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Chloride Supports O₂ Activation in the D201G Facial Triad Variant of Factor-Inhibiting Hypoxia Inducible Factor, an α -Ketoglutarate Dependent Oxygenase

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Abstract

α -Ketoglutarate (α KG) dependent oxygenases comprise a large superfamily of enzymes that activate O₂ for varied reactions. While most of these enzymes contain a nonheme Fe bound by a His₂(Asp/Glu) facial triad, a small number of α KG-dependent halogenases require only the two His ligands to bind Fe and activate O₂. The enzyme “factor inhibiting HIF” (FIH) contains a His₂Asp facial triad and selectively hydroxylates polypeptides; however, removal of the Asp ligand in the Asp201→Gly variant leads to a highly active enzyme, seemingly without a complete facial triad. Herein, we report on the formation of an Fe–Cl cofactor structure for the Asp201→Gly FIH variant using X-ray absorption spectroscopy (XAS), which provides insight into the structure of the His₂Cl facial triad found in halogenases. The Asp201→Gly variant supports anion dependent peptide hydroxylation, demonstrating the requirement for a complete His₂X facial triad to support O₂ reactivity. Our results indicated that exogenous ligand binding to form a complete His₂X facial triad was essential for O₂ activation and provides a structural model for the His₂Cl-bound nonheme Fe found in halogenases.

Graphical Abstract

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.inorgchem.8b01736. MALDI-TOF spectra, rate of CTAD hydroxylation, EXAFS, and O₂ consumption (PDF)

The authors declare no competing financial interest.

INTRODUCTION

The α -ketoglutarate (α KG) dependent oxygenases functionalize a wide variety of substrates using a nonheme Fe(II) coordinated by a His (Asp/Glu) facial triad.¹ The consensus mechanism of α KG dependent oxygenases is proposed to follow the HAG mechanism² involving H atom transfer to form a substrate radical, with rebound of an Fe-bound ligand (Scheme 1). Typical enzymes in this superfamily hydroxylate substrates by the transfer of an O atom from O₂, whereas a subset of α KG oxygenases perform halogenation chemistry (Scheme 1).³ As seen with both the hydroxylases and halogenases, the presence of the organic substrate (R-H) is necessary to trigger O activation^{4–7} leading to the formation of an Fe(IV)=O intermediate, giving rise to subsequent rebound chemistry.^{8,9} Gaining insight into the structural factors needed to trigger O₂ activation is of paramount interest to the field.

The resting enzyme form of hydroxylases contains a ferrous ion coordinated by a His₂ (Asp/Glu) facial triad, a bidentate α KG, and an aquo ligand. In contrast to the hydroxylases, halogenases naturally have a His₂(Ala/Gly) motif but bind a halide ion to support halogenase chemistry.¹⁰ The crystal structure of chloride-bound SyrB2 provided the first evidence of a halide directly coordinated to the metal in this family of enzymes,¹⁰ supporting earlier studies with model complexes which indicated that metal-bound ligands

were transferred in nonheme Fe mediated halogenation.¹¹ Protein structures of both CytC3 and SyrB2 revealed extended hydrogen-bonding networks which were thought essential for chloride binding to the active-site.^{10,12}

Several lines of inquiry have shown that a Cl⁻ ligand is essential for the rebound step in halogenases to form halogenated product, but it remains unclear whether a complete His₂X facial triad is essential for the initial step of O₂ activation. Electronic structure calculations of a model halogenase cofactor predicted a modest kinetic barrier for OH rebound, whereas Cl rebound would be barrierless, suggesting a kinetic advantage for halogenation.¹³ Reports demonstrate the potential to interconvert hydroxylases and halogenases through changing the disposition of substrate relative to the hydroxide and halide ligands.^{14–16} While the above demonstrate that the Cl⁻ ligand is essential for rebound chemistry, it is not clear whether or not a complete facial triad would be necessary for the preceding step of O₂ activation. As hydroxylation does not involve the facial triad ligands in an obvious manner, hydroxylating enzymes with incomplete His₂X facial triads are of interest,^{17–20} as these examples suggest that a complete facial triad is not necessary for O₂ activation chemistry.

