Substrate Positioning by Gln239 Stimulates Turnover in Factor Inhibiting HIF, an αKG-Dependent Hydroxylase

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Supporting Information

ABSTRACT: Noneheme Fe(II)/αKG-dependent oxygenases catalyze diverse reactions, typically inserting an O atom from O2 into a C–H bond. Although the key to their catalytic cycle is the fact that binding and positioning of primary substrate precede O2 activation, the means by which substrate binding stimulates turnover is not well understood. Factor Inhibiting HIF (FIH) is a Fe(II)/αKG-dependent oxygenase that acts as a cellular oxygen sensor in humans by hydroxylating the target residue Asn803, found in the C-terminal transactivation domain (CTAD) of hypoxia inducible factor-1. FIH-Gln239 makes two hydrogen bonds with CTAD-Asn803, positioning this target residue over the Fe(II). We hypothesized the positioning of the side chain of CTAD-Asn803 by FIH-Gln239 is critical for stimulating O2 activation and subsequent substrate hydroxylation. The steady-state characterization of five FIH-Gln239 variants (Ala, Asn, Glu, His, and Leu) tested the role of hydrogen bonding potential and sterics near the target residue. Each variant exhibited a 20–1200-fold decrease in kcat and kcat/KM(CTAD), but no change in KM(CTAD), indicating that the step after CTAD binding was affected by point mutation. Uncoupled O2 activation was prominent in these variants, as shown by large coupling ratios (C = [succinate]/[CTAD-OH] = 3–5) for each of the FIH-Gln239 → X variants. The coupling ratios decreased in D2O, indicating an isotope-sensitive inactivation for variants, not observed in the wild type. The data presented indicate that the proper positioning of CTAD-Asn803 by FIH-Gln239 is necessary to suppress uncoupled turnover and to support substrate hydroxylation, suggesting substrate positioning may be crucial for directing O2 reactivity within the broader class of αKG hydroxylases.

Nonheme Fe(II)/αKG-dependent oxygenases make up a large superfamily of enzymes catalyzing diverse reactions, including demethylations, hydroxylations, ring expansions, and epoxidations.1,2 Many of these enzymes have important connections is Factor Inhibiting HIF (FIH), because of the role in hypoxia inducible factor-1. FIH-Gln239 makes two hydrogen bonds with CTAD-Asn803, positioning this target residue over the Fe(II). We hypothesized the positioning of the side chain of CTAD-Asn803 by FIH-Gln239 is critical for stimulating O2 activation and subsequent substrate hydroxylation. The steady-state characterization of five FIH-Gln239 variants (Ala, Asn, Glu, His, and Leu) tested the role of hydrogen bonding potential and sterics near the target residue. Each variant exhibited a 20–1200-fold decrease in kcat and kcat/KM(CTAD), but no change in KM(CTAD), indicating that the step after CTAD binding was affected by point mutation. Uncoupled O2 activation was prominent in these variants, as shown by large coupling ratios (C = [succinate]/[CTAD-OH] = 3–5) for each of the FIH-Gln239 → X variants. The coupling ratios decreased in D2O, indicating an isotope-sensitive inactivation for variants, not observed in the wild type. The data presented indicate that the proper positioning of CTAD-Asn803 by FIH-Gln239 is necessary to suppress uncoupled turnover and to support substrate hydroxylation, suggesting substrate positioning may be crucial for directing O2 reactivity within the broader class of αKG hydroxylases.

Human cells sense O2 through the hypoxia inducible factor (HIF) pathway, which is controlled by a small number of αKG oxygenases, including FIH.15,16 HIF is an αβ dimeric transcription factor that regulates numerous genes involved in tissue development, controlling processes such as glycolysis, erythropoiesis, and angiogenesis.17–19 In the presence of O2, FIH hydroxylates the β-carbon of HIF1α-Asn803,20 which is found in the C-terminal activation domain (CTAD) of HIF1α. CTAD-Asn803 hydroxylation blocks recruitment of the cAMP response element-binding protein (CREBP), preventing HIF-dependent gene transcription.4,21 The connection between CTAD-Asn803 positioning and O2 reactivity is critical to understanding how substrate stimulates O2 activation in this enzyme superfamily, as well as illuminating FIH’s role as an O2 sensor.

