)FIH (PDB: 4Z2W[34], white), (Fe+ α KG)D201E (PDBID: to be assigned, green) and (Zn+ α KG)D201E (PDBID: to be assigned, cyan). Fe is shown in brown and Zn in grey. (B) Active site overlays of (Fe+ α KG)FIH/CTAD (PDB: 1H2L[33], white) and (Zn+ α KG)D201E/CTAD (cyan). Fe is shown in brown and Zn in grey. In the D201E variant, the Glu residue crowds the diffusible ligand site.

Figure 6. Comparison of the carboxylate location and exchangeable site angle ($O_{\text{keto}}\text{-Fe-O2}$) in (Fe+ α KG)FIH variants; WT-FIH (Asp²⁰¹) (PDBID 1H2N [33]); D201E (Glu²⁰¹) (PDBID to be assigned).

Further support for steric imposition upon the diffusible ligand site by the distal O-atom in the D201E variant was found by collecting the X-ray crystal structure of the CTAD-bound form of enzyme. The structure of (Zn+ α KG)D201E/CTAD was refined to 2.1 Å resolution with an $R_{\text{work}} = 17.0\%$ and $R_{\text{free}} = 21.0\%$. The (Zn+ α KG)D201E/CTAD structure is similar to the published structure of (Fe+ α KG)FIH/CTAD (PDB:1H2L, rmsd = 0.164 Å) [33]. There were few

changes in contacts to the facial triad carboxylate in the D201E variant when compared to WT-FIH (Figure 5B). In the presence of CTAD, both WT-FIH and D201E possessed a 5-coordinate metal center but the distal O-atom of Glu²⁰¹ was shifted closer to the metal by ~0.3 Å relative to WT, leading to a 2.9 Å Fe-O distance (Table 5). There were two significant differences caused by CTAD binding for both WT-FIH and the D201E variant: the distal O2 formed a polar contact with the backbone amide of CTAD-Asn⁸⁰³, and the exchangeable site angle (Figure 6) decreased by roughly 10°. While the Asp→Glu substitution did not alter polar contacts related to substrate binding, it did add steric hindrance to the exchangeable ligand site.

We propose that Asp²⁰¹ links the O₂ binding site to CTAD binding from both polar contacts and sterics within the active site. The Asp²⁰¹ residue of WT-FIH is well positioned to stabilize an H₂O ligand [32-34] prior to CTAD binding, whereas CTAD binding leads to new polar contacts between this residue and the CTAD-Asn⁸⁰³ as noted previously [33]. Substituting this facial triad carboxylate ligand in D201X variants maintained the same capacity for O₂ activation, even when eliminating the carboxylate group as a ligand (X = Gly, Ala), however led to poor coupling and increased autohydroxylation. As the conservative D201E substitution retained this polar carboxylate ligand in a similar orientation as found for WT-FIH in the presence and absence of CTAD but uncoupled significantly, these polar contacts were insufficient to explain the timing of O₂ reactivity in FIH. The narrowed approach angle to the diffusible ligand site for D201E appears to disfavor the bulkier H₂O from binding while also adding greater steric push that would orient bound O₂ toward a reactive orientation with αKG – we note that reorientation of NO was observed upon FIH/CTAD binding, and attributed to sterics from the CTAD-Asn⁸⁰³ sidechain [34]. Similarly, the poor coupling in the D201G and D201A

variants could simply reflect the absence of stabilizing polar contacts to the aquo ligand, leading to variants that bind H₂O poorly and are constitutively primed for O₂ activation.

Conclusion

The α KG oxygenases utilize a sequential chemical mechanism in which O₂ activation precedes the chemical reaction with substrate. The kinetic and structural data from D201X variants of FIH suggest a model for coupling of the two half reactions in which the native carboxylate residue of the His₂Asp facial triad forms essential polar and steric contacts linkage between substrate binding to enzyme and O₂ binding to Fe(II). As the distal O-atom of the facial triad carboxylate is a polar contact for an aquo ligand in the (Fe+ α KG)FIH state, properly orienting this residue may be crucial for holding that H₂O onto the Fe(II) site until CTAD binds. Once primary substrate binds, the distal O-atom is slightly pulled away from the aquo ligand[33] which allows O₂ to reach the Fe leading to decarboxylation of α KG. Altering the facial triad ligand in the D201X variants did not impede O₂ reactivity, but rather perturbed the structural linkage thereby disrupting the timing of O₂ activation relative to CTAD binding. This relied on the contacts between the D201X residue, the Fe(II)-OH₂ moiety, and incoming CTAD substrate; with any perturbation from the WT residue leading to uncoupled turnover in FIH. These results suggest that the sequential chemical mechanism found in the broad family of Fe(II)/ α KG-dependent oxygenases may be a result of second-sphere contacts to the facial triad carboxylate residue.

