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# Kinetic Analysis of Iron-Dependent Histone Demethylases: α-Ketoglutarate Substrate Inhibition and Potential Relevance to the Regulation of Histone Demethylation in Cancer Cells

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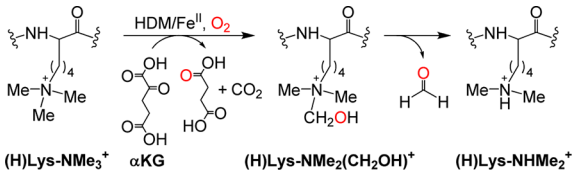
Supporting Information

**ABSTRACT:** The Jumonji C domain-containing histone demethylases (JmjC-HDMs) are α-ketoglutarate (αKG)-dependent, $O_2$-activating, non-heme iron enzymes that play an important role in epigenetics. Reported herein is a detailed kinetic analysis of three JmjC-HDMs, including the cancer-relevant JMJD2C, that was achieved by employing three enzyme activity assays. A continuous $O_2$ consumption assay reveals that HDMs have low affinities for $O_2$, suggesting that these enzymes can act as oxygen sensors in vivo. An interesting case of αKG substrate inhibition was found, and the kinetic data suggest that αKG inhibits JMJD2C competitively with respect to $O_2$. JMJD2C displays an optimal activity in vitro at αKG concentrations similar to those found in cancer cells, with implications for the regulation of histone demethylation activity in cancer versus normal cells.

Covalent modification of chromatin by histone methylation has wide-ranging effects on nuclear functions, such as transcriptional regulation, genome integrity, and epigenetic inheritance.[1] Until recently, histone methylation was believed to be a static modification; however, the identification of histone demethylase (HDM) enzymes has revealed that this epigenetic mark is dynamically controlled.[2] The Jumonji C domain-containing HDMs (JmjC-HDMs) catalyze the demethylation of methylated lysine residues through a hydroxylation reaction and belong to the large class of α-ketoglutarate (αKG)-dependent, $O_2$-activating, non-heme iron enzymes.[3] More than a dozen JmjC-HDMs have been reported and shown to exhibit both residue and methylation state specificity.[4] In addition, JmjC-HDMs have been implicated in cancer and stem cell biology. For example, it has recently been shown that PLU-1 is a HDM that plays an important role in the proliferation of breast cancer cells through transcriptional repression of tumor suppressor genes,[5] while the HDM GASC1 (or JMJD2C) was proposed to be linked to stem cell phenotypes in breast cancer.[6] Thus, it is of great interest to design specific inhibitors for the various HDM subfamilies by utilizing the enzymes' substrate specificities.[7] However, there are limited data available on the enzymology of HDMs. In this regard, we have successfully expressed and purified three HDMs, including the cancer-relevant JMJD2C, and obtained a detailed enzyme kinetic analysis by employing three different assays (vide infra). Interestingly, a case of αKG substrate inhibition was observed, which could have important implications for the regulation of HDM activity in cancer biology.

In the lysine demethylation reaction, HDMs hydroxylate the *N*-methyl substrate to a hydroxymethyl group, which is converted nonenzymatically to formaldehyde and the demethylated product (Scheme 1).[2] Our initial kinetic studies focused

**Scheme 1. Histone Demethylation by JmjC-HDMs, Shown for a Trimethyllysine Substrate**




on JMJD2A, the first HDM with a resolved crystal structure and one that has been used extensively in biochemical studies.[4] The pseudogene-encoded JMJD2E, with a sequence that is >80% similar to that of JMJD2A, has been often employed in enzyme activity studies as it has the highest activity when compared to those of the other members of the JMJD2 subfamily.[7a,b] Finally, JMJD2C is of great interest because of its implications in brain, breast, prostate, and esophageal cancers,[6,8] yet the kinetics of JMJD2C has not been fully investigated to date.[7c−e]

Truncated constructs of JMJD2A,[4b] JMJD2E,[7a] and JMJD2C,[7d] containing the catalytic JmjN and JmjC domains, were transformed into the *Escherichia coli* Rosetta II strain and expressed and purified using published procedures.[9] The enzymatic activity of the three HDMs was monitored by three complementary assays: a coupled formaldehyde dehydrogenase (FDH) NADH fluorescence assay, a discontinuous MALDI-TOF mass spectrometry (MS) assay, and a continuous $O_2$ consumption assay. Initially, kinetic parameters were obtained using an adapted version of the widely employed FDH-coupled assay,[4c,10] and a trimethylated H3K9me$_3$ octapeptide as the histone substrate analogue.[9] Demethylation of the peptide substrate was also confirmed by MALDI-TOF MS, and quantification of the peptide products was correlated to the extent of demethylation measured using the other enzymatic assays.[9] Since no direct continuous HDM activity