The consensus chemical mechanism for FIH is based upon an array of kinetic and spectroscopic studies of FIH and other αKG oxygenases. Kinetic studies of thymine hydroxylase, FIH, CAS, and TauD support the ordered, sequential binding of αKG and primary substrate followed by O2.22–25 Although αKG, O2, and an oxidizable compound are all substrates for these enzymes, we will refer to the oxidizable substrate as the “primary substrate”. Spectroscopic studies of CAS,26–28 TfdA,29 FIH,30 and TauD31 revealed that the Fe(II) released an aquo ligand after the primary substrate bound, creating a site for O2 binding. Binding and activation of O2 lead to the oxidative decarboxylation of αKG and the formation of a highly reactive ferryl intermediate (Scheme 1). Although the precise sequence of intermediates is not known, the ferryl intermediate has been observed in TauD32–34 and P4H,35 demonstrating that H atom abstraction by the ferryl intermediate occurs,36 with the next
step likely to be \( ^\bullet \)OH rebound to hydroxylate the primary substrate.

The most intriguing feature of the consensus mechanism is that binding the primary substrate stimulates \( \mathbf{O}_2 \) reactivity.\textsuperscript{22,37,38} Loss of an aquo ligand when the primary substrate is bound opens a coordination site for \( \mathbf{O}_2 \) binding, as observed upon binding of the primary substrate in several \( \alpha \text{KG} \)-dependent oxygenases, including CytC3, TauD, CAS, and FIH.\textsuperscript{26,30,31} Although aquo release is central to the widely accepted model for substrate-stimulated \( \mathbf{O}_2 \) activation,\textsuperscript{39} we note that simple ligand exchange is insufficient for \( \mathbf{O}_2 \) activation in these enzymes. For example, substrate binding to FIH leads to only fractional release of the aquo ligand,\textsuperscript{30} and mutagenesis suggests that hydrogen bond donors to the \( \alpha \text{KG} \) are necessary for full activity in this enzyme.\textsuperscript{40} Computational studies\textsuperscript{41−44} and mechanistic probes\textsuperscript{45−47} further point to turnover being limited by steps after \( \mathbf{O}_2 \) binds to the Fe(II). These and related observations lead us to propose that substrate-stimulated \( \mathbf{O}_2 \) reactivity arises from bonding changes throughout the active site, ranging from aquo release at the iron cofactor to altered contacts in the second coordination sphere.

A focus of this research in our lab is to identify those active site features that change upon substrate binding to stimulate \( \mathbf{O}_2 \) activation in \( \alpha \text{KG} \) oxygenases. Although the precise sequence of intermediates formed during turnover is not known, we define \( \mathbf{O}_2 \) activation as the steps between \( \mathbf{O}_2 \) binding and oxidative decarboxylation (Scheme 1) by virtue of the irreversible chemistry; this step is depicted as the nucleophilic attack of the putative ferric superoxide on the \( \alpha \)-keto position of \( \alpha \text{KG} \). On the basis of known crystal structures of FIH,\textsuperscript{14,48,49} we have used point mutagenesis to identify several essential second-coordination sphere interactions in FIH, including those hydrogen bonding to Fe(II) ligands, as well as FIH-Gln239, an anchor residue that forms two hydrogen bonds with the target residue, CTAD-Asn803 (Figure 1).\textsuperscript{14} Intriguingly, disruption of this two-point hydrogen bond in the FIH-Gln239 \( \rightarrow \) Asn point mutant led to a decrease in \( k_{\text{cat}} \) of 250-fold, but a negligible change in \( k_{\text{M(CTAD)}} \).\textsuperscript{40} This was attributed to a combination of steric hindrance near the open coordination site on Fe(II) and incorrect CTAD positioning for the HAT step. Subsequently, it was shown that an irreversible step associated with \( \mathbf{O}_2 \) activation was rate-limiting in wild-type FIH (WT-FIH),\textsuperscript{25} suggesting that the slower turnover for the Gln239 \( \rightarrow \) Asn variant could arise from slower \( \mathbf{O}_2 \) activation. This suggests the intriguing possibility that target residue position may stimulate \( \mathbf{O}_2 \) activation and that the overall structure of the active site is crucial for \( \mathbf{O}_2 \) activation.