Notes

The authors declare no competing financial interest.

ASSOCIATED CONTENT

The steady state kinetics for the D201E and D201G variants varying α KG; F_o-Fc map from structures, and autohydroxylation of D201A can be found in the supplemental information.

Supplementary data to this article can be found online at

Abbreviations

α KG, α -ketoglutarate

CTAD, C-terminal transactivation domain

FIH, factor-inhibiting HIF

HAT, hydrogen atom transfer

HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HIF, hypoxia inducible factor-1 α

MALDI-TOF-MS, Matrix assisted laser desorption ionization-time of flight-mass spectrometry

MCD, magnetic circular dichroism

MLCT, metal-to-ligand charge-transfer

MPH, MES-PIPES-HEPES buffer

NOG, N-oxalylglycine

P4H, prolyl-4-hydroxylase

TauD, taurine dioxygenase

TFA, trifluoroacetic acid

VTVH, variable temperature variable field

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Funding Sources

This research was supported by the U.S. National Institutes of Health (1R01-GM077413 to M.J.K). J.A.H and C.Y.T. were supported in part by the NIH Chemistry-Biology Interface Predoctoral Training Grant T32-GM008515. These crystallographic data were collected at the Advanced Photon Source on the Northeastern Collaborative Access Team beamline 24-ID-C, which is supported by a grant from the National Institute of General Medical Sciences (P41 GM103403) from the National Institutes of Health.

Acknowledgements

We thank Jeanne A. Hardy for support of the crystallography on this project, and Scott Garman for helpful comments. We also thank Kay Perry at beamline 24- ID-C at the Advanced Photon Source at Argonne National Laboratories for assisting us with remote collection. This research used resources of the Advanced Photon Source, a U.S. Department of Energy (DOE) Office of Science User Facility operated for the DOE Office of Science by Argonne National Laboratory under Contract No. DE-AC02-06CH11357.

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Table 1. Steady-state kinetic parameters of WT-FIH, D201G and D201E^a

	k_{cat}^{app} (min ⁻¹) ^b	$k_{cat}/K_{M(CTAD)}^{app}$ (μM ⁻¹ min ⁻¹) ^b	$K_{M(CTAD)}^{app}$ (μM) ^b	$K_{M(αKG)}^{app}$ (μM) ^c
WT FIH	33 ± 2	0.42 ± 0.08	78 ± 17	20 ± 5
D201E	1.6 ± 0.2	0.022 ± 0.002	70 ± 15	26 ± 8
D201G	23 ± 2	0.34 ± 0.05	71 ± 12	23 ± 6
D201A	<i>d</i>	<i>d</i>	<i>d</i>	<i>d</i>

a. Based on $K_{M(O_2)}$, each variant had a different degree of O₂ saturation. WT, 52%; D201E, 75%; D201G, 65%.

b. Reactions contained ascorbate (2 mM), αKG (100 μM), FeSO₄ (25 μM) and CTAD (0-260 μM) in 50 mM HEPES containing 100 mM NaCl, pH 7.00, 37.0 °C.

c. Reactions contained ascorbate (2 mM), αKG (0-200 μM), FeSO₄ (25 μM) and CTAD (80 μM) in 50 mM HEPES containing 100 mM NaCl, pH 7.00, 37.0 °C.

d. No hydroxylation activity detected; Estimated hydroxylation detection limit <0.005 min⁻¹, if active.

Table 2. O₂ Activation Kinetic parameters for FIH and Asp²⁰¹ variants^a.

	k_{cat}^{app} (min ⁻¹)	$k_{cat}/K_{M(O_2)}^{app}$ (μM ⁻¹ min ⁻¹)	$K_{M(O_2)}^{app}$ (μM)
WT FIH ^b	33 ± 3	0.17 ± 0.03	200 ± 40
D201E	0.81 ± 0.02	0.014 ± 0.002	70 ± 15
D201G	20 ± 1.0	0.18 ± 0.02	115 ± 20

a. Reactions contained ascorbate (2 mM), αKG (100 μM), FeSO₄ (25 μM), CTAD (80 μM) and O₂ (0-950 μM) in 50 mM HEPES containing 100 mM NaCl pH 7.00, 37.0 °C.

b. From Ref. ([25])

Table 3. Coupling ratios for facial triad variants of FIH.