FIH (factor-inhibiting hypoxia inducible factor) is an α KG dependent oxygenase which hydroxylates the side chain of Asn803 within the C-terminal transactivation domain (CTAD) of the hypoxia inducible factor (HIF-1 α). While the Fe cofactor of WT-FIH has the His₂Asp facial triad, prior reports indicated that Asp201 $\rightarrow$ (Ala/Glu/Gly) variants of FIH supported intriguing O₂ activation and hydroxylation chemistry making these variants excellent starting points to test the link between His₂X facial triads and O₂ activation. Here, we show the exogenous anion ligand (X) has remarkable effects in the activity of the Asp201 $\rightarrow$ (Ala/Glu/Gly) variants. We demonstrate that exogenous anion binding to form a complete His₂Cl facial triad is both selective and essential for the Asp201 $\rightarrow$ Gly variant of FIH to support O₂ activation chemistry. We also provide metrical details of the Fe–Cl cofactor in the Asp201 $\rightarrow$ Gly variant, revealing an excellent mimic of the halogenase geometry found in SyrB2. These results indicate that an elaborate halide binding pocket is not needed to replicate the chloroferrous cofactor of halogenases in FIH and more importantly indicated that an anionic ligand was essential to complete the His₂X facial triad for O₂ activation.

METHODS

Materials.

All materials, except for the synthetic peptide CTAD and the FIH variants, were purchased from commercial vendors and used without further purification. CTAD was purchased from EZBiolab (Carmel, IN, USA) as desalted peptide and purified using RP-HPLC to >95% purity. The CTAD peptide sequence, DESGLPQLTSYDAEVNAPIQGSRNLLQGEELLRALDQVN, corresponds to that of the C-terminal activation domain of human HIF-1 α (788–826) with the **Asn803** residue targeted by FIH in bold, and the Cys800 $\rightarrow$ Ala variant underlined. The C800A variant was used in order to avoid potential complications from disulfide formation.

Protein Expression and Purification.—WT-FIH and the Asp201→(Ala/Glu/Gly) variants were overexpressed in *Escherichia coli* with an N-terminal His₆ tag and purified as previously described.²¹

Activity Assays.—Activity assays used to screen for the halide dependent hydroxylation of CTAD contained α KG (100 μ M), FeSO₄ (25 μ M), CTAD (100 μ M), ascorbic acid (2 mM), WT FIH, or an Asp201 variant (0.5–2.5 μ M) and NaX (X = F, Cl, Br, or I; 100 mM) in 50 mM HEPES pH 7.00. Similarly, the reaction buffer for activity assays that screened for anion-dependent modification of CTAD contained α KG (100 μ M), FeSO₄ (25 μ M), CTAD (100 μ M) ascorbic acid (2 mM), FIH (0.5–2.5 μ M), and the sodium salt of various anions (2 mM) in 50 mM HEPES pH 7.00. All reactions were initiated by the addition of enzyme and were performed at 37.0 °C.

To determine the enzyme activity, reaction mixture aliquots (5 μ L) were quenched in 75% acetonitrile/0.2% TFA (trifluoroacetic acid) saturated with 3,5-dimethoxy-4-hydroxycinnamic acid (20 μ L) over a predefined time course spanning several minutes. Each quenched aliquot was analyzed for peptide hydroxylation and other covalent modifications using a Bruker MicroFlexII MALDI-TOF-MS (Figures S1 and S2). Initial rates were calculated based on the rate of product formation (Figure S3).

O₂ Consumption Assays.—The rate of oxygen consumption was monitored using a Clark-type electrochemical O₂ sensor. Each day, a new membrane was prepared and the electrode was calibrated using air saturated water and sodium dithionite. Ascorbate (50 μ M), α KG (100 μ M), FeSO₄ (25 μ M), and CTAD (80 μ M) in 50 mM HEPES pH 7.00 were equilibrated in the reaction vessel at 37.0 °C until a stable baseline was achieved. The reaction was then initiated with cold enzyme (1–10 μ M) using a gastight Hamilton syringe. O₂ consumption over time was recorded until the rate of O₂ consumption approached the baseline slope.

UV–vis Absorption Spectroscopy.—All reagents were prepared anaerobically using multiple cycles of evacuation and N₂ flush. Samples consisting of FIH (400 μ M), FeSO₄ (375 μ M), and α KG (400 μ M) in 50 mM HEPES pH 7.00 at 23 °C containing either a sodium salt halide (100 mM) or pseudohalide (2 mM) were prepared in an anaerobic glovebox. UV–vis spectra of each sample were recorded anaerobically using an Avantes UV–vis spectrometer with an integration time of 25 ms and averaging of 25 spectra. The spectrum of (Fe)FIH was subtracted from the spectrum of (Fe + α KG)FIH to produce the reported spectra.