This study tests the role of Gln239 in substrate hydroxylation in \( \alpha \text{KG} \) oxygenases. As FIH hydroxylates a specific target residue within a large peptide (CTAD-Asn803), our focus was directed at this target residue pocket, formed by the side chains of FIH residues Tyr102, His199, Arg238, and Gln239 (Figure 1).\textsuperscript{14} Five FIH-Gln239 \( \rightarrow \) X variants were prepared (X = Ala, Asn, Glu, His, and Leu) to vary the bulk and hydrogen bonding potential within the target residue pocket. Although these variants exhibited significantly reduced steady-state rate constants that decreased monotonically with increasing residue bulk, CTAD binding affinity was unaffected by mutation. In contrast to the case in WT-FIH, \( \mathbf{O}_2 \) activation was appreciably uncoupled from CTAD hydroxylation in the variants; uncoupled \( \mathbf{O}_2 \) activation was partially suppressed in D\textsubscript{2}O. These data establish that the proper orientation of CTAD-Asn803 by FIH-Gln239 is required for substrate hydroxylation,
most likely because of the need for the proper target residue positioning during steps after O₂ activation.

### EXPERIMENTAL PROCEDURES

**Materials.** All reagents were purchased from commercial vendors and were not further purified, with the exception of the 39-mer CTAD peptide. The 39-mer CTAD peptide corresponding to the C-terminal activation domain of human HIF1α (HIF1α⁷⁸⁸–⁸⁸⁶) contained a Cys⁸⁸⁰ → Ala change (underlined) (DESLPQLTSYDAEVEPIIQGSRNLLQGEELLRALDQVN). This was purchased as a desalted peptide from EZBiolab (Carmel, IN) with free N- and C-termini. The CTAD-Asn803 change and was purchased at >95% purity from EZBiolab with μPuri (DESGLPQLTSYDAEVEPIIQGSRNLLQGEELLRALDQVN). All mutations were sequenced (Genewiz) to obtain >95% pure CTAD. The 19-mer CTAD peptide was prepared in D₂O. Deuterium oxide (D₂O, 99.9%) was purchased from Cambridge Isotope Laboratories (Andover, MA) and used as received. Working FIH stock solutions were made by diluting high-concentration stocks from H₂O into D₂O containing 50 mM HEPES (pD 7.00). Assays were performed in 50 mM HEPES (pD 7.00), with a final D₂O percentage estimated to be 96%. SKIEs were calculated from the direct comparison of kinetic parameters observed in buffers containing H₂O and D₂O: e.g., D₂O/kcat = kcat(D₂O)/kcat(H₂O).

**Succinate Quantification.** The coupling between the two half-reactions was determined by monitoring the production of succinate and CTADOH concentrations in several quench points from a common reaction. Reactions of αKG (500 μM), Fe₅S₉ (25 μM), CTAD (350 μM), and FIH (5–10 μM) were conducted at 37.0 °C and analyzed similarly using previously reported procedures. As HEPES interfered with the succinate analysis, the reaction buffer consisted of 50 mM Tris (pL 7.00). A Hamilton PRP-X300 anion exclusion column was used to separate the succinate produced from the quenched reactions, and UV detection at 210 nm was used to determine the succinate concentration. Using aliquots from the same quenched assay, a Bruker Daltonics Omniflex MALDI-TOF MS was used to determine the CTAD⁹⁸ concentration. The coupling ratio (C) was determined by taking the ratio of the rate of succinate formation and the rate of CTAD⁹⁸ formation from matched time points.