Variant	$C \times k_{\text{cat}}/K_{\text{M}(\text{O}_2)}^{\text{app}}$ ($\mu\text{M}^{-1} \text{min}^{-1}$)	$k_{\text{cat}}/K_{\text{M}(\text{O}_2)}^{\text{app}}$ ($\mu\text{M}^{-1} \text{min}^{-1}$)	C
WT (Asp ²⁰¹)	0.18 ± 0.03	0.17 ± 0.03	1.05 ± 0.05
D201G	0.36 ± 0.05	0.18 ± 0.02	2.0 ± 0.20
D201A	----	----	$>> 80^{\text{a}}$
D201E	0.28 ± 0.06	0.014 ± 0.002	20 ± 3
$C = (\text{moles succinate})/(\text{moles CTAD-OH})$			

a. Estimated based on moles of succinate produced and the limit of detection for hydroxylation.

Table 4. Data collection and refinement statistics for the FIH D201E variant. Values in parentheses represent the highest resolution shell.

Data set	(Zn ²⁺ +αKG)D201E	(Fe ²⁺ +αKG)D201E	(Zn ²⁺ +αKG)D201E/CTAD
PDB ID	5JWL	5JWK	5JWP
λ (Å)	1.08	1.08	1.08
Space group	<i>P</i> 4 ₁ 2 ₁ 2	<i>P</i> 4 ₁ 2 ₁ 2	<i>P</i> 4 ₁ 2 ₁ 2
Unit cell (Å, °)	a = b = 86.17, c = 149.06 α = β = γ = 90	a = b = 86.13, c = 149.21 α = β = γ = 90	a = b = 86.22, c = 14.90 α = β = γ = 90
Resolution (Å)	50 – 2.4 (2.44 – 2.40)	50 – 2.3 (2.34 – 2.30)	34 – 2.1 (2.14 – 2.1)
R _{sym} (%)	13.0 (100)	12.4 (88)	7.0 (100)
Redundancy	9.0 (9.5)	8.2 (7.9)	5.2 (5.3)
Mean I/σ (I)	27 (2.7)	23.4 (2.4)	24.8 (2.3)
Completeness	98.2 (100)	99.4(100)	99.5 (99.8)
Refinement			
Resolution range (Å)	37 – 2.4	37 – 2.3	34 – 2.1
Number of Reflections	22314	25594	28327
Average B factor (Å ²)	67	66	52
R _{work} /R _{free} (%)	18.0/22.6	18.6/22.7	17.0/21.0
RMS deviations			
Bond lengths (Å)	0.016	0.016	0.019
Bond angles (°)	1.755	1.866	1.904
Ramachandran plot, residues in (%)			
Preferred region	92.9	94.4	96.0
Allowed region	7.1	5.6	4.0
Disallowed region	0	0	0

Table 5. Selected bond lengths (Å) and angles for (Fe+αKG)WT-FIH and (Fe+αKG)D201E.

	WT-FIH ^a	D201E ^b	WT-FIH/ CTAD ^c	D201E/ CTAD ^d
M - O ₁₍₂₀₁₎	1.9	1.9	2.1	1.9
M - O ₂₍₂₀₁₎	3.1	3.2	3.4	2.9
O ₂₍₂₀₁₎ - N ₁₍₂₃₈₎	3.6	3.5	3.2	3.7
O ₂₍₂₀₁₎ - O _{keto}	5.2	4.9	5.3	4.6
O _{keto} -Fe-O ₂₍₂₀₁₎	151.1°	134.4°	144.2	126.7°
O ₂₍₂₀₁₎ - N ₈₀₃ (backbone amide)			3.1	2.9

a. (Fe+αKG)FIH; PDBID 1H2N [33]

b. (Fe+αKG)D201E; PDBID 5JWK, this work

c. (Fe+αKG)FIH/CTAD PDBID 1H2L[33]

d. (Zn+αKG)D201E/CTAD; PDBID 5JWP, this work

Figure/Scheme Captions

Figure 1. Anaerobic UV-Vis spectra of WT-FIH and the D201X variants for the (Fe+ α KG) form of WT-FIH (line), D201E (dashed), D201A (dotted) and D201G (dot/dash); FIH (400 μ M), FeSO₄, (375 μ M), α KG (400 μ M).