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**Table 1. Kinetic Parameters for the Three JmjC-HDMs, JMJD2A, JMJD2E, and JMJD2C[a]**

| | | H3K9me$_3$ | $O_2$ | αKG |
|---|---|---|---|---|
| HDM | assay | $K_m^{app}$ (μM), $k_{cat}^{app}$ ($min^{-1}$)[b] | $K_m^{app}$ (μM), $k_{cat}^{app}$ ($min^{-1}$) | $K_m^{app}$ (μM), $K_i^{app}$ (mM)[d] |
| JMJD2A | $O_2$ consumed | 31 ± 3, 2.5 ± 0.1[b] | 57 ± 10, 2.5 ± 0.1 | 10 ± 1, 10 ± 2[d] |
| | coupled FDH | 104 ± 16, 1.4 ± 0.1[b] | NM[c] | 21 ± 4, 13 ± 3[d] |
| JMJD2E | $O_2$ consumed | 38 ± 3, 3.3 ± 0.1[b] | 197 ± 16, 4.0 ± 0.1 | 21 ± 2, 12 ± 1[d] |
| | coupled FDH | 224 ± 15, 2.1 ± 0.1[b] | NM[c] | 37 ± 7, 11 ± 3[d] |
| JMJD2C | $O_2$ consumed | 32 ± 3, 2.1 ± 0.1[b] | 158 ± 13, 2.6 ± 0.1 | 12 ± 2, 4.3 ± 0.6[d] |
| | coupled FDH | 76 ± 11, 0.70 ± 0.03[b] | NM[c] | 22 ± 5, 3.4 ± 0.6[d] |

[a]See the Supporting Information for assay conditions. [b]$k_{cat}^{app}$ values were determined at 258 μM $O_2$. [c]Not measured. [d]αKG $K_i^{app}$ values reported at 258 μM $O_2$.

assay has been developed to date, we have employed for the first time an $O_2$ consumption assay to measure in real-time the HDM enzymatic activity by using a Clark oxygen electrode.[9,11]

Using the assays described above, we have determined the kinetic parameters for the three JmjC-HDMs with respect to all three substrates: the ARK(me$_3$)STGGK peptide substrate, $O_2$, and αKG. For JMJD2A and JMJD2C, the FDH-coupled assay yielded $K_m$ values for the peptide substrate (104 ± 16 μM for JMJD2A and 76 ± 11 μM for JMJD2C) that are similar to those found in the literature,[4a,7e,12] while for JMJD2E, a slightly larger $K_m$ value was obtained [224 ± 15 μM (Table 1)].[a] Interestingly, the use of the $O_2$ consumption assay reveals significantly reduced peptide $K_m$ values for JMJD2A (31 ± 3 μM), JMJD2E (38 ± 3 μM), and JMJD2C (32 ± 3 μM), as well as turnover numbers that are similar or higher than those from other studies.[4a,7e,12] The $O_2$ consumption assays can be performed under saturating conditions for all substrates and thus provide a unique opportunity to obtain true $k_{cat}$ values for the three HDMs (Table 1). Most importantly, the observed activity of JMJD2C is higher than that reported previously[7e] and comparable to those of JMJD2A and JMJD2E.[7] Thus, JMJD2C can now be used in inhibition studies employing the $O_2$ consumption assay, especially given its direct implication in cancer biology.[6] Overall, the $O_2$ consumption assay seems to allow for a superior characterization of HDMs versus the FDH-coupled assay, because the obtained $K_m$ values for the peptide substrate are more in line with the expected affinities for the natural substrate in vivo, while higher turnover numbers were also found for all three HDMs investigated.[9]

Using the $O_2$ consumption assay, we have measured for the first time the $K_m(O_2)$ values for HDMs and found that these enzymes have relatively low apparent affinities for $O_2$, with $K_m$ values near or above the normal cellular $O_2$ concentration [57 ± 10 μM for JMJD2A, 197 ± 16 μM for JMJD2E, and 158 ± 13 μM for JMJD2C (Figure 1)].[13] Such $O_2$ affinities suggest the enzymatic activity can be altered by small changes in $O_2$ concentration, and thus, the JmjC-HDMs can act as oxygen sensors in vivo, as observed previously for other αKG-dependent non-heme iron oxygenases involved in the hypoxic response.[13] In contrast, the enzymes' affinities for αKG are high compared to those for $O_2$, and in line with values reported previously.[7a,14] For all three HDMs investigated, the αKG $K_m$ values are between 10 and 37 μM and differ by <2-fold between the FDH-coupled assay and the $O_2$ consumption assay, with the latter technique providing slightly lower $K_m$ values (Table 1).