**Fluorescence Spectroscopy.** The FIH–CTAD binding constants were measured through quenching of the intrinsic tryptophan fluorescence of (Co + αKG)FIH upon CTAD binding at room temperature (~20 °C). The fluorescence cuvette contained FIH (1.5 μM), CoSO₄ (25 μM), αKG (500 μM), and 50 mM HEPES (pH 7.05). This solution was titrated with 50 mM HEPES (pH 7.05) containing CTAD (1 mM), FIH (1.5 μM), CoSO₄ (25 μM), and αKG (500 μM). All titrations were performed aerobically. After each addition of titrant, samples were gently mixed and allowed to equilibrate for 5 min before being excited at 295 nm. The fluorescence intensities at 330 nm were plotted versus the total CTAD concentration and fit using eq 1

\[
\frac{I - I_0}{I_t - I_0} = \frac{[E] + [S] + K_D}{\sqrt{([E] + [S] + K_D)^2 - 4[E][S]^n}} \left(\frac{2[E]}{2[E]}\right)
\]

where I is the measured fluorescence intensity, [E] is the protein concentration, [S] is the total CTAD concentration, n is the number of binding sites, and K_D is the binding affinity. The initial intensity (I₀) and final intensity (I_t) were obtained from measured spectra.

### RESULTS

Variants of FIH-Gln239 were used to test the effect of target residue positioning on substrate hydroxylation in FIH. The variants were designed to vary hydrogen bonding potential (Gln239 → Glu and Gln239 → His) and cavity size (Gln239 → Ala, Gln239 → Asn, and Gln239 → Leu) in the target residue pocket of FIH. The Gln239 variants were kinetically characterized in the steady state with CTAD as the varied substrate, giving the apparent rate constants kcat and kcat/KM(CTAD). The kinetic characterization revealed significantly diminished rate constants for turnover, leading us to determine...
the binding affinity of CTAD as well as the coupling ratio of the two half-reactions for each point mutant.

**Kinetic Characterization of Gln\(^{239}\) → X Variants.** We hypothesized the positioning of CTAD-Asn\(^{303}\) by FIH-Gln\(^{239}\) was necessary to support turnover and therefore focused our studies on steady-state characterization by monitoring CTAD\(^{304}\) formation via MALDI-TOF. Although \(O_2\) uptake was the first method that we considered, the slow turnover for FIH makes high-precision kinetic determinations by this method challenging. Assays using fixed concentrations of \(\alpha\)KG (500 \(\mu\)M) and \(O_2\) (217 \(\mu\)M) and varied concentrations of CTAD (15–300 \(\mu\)M) were used to measure initial rates, which where then fit to the Michaelis–Menten equation to obtain the apparent steady-state rate constants, \(k_{\text{cat}}\) and \(k_{\text{cat}}/K_{M(\text{CTAD})}\). The Michaelis constant for \(\alpha\)KG was determined for each variant \([K_{M(\alpha\text{KG})} = 4–7 \ \mu\text{M}],\) which was slightly lower than that for WT-FIH \([K_{M(\alpha\text{KG})} = 16 \ \mu\text{M}].\) Because of the \(O_2\) concentration is subsaturating, the apparent \(k_{\text{cat}}\) encompasses all steps after CTAD binding, including those involved in \(O_2\) binding and activation. All of the \(\text{Gln}^{239} \rightarrow \text{X}\) variants exhibited a significant decrease in \(k_{\text{cat}}\) (Figure 2). The \(\text{Gln}^{239} \rightarrow \text{Ala}\) \((k_{\text{cat}} = 1.27 \pm 0.10 \ \text{min}^{-1})\) variant was most active, as the \(k_{\text{cat}}\) decreased 20-fold relative to that of WT-FIH, whereas the \(k_{\text{cat}}\) for Gln\(^{239} \rightarrow \text{Asn}\) \((0.14 \pm 0.02 \ \text{min}^{-1})\) decreased 200-fold. The \(k_{\text{cat}}\) for variants capable of one-point hydrogen bonding decreased >1200-fold: Gln\(^{239} \rightarrow \text{His}\) \((0.023 \pm 0.003 \ \text{min}^{-1})\) and Gln\(^{239} \rightarrow \text{Glu}\) \((0.024 \pm 0.002 \ \text{min}^{-1})\). We were unable to observe hydroxylation from the Gln\(^{239} \rightarrow \text{Leu}\) variant.