X-ray Absorption Spectroscopy.—A sample of 1 mM FeSO₄ was prepared in 50 mM HEPES, pH 7.00, 100 mM NaCl, and metal concentration was determined using a PerkinElmer Optima DV4300 ICP-OES instrument. This metal sample was used to reconstitute the Asp201→Gly variant of FIH anaerobically with FeSO₄ (0.9 equiv), N-oxalylglycine (NOG) (1.0 equiv), and excess NaCl (100 equiv) in 50 mM HEPES, pH 7.00.

The X-ray absorption spectroscopy (XAS) data set was collected at the Stanford Synchrotron Radiation Laboratory (SSRL) on beamline 9–3 using a 20 element Ge X-ray

fluorescence detector on a sample containing 1 mM protein held at liquid He temperature. Beamline setup and ring conditions are as previously described²² and are further detailed in the Supporting Information.

XANES analysis was performed using OriginPro 2015 to fit the edge background and pre-edge features as pseudo-Voigt peaks, as previously described.²³ These features were analyzed assuming O_h local symmetry for the Fe site. Data processing and EXAFS analysis were performed as previously described using the SixPack,²⁴ Artemis, and FEFF6^{25,26} software packages, and further information regarding these procedures is available in the Supporting Information. Assessment of the goodness of fit was performed as previously described.²²

Reported data sets were fit with separate sets of r_{eff} or the change in metal–ligand bond distance with respect to the input path distance. Each fit was initiated with a universal E_0 (7125 eV for Fe) and $E_0 = 0$ eV, which was allowed to vary for each fit. Histidine ligands were fit as geometrically rigid imidazole rings.^{27–29} Multiple-scattering parameters for imidazole ligands bound to Fe were generated using the FEFF6 software package with the imidazole input obtained from average bond lengths and angles gathered from crystallographic data, as previously described.^{30,31}

Metal Titrations.

The K_d of metal binding to FIH was measured by intrinsic tryptophan fluorescence quenching using a PTI fluorimeter.²¹ Samples containing 20 μM FIH, 500 μM αKG , and 1 mM sodium citrate in 50 mM HEPES pH 7.00 were prepared in a fluorescence cuvette. Sodium citrate was used to buffer the free metal in solution. The titrating solution contained 1 mM CoSO_4 and 1 mM sodium citrate in 50 mM HEPES pH 7.00. Co(II) was used as an Fe(II) mimic to avoid auto-oxidation. Increasing amounts of metal were added to the cuvette solution and incubated for 7 min at room temperature while stirring. Following incubation, samples were excited at 285 nm with emission monitored from 300 to 400 nm. The intensity of the fluorescence peak at 330 nm after each metal addition was used to determine the relative fluorescence intensity, which was fit to the following equation to determine the $K_d(\text{Co})$ binding for each protein variant,³² using the log of the concentration of metal (x), the metal binding affinity (K_d), the Hill coefficient (B) and the fluorescence intensity at 330 nm (y).

$$y = y_{\min} + \frac{y_{\max} - y_{\min}}{1 + 10^{(x - \log K_d)B}}$$

The stability constant of the free Co^{2+} ion, $[\text{Co}(\text{H}_2\text{O})_6]^{2+}$, in citrate was used to calculate the buffered concentration of free Co^{2+} in solution.³³

RESULTS AND DISCUSSION

The reactivities of WT FIH and Asp201→(Ala/Gly/Glu) variants were previously measured in the presence of buffers containing chloride anions, such as Tris/HCl buffer¹⁹ or HEPES

with 100 mM NaCl to control ionic strength.²⁰ When preparing protein samples for structural analysis by XAS, we observed that initial activity screens of the Asp201→Gly variant in HEPES buffer showed batch-to-batch variation in the rate for hydroxylating the CTAD peptide. We subsequently found that prolonged dialysis into chloride-free HEPES buffer led to a protein that had a low rate for CTAD^{OH} formation, suggesting that chloride was essential for O₂ activation by this variant. This raised the potential for Cl⁻ as an essential exogenous anion to complete a His₂Cl facial triad in this variant and for anion-selective rescue of activity for the other enzyme variants. This was intriguing as anion rescue failed in two α KG-dependent peptidyl hydroxylases,^{17,19} and limited rescue of activity was observed in α KG-dependent hydroxylases utilizing smaller substrates.^{15,18,34}

Anion Dependent Oxygen Activation.