Figure 2. Steady-state kinetics of FIH variants with CTAD as the varied substrate. WT (■) D201G (●) Inset: D201E (▼) D201A (◆). All assays contained saturating concentrations of FeSO₄ (25 μ M), α KG (100 μ M), and ascorbate (2 mM), with an ambient O₂ concentration (217 μ M) and were performed in 50 mM HEPES containing 100 mM NaCl, pH 7.00, 37°C.

Figure 3. Steady-state kinetics varying O₂ for WT (■) and D201G (●) (Insert) Steady-state kinetics varying O₂ for D201E (▼). All assays contained ascorbic acid (2mM), α KG (100 μ M), FeSO₄ (25 μ M) and CTAD (80 μ M).

Figure 4. Autohydroxylation following exposure to air of FIH variants (100 μ M) mixed anaerobically with FeSO₄ (100 μ M) and α KG (500 μ M) in 50 mM HEPES pH 7.00 containing 100 mM NaCl. (A) Timecourse for WT-FIH and the D201E variant; inset, D201E spectra. (B) Timecourse for the D201G variant; inset, D201G spectra.

Figure 5. (A) Active site overlays of (Fe+ α KG)FIH (PDB: 4Z2W[34], white), (Fe+ α KG)D201E (PDBID: to be assigned, green) and (Zn+ α KG)D201E (PDBID: to be assigned, cyan). Fe is shown in brown and Zn in grey. (B) Active site overlays of (Fe+ α KG)FIH/CTAD (PDB: 1H2L[33], white) and (Zn+ α KG)D201E/CTAD (cyan). Fe is shown in brown and Zn in grey. In the D201E variant, the Glu residue crowds the diffusible ligand site.

Figure 6. Comparison of the carboxylate location and exchangeable site angle (O_{keto}-Fe-O₂) in (Fe+ α KG)FIH variants; WT-FIH (Asp²⁰¹) (PDBID 1H2N [33]); D201E (Glu²⁰¹) (PDBID to be assigned).

Scheme 1. Consensus Mechanism for FIH

Figure 1. Anaerobic UV-Vis spectra of WT-FIH and the D201X variants for the (Fe+ α KG) form of WT-FIH (line), D201E (dashed), D201A (dotted) and D201G (dot/dash); FIH (400 μ M), FeSO₄, (375 μ M), α KG (400 μ M).

Figure 2. Steady-state kinetics of FIH variants with CTAD as the varied substrate. WT (■) D201G (●) Inset: D201E (▼) D201A (◆). All assays contained saturating concentrations of FeSO₄ (25 μM), αKG (100 μM), and ascorbate (2 mM), with an ambient O₂ concentration (217 μM) and were performed in 50 mM HEPES containing 100 mM NaCl, pH 7.00, 37°C.

Figure 3. Steady-state kinetics varying O₂ for WT (■) and D201G (●) (Insert) Steady-state kinetics varying O₂ for D201E (▼). All assays contained ascorbic acid (2mM), αKG (100 μM), FeSO₄ (25 μM) and CTAD (80 μM).

Figure 4. Autohydroxylation following exposure to air of FIH variants (100 μ M) mixed anaerobically with FeSO_4 (100 μ M) and α KG (500 μ M) in 50 mM HEPES pH 7.00 containing 100 mM NaCl. (A) Timecourse for WT-FIH and the D201E variant; inset, D201E spectra. (B) Timecourse for the D201G variant; inset, D201G spectra.

Figure 5. (A) Active site overlays of (Fe+αKG)FIH (PDB: 4Z2W[34], white), (Fe+αKG)D201E (PDBID: to be assigned, green) and (Zn+αKG)D201E (PDBID: to be assigned, cyan). Fe is shown in brown and Zn in grey. (B) Active site overlays of (Fe+αKG)FIH/CTAD (PDB: 1H2L[33], white) and (Zn+αKG)D201E/CTAD (cyan). Fe is shown in brown and Zn in grey. In the D201E variant, the Glu residue crowds the diffusible ligand site.

Figure 6. Comparison of the carboxylate location and exchangeable site angle ($O_{\text{keto}}\text{-Fe-O2}$) in (Fe+ α KG)FIH variants; WT-FIH (Asp²⁰¹) (PDBID 1H2N [33]); D201E (Glu²⁰¹) (PDBID to be assigned).

Scheme 1. Consensus Mechanism for FIH

Graphical Abstract Synopsis

The facial triad carboxylate structurally links O₂ and substrate binding in the factor-inhibiting hypoxia inducible factor-1 α (FIH), an α -ketoglutarate dependent oxygenase.