Steps from CTAD binding through the first irreversible step (decarboxylation) comprise \(k_{\text{cat}}/K_{M(\text{CTAD})}\) (Scheme 2). The effect of each point variant on \(k_{\text{cat}}/K_{M(\text{CTAD})}\) was nearly identical to their effect on \(k_{\text{cat}}\), indicating that the variants affected a step that was separate from CTAD binding.

We tested the activity of FIH-Gln\(^{239} \rightarrow \text{Asn}\) using a 19-mer CTAD peptide containing the complementary CTAD-Asn\(^{303}\) → Gln point mutation, which switched the residues at this interface. This switch mutation was designed to restore the bulk and hydrogen bonds observed between WT-FIH and WT-CTAD. However, the activity level was below our detection limit (0.002 \text{min}^{-1}), as hydroxylated CTAD-Asn\(^{303}\) → Gln was not detected upon being incubated with FIH-Gln\(^{239} \rightarrow \text{Asn}\). WT-FIH was similarly unreactive toward this variant CTAD, as WT-FIH hydroxylated the 19-mer WT-CTAD with an appreciable rate, but did not hydroxylate the variant CTAD (Table 1).

**Binding Affinity of CTAD for Gln\(^{239} \rightarrow \text{X}\) Variants.** The binding affinity of each FIH variant for CTAD was measured by titration using the intrinsic tryptophan fluorescence of FIH. A solution containing CTAD (1 mM) was titrated into a solution containing FIH (1.5 \(\mu\)M) while the fluorescence at 330 nm was monitored (\(\lambda_{\text{ex}} = 295 \ \text{nm}\)); both solutions were anaerobic and contained CoSO\(_4\) (25 \(\mu\)M) and \(\alpha\)KG (500 \(\mu\)M). The change in fluorescence intensity (330 nm) was plotted as a function of CTAD concentration and fit to eq 1. The experimentally determined \(K_{D}\) for each point mutant (Table 2) was similar to that of WT-FIH (78 ± 7 \(\mu\)M), indicating the thermodynamics of CTAD binding was not affected by point mutation.

**Uncoupled Turnover in the Gln\(^{239} \rightarrow \text{X}\) Variants.** The kinetic parameters of the Gln\(^{239} \rightarrow \text{X}\) mutations led us to explore the coupling of \(O_2\) activation to substrate hydroxylation. We hypothesized that if the conformational state of CTAD-Asn\(^{303}\) were incorrect for HAT, then the two half-reactions would uncouple to produce more succinate than hydroxylated product (CTAD\(^{303}\)). Quenched aliquots from reaction mixtures containing saturating concentrations of \(\alpha\)KG (500 \(\mu\)M) and CTAD (350 \(\mu\)M) in 50 mM Tris (\(pH\) 7.00) were analyzed for CTAD\(^{303}\) via MALDI-TOF MS and succinate via HPLC. Tris buffer was used for these assays to minimize the background signal in the HPLC chromatograms that arose due to buffer components.

The coupling values for the Gln\(^{239} \rightarrow \text{X}\) (X = Ala, Asn, Glu, and His) variants were obtained by taking the ratio of the rates of formation for succinate and CTAD\(^{303}\). Variants produced three to five succinates per equivalent of CTAD\(^{303}\); succinate formation was observed for the Gln\(^{239} \rightarrow \text{Leu}\) variant \([k_{\text{obs(suc)}} = 0.08 \ \text{min}^{-1}]\), indicating \(O_2\) activation occurred even though CTAD hydroxylation was not detected for this variant (Table 3). This uncoupling is similar to the values found previously for second-coordination sphere variants of FIH.40

The coupling of WT, Gln\(^{239} \rightarrow \text{Ala}\), and Gln\(^{239} \rightarrow \text{Asn}\) in deuterated buffer was used to determine if the coupling ratio changed between protonated and deuterated buffers. The coupling for WT FIH in H\(_2\)O (\(C = 1.0 \pm 0.1\)) and D\(_2\)O (\(C = 1.0 \pm 0.1\)) was in agreement with our previous work,35 showing WT remains tightly coupled under all tested conditions. However, the coupling ratio in D\(_2\)O for Gln\(^{239} \rightarrow \text{Ala}\) (\(C = 1.4 \pm 0.2\)) and Gln\(^{239} \rightarrow \text{Asn}\) (\(C = 2.2 \pm 0.2\)) approached unity, indicating that solvent deuteration led to more tightly coupled turnover for these variants.