FIH variants were screened for anion selective hydroxylation of the CTAD peptide, which was used in place of the full-length HIF-1 α protein as a substrate for FIH.^{21,35} Each FIH variant was exhaustively dialyzed into 50 mM HEPES/NaOH, pH 7.00 to remove loosely associated anions from the apoprotein sample, and then tested for activity at 37.0 °C. Reactions were monitored over several minutes by MALDI-TOF-MS in order to detect the +16 mass gain due to peptide hydroxylation.

MALDI analysis revealed that all variants produced the hydroxylated CTAD peptide (CTAD^{OH}) albeit with widely different rates for product formation (Figure 1A). As we used MALDI for product analysis, we could observe whether products contained an additional O atom (+16) or halogen atom (eg: replacing an H with a Cl would increase mass by +34 and +36); the only product observed was the +16 product corresponding to peptide hydroxylation (Figure S2). WT-FIH exhibited robust hydroxylation activity under all tested conditions (Figure 1A); notably, NaX was not required for activity; we inferred that the diminution of activity in the presence of NaI may arise from quenching of the ferryl intermediate by iodide. Notably, the conservative Asp201→Glu variant, which bound the Fe by a His₂Glu facial triad, exhibited anion independent hydroxylation (Figure 1A) albeit at roughly 10% the rate of WT-FIH.²⁰

Both of the variants containing a nonpolar side chain at position 201, Asp201→Ala/Gly, exhibited anion selective hydroxylation activity with contrasting anion requirements (Figure 1A). The Asp201→Gly variant was nearly as active as WT-FIH toward hydroxylating CTAD, provided that NaCl was present. The Asp201→Ala variant, mimicking the ligands found in α KG-dependent halogenases such as SyrB2, hydroxylated CTAD only in the presence of F⁻. The anion-selectivity of the Asp201→Ala/Gly variants indicated that an exogenous anion was essential for substrate hydroxylation, implying that a complete His₂X facial triad formed in these enzyme variants. This further suggested a complementarity between the size of the anion and the bulk of the noncoordinating Asp201→Ala/Gly side chain.

As forming CTAD^{OH} requires successful completion of both half reactions, it was possible that the exogenous anion was needed either for the initial O₂ activation or for the subsequent peptide hydroxylation. To gain insight into which step in the consensus mechanism was influenced by the anion, O₂ consumption by the Asp201→Gly variant was assessed using a

Clark-type O₂ sensor. The O₂ sensor is an electrochemical cell, requiring KCl electrolyte under an O₂-permeable membrane, which separates the enzyme reaction from the electrode. The rate of O₂ consumption by Asp201→Gly was significantly enhanced by the addition of chloride to the assay buffer (Figure 1B) which showed that the chloride anion stimulated the O₂ consumption step. The relative rates of O₂ consumption in the presence of other NaX salts were consistent with the relative rates of CTAD hydroxylation, indicating anion binding was essential for the initial O₂ activation half-reaction.

The α KG dependent halogenase, SyrB2, supported incorporation of alternate anions into the product, including Cl⁻, N₃⁻, and NO₂⁻,³⁴ suggesting that richer chemistry may be possible with varied exogenous ligands in enzymes with a His₂X facial triad. The anion-selective FIH variants Asp201→Ala/Gly were screened for anion incorporation using the sodium salts of several monoanions including pseudohalides, using MALDI-TOF analysis to identify the product masses. Only two of the tested anions supported hydroxylation activity above a basal level to form CTAD^{OH}, but none of the tested anions were incorporated into the product (Figure 2A). The Asp201→Gly variant had significant hydroxylation activity for X⁻ = N₃⁻ and interestingly, both the Asp201→Gly and Asp201→Ala variant showed near WT activity when X⁻ = OCN⁻. The higher activity with OCN⁻ may reflect the increased charge asymmetry of OCN⁻ relative to N₃⁻.