**Solvent Kinetic Isotope Effects (SKIEs).** SKIEs on both \(k_{\text{cat}}\) and \(k_{\text{cat}}/K_{M(\text{CTAD})}\) were used to test the importance of solvent-dependent steps during turnover. Initial rates from steady-state assays using saturating \(\alpha\)KG concentrations (500 \(\mu\)M), ambient \(O_2\) concentrations (217 \(\mu\)M), and varied concentrations of CTAD (15–300 \(\mu\)M) were fit to the Michaelis–Menten equation (Figure 3). Turnover was faster in D\(_2\)O with both WT-FIH and each variant, leading to an inverse SKIE on \(k_{\text{cat}}\) and \(k_{\text{cat}}/K_{M(\text{CTAD})}\) (Table 4). However, these SKIEs must be considered in the context of the solvent-dependent uncoupling observed for the variants.31
Scheme 2. Minimal Chemical Scheme for Uncoupling

Table 1. Initial Rates for 19-mer Peptides CTAD and CTAD-N803Q

|          | WT-CTAD (min⁻¹) | CTAD-N803Q (min⁻¹) |
|----------|-----------------|--------------------|
| WT       | 0.90            | <0.005             |
| Q239N    | <0.005          |                    |

*Assays contained ascorbate (2 mM), αKG (500 μM), FeSO₄ (25 μM), and 19-mer CTAD (400 μM) in 50 mM HEPES (pH 7.00) at 37°C. The CTAD peptide used contained 19 residues. *No activity detected; estimated detection limit, if active.

**DISCUSSION**

The ordered sequential consensus mechanism for αKG oxygenases leads to coupled turnover when primary substrate binding stimulates reactivity toward O₂, a phenomenon termed substrate-induced activity enhancement, priming, or triggering by different groups. As primary substrate does not directly bind to the Fe(II), altered local contacts within the active site likely stimulate O₂ activation. Although the idea of stimulated O₂ activation refers to the empirical observation of increased turnover rates induced by substrate binding, the dominant model used to explain this focuses on aquo release, which creates an open coordination site for O₂ binding. In our opinion, broader changes within the active site are correlated with this effect, such as the position of the primary substrate.

FIH is notable in that enzyme—substrate contacts are quite extensive because the substrate is a large peptide (CTAD), with the target residue positioned above the Fe by a two-point hydrogen bond to the side chain of an anchoring residue, FIH-Gln239.14,55 This study varied the sterics and H-bonding potential of this anchor residue to test its role in hydroxylating CTAD-Asn803.

**CTAD Hydroxylation Is Slowed by Gln²³⁹ Variants.** Each of the Gln²³⁹ → X variants (X = Ala, Asn, Glu, His, or Leu) altered the hydrogen bond potential and/or bulk of the target residue pocket, disrupting the positioning of the target residue. This incorrect positioning could have impacted any one of several steps within the kinetic mechanism, which may

Table 2. Apparent Kinetic Parameters for FIH and Its Variants

|          | kₐ (min⁻¹)b | kₐ/KₐCTAD (μM⁻¹ min⁻¹)b | KₐCTAD (μM)c | KₐCTAD (μM)d |
|----------|-------------|--------------------------|--------------|--------------|
| WT       | 30 ± 2.5c   | 0.4 ± 0.1d               | 70 ± 20f     | 78 ± 7g      |
| Q239A    | 13 ± 0.1f   | 0.021 ± 0.002e           | 61 ± 10i     | 100 ± 16j    | 5.0 ± 0.5k |
| Q239N    | 0.14 ± 0.02l | 2.0 × 10⁻⁵ ± 8 × 10⁻⁴m   | 74 ± 30n     | 98 ± 10o     | 4.0 ± 0.4p |
| Q239H    | 0.023 ± 0.003o | (1.4 ± 1) × 10⁻⁹q      | 68 ± 18r     | 64 ± 14s     | 7.0 ± 1.4t |
| Q239E    | 0.024 ± 0.002u | 3.4 × 10⁻⁹ ± 7 × 10⁻⁵v  | 71 ± 10w     | 75 ± 15x     | 4.7 ± 2.0y |
| Q239L    | <0.005z     | <8 × 10⁻⁶|               | ND  a        | 80 ± 8b      | ND  a      |

*In 50 mM HEPES (pH 7.00) at 37°C. *Assays in which CTAD was the varied substrate, in ascorbate (2 mM), αKG (500 μM), FeSO₄ (25 μM), and CTAD (0–300 μM). *Determined using intrinsic tryptophan fluorescence with Co-substituted enzyme. *Assays in which αKG was the varied substrate, in ascorbate (2 mM), αKG (2–200 μM), FeSO₄ (25 μM), and CTAD (100 μM). *From ref 25. *From ref 30. *No activity detected; estimated detection limits as reported. *Not determined.
be distinguished through analysis of steady-state kinetic parameters and coupling ratios. The significant reduction in $k_{cat}$ and $k_{cat}/K_{M}$ relative to those of WT-FIH indicated that the anchor residue played a prominent role in supporting turnover. Keeping in mind the observation that the binding affinity of CTAD was unchanged from that of WT-FIH (Table 2), we are led to conclude that the predominant role of FIH-Gln39 is to position substrate for a chemical step rather than to bind CTAD.

Although it may seem surprising that the anchor residue FIH-Gln39 contributes very little to the CTAD binding affinity, this is consistent with prior studies of CTAD variants. As the length of the CTAD has been shown to have a significant affect on the $K_M$ and the binding affinity of WT-FIH for CTAD is indistinguishable from the Michaelis constant in CTAD binding is the surface contact with FIH, with only minor contributions from the target residue pocket. Alanine scanning point mutagenesis of CTAD revealed that CTAD-Val802 was the most significant residue for CTAD binding, with a 2-fold increase in the $K_M$ for the CTAD-Val802 → Ala variant. Molecular dynamics studies suggested that this mutation led to reorientation of Asn803, perhaps because of disruption of the tight turn conformation in residues 801–803 of CTAD. Further support for a minimal impact of FIH-Gln39 on CTAD binding is the observation that FIH hydroxylates substrates with target residues other than asparagine. The structural features of these substrates suggest the overall contact between FIH and the CTAD peptide is important in determining substrate binding to FIH.

**Inverse SKIEs and Coupling.** We recently reported inverse SKIEs for WT FIH, on both $k_{cat}$ and $k_{cat}/K_{M}$ (Table 4). This was due to the isotopically sensitive metal–aquo fractionation prior to a rate-limiting step for WT-FIH. Importantly, WT-FIH exhibited fully coupled turnover, such that O$_2$ activation always led to substrate hydroxylation. Consequently, it was deduced that the rate-limiting step for $k_{cat}$ and $k_{cat}/K_{M}$ was an irreversible step immediately after aquo release. This step is depicted as the oxidative decarboxylation of αKG in Scheme 1.

For each of the Gln239 → X variants, inverse SKIEs were measured on both $k_{cat}$ and $k_{cat}/K_{M}$ when determined from the rate of CTAD oxidation (formation of Table 4). Although the SKIE data resembled those reported for WT-FIH, turnover for these variants was significantly uncoupled, which precluded the use of SKIEs to diagnose rate-limiting steps in the steady state. Nevertheless, uncoupling in the variants depended on solvent isotopic composition (Table 3), suggesting that the ferryl intermediate could form even when CTAD was improperly positioned. The fact that C approached unity in D$_2$O for these variants suggested that the main effect of the Gln239 → X change was to perturb the hydroxylation step.