There have been very few examples of exogenous ligands binding to and rescuing activity in α KG-dependent oxygenases. The H99A variant of taurine dioxygenase from Escherichia coli (TauD) was reported by different researchers to be nearly inactive¹⁸ or to retain 40% the activity of WT-TauD.³⁶ This discrepancy may reflect differences in protein purification procedures and/or the variable incorporation of an exogenous ligand such as H₂O or NH₃ that could arise from the specific protein handling methods. The most prominent example of exogenous anion binding to the Fe cofactor is from the α KG-dependent halogenase SyrB2, which showed tolerance for a variety of anions to bind in place of the natural Cl⁻ anion.³⁴ However, SyrB2 naturally binds Cl⁻ to form a His₂Cl facial triad, and is therefore likely to tolerate other ligands. On the other hand, the rescuing activity of a natural hydroxylase following mutation of the Asp/Glu facial triad ligand is rare. The Asp101→Ala variant of taurine dioxygenase (TauD) exhibited a trace level of turnover as isolated, which was increased by binding formate, implying that formate bound to the Fe. However, the overall rate of turnover remained low (<<1%) relative to WT-TauD.¹⁸ Facial triad variants of prolyl-4-hydroxylase were inactive and could not be rescued by addition of exogenous ligands.¹⁷ The single success was the N- substituted l-amino acid dioxygenase (SadA), which normally forms hydroxylated products. The Asp157→Gly variant of SadA was able to both bind Cl⁻/Br⁻ as well as perform alternate rebound chemistry, forming a mixture of products containing R-OH and R-X bonds.¹⁴ Although structural data for this SadA variant was not presented, the chemical competence and requirement of X⁻ clearly implicated formation of a complete His₂X facial triad.

Anion Binding to the Asp201→Gly Variant.

Anion binding to the facial-triad variants was monitored by UV-vis absorption spectroscopy in an anaerobic glovebox using a diode-array spectrophotometer. Asp201→(Ala/Glu/Gly)

variants reconstituted with Fe(II) and α KG exhibited modest shifts in the metal-to-ligand charge-transfer (MLCT) band from that of WT-FIH ($\lambda = 500$ nm),^{21,37} max with the largest shift observed for the Asp201→Gly variant ($\lambda_{\text{max}} = 485$ nm). These electronic transitions indicated that there were modest changes in the orbital energies of the Fe(II) + α KG chromophore due to removal of the facial triad Asp201 ligand. The MLCT transitions shifted by less than 5 nm upon addition of 100 mM NaCl to the FIH variants presumably due to displacement of an aquo ligand, as similarly small shifts were reported for Cl[−] binding to the α KG-dependent halogenase SyrB2,³⁴ reflecting the comparable positions in the spec-trochemical series for H₂O and Cl[−] ligands. Addition of OCN[−] or N₃[−] to the Asp201→Gly variant red-shifted the MLCT transition by roughly 15 nm, indicating that these pseudo halide ligands bound to the Fe(II), forming a His₂X facial triad within the FIH active site (Figure 2B).

X-ray Absorption Spectroscopy of (Fe+NOG+Cl)-Asp201→Gly.

Structural data supporting the His₂Cl facial triad were obtained by Fe K-edge X-ray absorption spectroscopy on the Asp201→Gly variant of FIH constituted anaerobically with FeSO₄ (1.0 equiv), N-oxalyl-glycine (NOG, 0.9 equiv), and excess NaCl (100 equiv) in 50 mM HEPES, pH 7.00 (Figure 3). NOG is isostructural to α KG and has been used in several crystallographic studies of FIH to define the structure of the cofactor while avoiding oxidation. The X-ray absorption edge energy reflects the electron density around the metal center and can indicate the metal oxidation state when compared to similar coordination environments. The edge energy (7123.3 eV) was 1.7 eV lower than that observed for oxidized, Fe(III)WT-FIH (7125.0 eV),²¹ indicating a more reduced Fe site in the D201G-FIH complex, where the 1.7 eV shift is consistent with a reduction to Fe(II).

X-ray absorption near-edge structure (XANES) analysis was used to determine the coordination number and geometry of the metal center (Figure 3A, inset). Metal ions such as Fe(II) have vacancies in the 3d orbitals and exhibit pre-edge features involving 1s → 3d electronic transitions, the number and intensity of which vary with coordination number and geometry. The pre-edge data for FIH reveals partially resolved features arising from multiple excited states.²³ Three ligand field excited states are expected to arise from the 1s → 3d transition of the ⁵T_{2g} ground state of O_h high-spin Fe(II) centers (⁴T, ⁴1g T_{2g}, and ⁴T_{1g}) and can generally be fit to the data between 7111 and 7114 eV. In the case of FIH, only two peaks are required to fit the data, similar to the situation for solid state FeI₂, where transitions to the lower energy excited states are not resolved. These two peaks are observed at 7111.1 and 7113.0 eV and have areas of 5.0×10^{-2} eV and 0.9×10^{-2} eV, respectively, giving a total pre-edge peak area of 5.9×10^{-2} eV, which is somewhat greater than typically observed for O_h high-spin Fe(II) ($3.1\text{--}4.4 \times 10^{-2}$ eV), possibly reflecting a lowering of the symmetry that would allow for a small amount of 3d-4p mixing. The peak intensity ratio (area of lowest energy peak/total area) is 0.83 and is similar to that observed for high-spin model Fe(II) complexes (0.72–0.85), as is the energy difference between the resolved peaks (1.9 eV; 1.8–2.4 eV in models). The results of the XANES analysis are consistent with a 6-coordinate distorted O_h Fe(II) center, as expected for the His₂Cl facial triad, a bidentate NOG, and a coordinated aquo ligand.

Extended X-ray absorption fine structure (EXAFS) analysis was used to measure the distance to Fe-bonded atoms and to test for the presence of Cl in the coordination sphere. EXAFS provides information on the atomic number of scattering atoms, readily distinguishing period 2 (N/O) ligands from period 3 (S/Cl) ligands, as well as a second estimate on the coordination number. The EXAFS data set was fit using progressively more detailed models to arrive at a best fit. The first fit attempted used a single shell of N donor atoms with coordination numbers ranging from 4–6 followed by a fit with a single shell of S-donor atoms with coordination number between 4 and 6. Neither single shell fit generated a fit with a R-value below 0.05. The data was then fit with a mixed shell of N and S donors, with all possible combinations of N and S donors at various distances with coordination number 6. This improved the fit, and the best fit ($R = 0.0601$) assuming uniform shells, obtained for 5N/O ($2.12(1) \text{ \AA}$) 1S/Cl($2.40(3) \text{ \AA}$), is shown in Table S1. Although this fit demonstrates that a Cl ligand is in the primary coordination shell of D201G, it did not include the crystallographic information about ligand composition.

As considering the scattering from only primary ligands in the mixed shell fit did not result in a fit with a R-value of less than 0.05, knowledge from the crystallographic structure of D201G was used as a model for the ligand compositions for EXAFS fits. Scattering from two rigid imidazole rings for His199 and His279 was next included, leading to an EXAFS fit that was consistent with the XANES analysis and featured one S/Cl-donor ligand, three N/O-donors, and two imidazole rings ($R = 0.03$). However, this fit did not accurately portray the ligand composition as NOG is a bidentate ligand with a rigid second coordination sphere of atoms that are likely to contribute to scattering. NOG was subsequently included in the model, reducing the R to 0.024 for the best fit (Table 1).

The best fit of the EXAFS corresponded to an $\text{Fe}(\text{His})_2(\text{O/N})_3\text{Cl}$ center, agreeing with the pseudo-octahedral geometry shown by XANES. Notably, it was not possible to obtain an acceptable fit ($R < 0.05$) without including a Cl scattering atom in the model (Table S1).

It is important to note that including rigid ligands (Imd and NOG) adds parameters to the fit but also makes these ligands distinct from a simple N/O donor atom.³⁹ The assessment of goodness of fit included both the R value as well as the reduced χ^2 value, which accounted for the additional parameters; the best fit reported in Table 1 had the lowest R and χ^2 of the fits, suggesting that we did not overparameterize.

Interestingly, the EXAFS fit (Table 1) indicated that the bond lengths for (Fe+Cl+NOG)D201G are comparable to those reported for WT-FIH based on crystallography. For example, structures for (Fe+ α KG)WT-FIH (PDBID 1MZF,⁴⁰ 1H2N,⁴¹ 4Z2W⁷) reported bond lengths for Fe–N_{His} ($1.97\text{--}2.24 \text{ \AA}$) and Fe– α KG ($1.87\text{--}2.34 \text{ \AA}$) in good agreement with the bond lengths in Table 1 for D201G. Further, the bond lengths for the metal cofactor of D201G were reported previously, albeit without bound chloride.¹⁹ Refinement of X-ray diffraction data for (Zn+ α KG)D201G/CTAD¹⁹ showed the D201G metal cofactor bonded to five ligands (His199, His279, a bidentate α KG, and H₂O) in a highly distorted square planar geometry ($\tau = 0.3$, whereas $\tau = 0$ for ideal square planar).⁴² Notably, the Fe–N_{His} distances were asymmetric (2.11 and $2.26 \text{ \AA}$) by X-ray diffraction, which mirrors the asymmetry reported by EXAFS for the (Fe+Cl+NOG)D201G sample (Table 1).

The Fe–Cl distance of the Asp201→Gly variant (2.36 Å) compares well with Fe–Cl distances found in the α KG-dependent halogenases SyrB2³⁸ (2.31 Å, EXAFS) and WelO5¹⁵ (2.1–2.3 Å, crystallography) indicating that the Asp201→Gly variant of FIH replicated the structure of the halogenase cofactor (Table 2). Further, as the O₂ activation chemistry (Figure 1) and the other metrical details match the halogenase cofactors (Table 2), the Asp201→Gly variant is an excellent mimic of the halogenase structures.