**Sterics and H-Bonding Impact Substrate Hydroxylation.** A simple model to explain how the target residue pocket impacts productive turnover is one in which multiple conformational states of the target residue are adopted but only one conformation supports hydroxylation. X-ray crystal structures of (M+αKG)FIH bound to CTAD$_{14}$ or Notch-derived peptides$^{55}$ revealed that the target residue adopted a specific rotameric conformation, with a side chain torsional angle (HIN–C$_{α}$–C$_β$–C$_γ$) of −71°. This is observed for both Notch target residues Notch-Asn$^{210}$ and Notch-Asn$^{945}$ [Protein Data Bank (PDB) entries 3P3P and 3P3N, respectively]. As a good deal of flexibility near Gln239 was observed crystallographically for (Fe+αKG)FIH when CTAD was absent (PDB entry 1MZF),$^{58}$ changing the hydrogen bonding potential and packing density of this anchor residue should alter the target residue position above the Fe(II). The significant reduction in catalytic efficiency for each point mutant strongly suggests that the major role of FIH-Gln39 is to stabilize the proper rotamer of CTAD-Asn$_{803}$ that can undergo hydroxylation during turnover.

The kinetic data further suggest that packing near the target residue may also impact O$_2$ activation in FIH. The overall trend in the kinetic parameters measured by coupled turnover (Table 2) was dominated by bulk, as the kinetic parameters for the

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**Table 4. Apparent Kinetic Parameters in D$_2$O and SKIEs for FIH and Its Variants**

|        | $k_{cat}$ (min$^{-1}$)$^b$ | $k_{cat}/K_{M}$ (μM$^{-1}$ min$^{-1}$)$^b$ | Δ$^3$O$^3_{K_{cat}}$ | Δ$^3$O$^3_{K_{cat}/K_{M}}$ |
|--------|------------------------|---------------------------------|---------------------|------------------------|
| WT$^a$ | 59 ± 2                 | 1.09 ± 0.11                    | 0.51 ± 0.07          | 0.40 ± 0.07             |
| Q239A  | 2.55 ± 0.21            | 0.044 ± 0.011                  | 0.50 ± 0.05          | 0.48 ± 0.15             |
| Q239N  | 0.27 ± 0.02            | 0.005 ± 0.001                  | 0.50 ± 0.06          | 0.41 ± 0.18             |
| Q239H  | 0.050 ± 0.003          | 2.0 × 10$^{-3}$ ± 8 × 10$^{-4}$ | 0.46 ± 0.07          | 0.17 ± 0.06             |
| Q239E  | 0.046 ± 0.002          | (8 ± 3) × 10$^{-4}$            | 0.52 ± 0.05          | 0.41 ± 0.16             |
| Q239L  | ND$^f$                 | ND$^f$                          | ND$^f$               | ND$^f$                 |

$^a$In 50 mM HEPES (pD 7.00) at 37.0 °C. $^b$Determined from assays with CTAD as the varied substrate, in ascorbate (2 mM), αKG (500 μM), FeSO$_4$ (25 μM), and CTAD (0–250 μM); $^{3}D_{D_{2}O} = 0.96$. $^{Δ3}O_{K_{cat}} = k_{cat}(D_{2}O)/k_{cat}(H_{2}O)$; $^{Δ3}O_{K_{cat}/K_{M}} = [k_{cat}/K_{M}(D_{2}O)]/[k_{cat}/K_{M}(H_{2}O)]$. $^f$From ref 25. $^f$Not determined.
Gln\textsuperscript{239} → X point variants decreased monotonically in a series: X = (Ala > Asn > Glu and His ⇒ Leu). As the kinetic parameters of the variants listed in Table 2 are functions of all steps leading to CTAD hydroxylation, it is not possible to separately identify the impact of the variants on O\textsubscript{2} activation. However, the coupling data directly measured succinate production (Table 4), which reports directly on O\textsubscript{2} activation. The rates of succinate production clearly showed that each variant produced succinate much more slowly that WT-FIH, suggesting that O\textsubscript{2} activation was slowed in these variants. As the variant produced succinate much more slowly that WT-FIH, this di...
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