Metal Binding in Asp201 Variants.

The presence of an Fe–Cl bond in the Asp201→Gly variant raised the question of whether mutations at this site impair metal binding and if the anionic ligand would increase metal binding affinity. Comparison of crystal structures of D201G (Zn bound), D201A (Fe bound), and WT FIH showed that the metal retained a pseudo-octahedral geometry in the active site by binding bicarbonate, implying that the aspartate ligand was not required for metal binding.¹⁹ Additional crystallographic studies with D201E (Zn and Fe bound structures) confirmed this conservative mutation retained polar contacts from the facial triad and the ability to bind metal.²⁰ Previous studies conducted by Hangasky et al. demonstrated the binding of α KG to Fe(II) by the Asp201→(Ala/Glu/Gly) variants using UV–vis absorption spectroscopy to observe the expected $\text{Fe}^{2+}(\text{t}_{2\text{g}}) \rightarrow \alpha\text{KG}(\pi^*)$ MLCT of the (Fe+ α KG)FIH chromophore.²⁰ While the above indicates that the M+ α KG (M = Fe²⁺, Zn²⁺) can bind to each of the Asp201→(Ala/Glu/Gly) variants, the binding affinity of these variants has not been reported.

Intrinsic tryptophan fluorescence quenching was monitored using a PTI fluorimeter to determine the equilibrium dissociation constant (K_d) of metal binding to the Asp201→(Ala/Glu/Gly) variants in the presence of a large excess of α KG (Figure 4). Co(II) was used as an Fe(II) mimic in order to conduct the assays aerobically, as was done previously for WT FIH.⁴³ Samples were excited at 285 nm, and emission was monitored at 330 nm as increasing amounts of Co(II) were titrated into the protein solution. We observed an 80-fold decrease in the K_d (Co) for the Asp201→Ala variant relative to that of WT FIH ($K = 2.0(2) \times 10^{-6}$ M) (Table 3). This difference in K_d (Co) is much greater than the 6-fold increase in K_d seen between the α KG dependent oxygenase TfdA and its Asp116→Gly active site variant.¹⁸ The lower metal binding affinity for the Asp201→Ala variant suggests that the Ala substitution sterically impinges on the metal center and may explain the inability of this variant to hydroxylate CTAD.

We observed that the metal binding affinities for the Asp201→Glu and Asp201→Gly variants were very similar to that of WT FIH, varying by less than 3-fold (Table 3). This suggests that these variants likely retain pseudo-octahedral geometry about the bound metal ion. Further, the high metal binding affinity for these variants is consistent with their competence to hydroxylate CTAD.

CONCLUSION

The Asp201→Gly variant of FIH binds Fe with a His₂Cl facial triad and activates O₂ only in the presence of specific anions. This suggests that an anionic ligand in the His₂X facial triad is required for turnover in the α KG dependent oxygenases. Previous studies of this enzyme

family have identified the requirement of a Cl^- anion in the active site to form halogenated product; however, the role of the His_2X facial triad in O_2 activation was not explored. The data presented here provide evidence that a full His_2X triad is an absolute requirement for O_2 activation in the αKG oxygenases. The XAS metrics for the $\text{Fe}-\text{Cl}$ bond provide insight into the requirements for the His_2Cl facial triad structure found in halogenases. This study further indicates that an extended anion binding site is not needed to form an $\text{Fe}-\text{Cl}$ center, as suggested from crystallography of CytC3.^{38,44} Overall, this work advances knowledge of the requirements for anion binding to trigger O_2 reactivity and the role of the His_2Cl facial triad structure in the αKG dependent oxygenases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Activity screens of WT FIH, Asp201→Glu, Asp201→Gly, and Asp201→Ala in the presence of sodium halides (100 mM) and 80 μ M CTAD. (A) The initial rate for CTAD^{OH} formation as measured by MALDI-TOF. (B) O₂ consumption of Asp201→Gly in the presence of the indicated NaX salts.

Figure 2.

Anion-dependent activity: (A) Activity screens of WT FIH, Asp201→Glu, Asp201→Gly, and Asp201→Ala in the presence of pseudo halides (2 mM). Each reaction contained α KG (100 μ M), FeSO₄ (25 μ M), CTAD (100 μ M), and ascorbic acid (2 mM). The rate for CTAD hydroxylation is denoted. (B) Anaerobic UV-vis spectra of the MLCT region for pseudo halides (2 mM) in complex with (Fe+ α KG)FIH variants (400 μ M) constituted with FeSO₄ (375 μ M) and α KG (400 μ M) in 50 mM HEPES pH 7.00.

Figure 3.

Iron K-edge XAS of Fe(II) D201G-FIH (1 mM), NOG (1 mM), and NaCl (100 mM) in 50 mM HEPES pH 7.0. (A) XANES spectrum. The left inset is an expansion of the $1s \rightarrow 3d$ features. The right inset shows the baseline-corrected pre-edge XANES features (blue) fit with two peaks (black), with the individual components shown in red and green. (B) Unfiltered k -space data and fit. (C) The Fourier-transformed EXAFS spectrum (data, blue; fit, black). The fit shown was obtained for 1 N/O @ 2.09(1) Å ($\sigma^2 = 0.004(1) \text{ Å}^2$) + 1 Cl @ 2.36(4) Å ($\sigma^2 = 0.005(4) \text{ Å}^2$) + 1 Imd @ 1.95(4) Å ($\sigma^2 = 0.003(3) \text{ Å}^2$) + 1 Imd @ 2.16(4) Å ($\sigma^2 = 0.003(3) \text{ Å}^2$) + 1 NOG @ 2.01(9) and 2.11(9) ($\sigma^2 = 0.006(6) \text{ Å}^2$); $R = 0.024$.

Figure 4.

$K_d(\text{Co})$ determination based on intrinsic tryptophan fluorescence quenching for Asp201→Ala (green), Asp201→Glu (blue), Asp201→Gly (red) and WT FIH (black). The Asp201→Gly sample contained 100 mM NaCl in the assay buffer. Assays contained the following: 20 μM FIH, 500 μM αKG , and 10 mM sodium citrate in 50 mM HEPES pH 7.0. The titrating solution was composed of 1 mM CoSO_4 , and 1 mM sodium citrate or 10 mM CoSO_4 , and 10 mM sodium citrate in 50 mM HEPES pH 7.0.

Scheme 1. Mechanism of Fe(II)/αKG Hydroxylases and Fe(II)/αKG Halogenases^a

^aR = substrate; for hydroxylases X = Asp/Glu; for halogenases X = halide.

Table 1.Best Fit to EXAFS Data for the Asp201→Gly Variant of FIH^a

n	Radius (Å)	σ^2 ($\times 10^{-3}$ Å ²)	E_0 (eV)	R factor	n _{var.}
1 N/O	2.09(1)	4(1)	-10(2)	0.0236	11
1 Cl/S	2.36(4)	5(4)			
1 Imd	1.95(4)	3(3)			
1 Imd	2.16(4)	3(3)			
1 NOG	2.01(9)	6(6)			
	2.11(9)				

^aFIH D201G Fe(II)+NOG.

Table 2.

Ligand Bond Lengths in FIH-Asp201→Gly Compared to Halogenases

protein	Cl	N/O
SyrB2 Fe(II)+aKG ^a	2.31	2.12
SyrB2 Fe(II)+aKG ^b	2.47	2.03–2.22
WeIO5 Fe(II)+aKG ^c	2.09–2.26	2.04–2.48
WeIO5 Fe(II)+aKG + substrate ^c	2.32–2.34	2.05–2.56
FIH D201G Fe(II)+NOG ^d	2.36	1.95–2.11

^aMatthews, M. L.; Krest, C. M.; Barr, E. W.; Vaillancourt, F. H.; Walsh, C. T.; Green, M. T.; Krebs, C.; Bollinger, J. M. *Biochemistry* 2009, 48 (20), 4331–4343.

^bBlasiak, L. C.; Vaillancourt, F. H.; Walsh, C. T.; Drennan, C. L. *Nature* 2006, 440 (7082), 368–371.

^cMitchell, A. J.; Zhu, Q.; Maggiolo, A. O.; Ananth, N. R.; Hillwig, M.L.; Liu, X. Y.; Boal, A. K. *Nat. Chem. Biol.* 2016, 12 (8), 636–640.

^dPresent work.

Table 3.**K_d(Co) Values**

	K_d(Co), ×10⁻⁶ M	B
WT	2.0 ± 0.2	-1.9 ± 0.09
D201A	157 ± 30	-0.8 ± 0.1
D201E	5.5 ± 0.8	-1.6 ± 0.07
D201G	4.5 ± 0.7	-1.3 ± 0.2