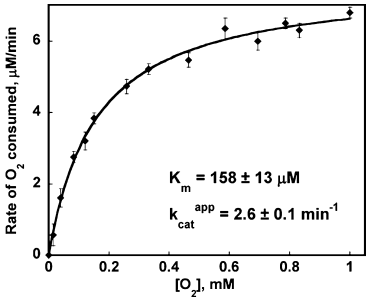


**Figure 1.** Michaelis–Menten plot for JMJD2C obtained using a Clark oxygen electrode and varying $O_2$ concentrations.[9]

Interestingly, we found a mild αKG substrate inhibition effect for JMJD2A and JMJD2E when αKG was present in high concentrations (>1 mM).[9] The apparent $K_i$ values were obtained using the FDH-coupled assay (for JMJD2A, $K_i^{app}$ = 13 ± 3 mM; for JMJD2E, $K_i^{app}$ = 11 ± 3 mM) and the $O_2$ consumption assay [for JMJD2A, $K_i^{app}$ = 10 ± 2 mM; for JMJD2E, $K_i^{app}$ = 12 ± 1 mM (Table 1)]. Interestingly, the αKG inhibitory effect is significantly stronger in the case of JMJD2C [with the FDH-coupled assay, $K_i^{app}$ = 3.4 ± 0.6 mM (Figure 2);

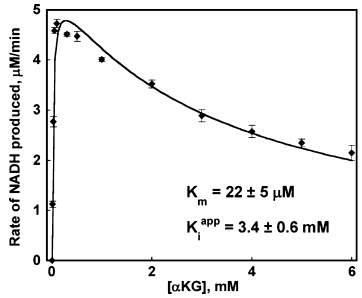


**Figure 2.** Inhibition of JMJD2C by αKG, measured by the FDH-coupled assay.[9]

with the $O_2$ electrode assay, $K_i^{app}$ = 4.3 ± 0.6 mM (Figure S5 of the Supporting Information)]. Although previous studies reported the inhibition of other αKG-dependent Fe(II) oxygenases by αKG,[15] the type of inhibition with respect to the other substrates was not investigated in detail. In this regard, the inhibition of JMJD2C by αKG was measured at constant αKG concentrations (300 μM, 2 mM, 4 mM, and 5 mM) and variable $O_2$ concentrations. It was found that the $K_m$ value for $O_2$ increased with an increasing αKG concentration, while the $V_{max}$ value stayed relatively constant (Figure S8 of the Supporting Information).[9] In addition, a double-reciprocal plot of the inverse of the rate of reaction versus the inverse of the $O_2$ concentration reveals that the linear plots corresponding to the different αKG concentrations intersect on the *y*-axis, suggesting that αKG is a competitive inhibitor with respect to $O_2$ (Figure 3).[b] Importantly, a competitive inhibition of $O_2$ by αKG has


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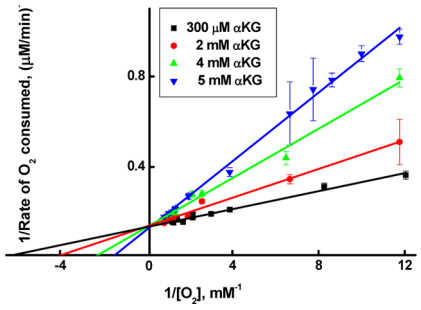


**Figure 3.** Double-reciprocal plot of 1/rate of $O_2$ consumed vs $1/[O_2]$ obtained using the $O_2$ consumption assay, suggesting αKG competitive inhibition of JMJD2C with respect to $O_2$.

not been observed for any αKG-dependent oxygenase to date.[7i,15] Moreover, the more pronounced αKG inhibition of JMJD2C versus JMJD2A and JMJD2E is expected to impact the different activity profiles in vivo for these enzymes.

The observed αKG substrate inhibition of JMJD2C could have important implications in cancer biology.[8] In normal healthy cells, the expression and activity of JMJD2C are believed to be highly regulated.[6,8] Given the observed in vitro inhibition by αKG, the activity of JMJD2C could be regulated in healthy tissue through a high cellular αKG concentration, which does not allow for an optimal demethylase activity of JMJD2C. Interestingly, there is a large difference in the level of αKG in healthy brain cells and glioblastomas. Whereas in healthy brain tissue the αKG concentration ranges from 1 to 3 mM,[16] it has been reported that the αKG concentration in gliomas and glioblastoma multiformes is 100−300 μM.[17] Indeed, we find that JMJD2C displays its highest activity in vitro at an αKG concentration of ∼300 μM (Figures S3 and S5 of the Supporting Information),[9] suggesting that a decreased concentration of αKG could lead to an increased HDM activity in cancer versus normal cells.[18]

In conclusion, a detailed kinetic analysis of three JmjC-HDMs, including the cancer-relevant JMJD2C, was achieved by employing three enzyme activity assays. Using a continuous $O_2$ consumption assay, we found that HDMs have affinities for $O_2$ near the cellular $O_2$ concentration, suggesting that HDMs may act as oxygen sensors in vivo. Importantly, we have observed a case of αKG substrate inhibition, and the kinetic data suggest that αKG inhibits JMJD2C competitively with respect to $O_2$. The concentration of αKG at which JMJD2C displays optimal activity in vitro is similar to the concentration of αKG in cancer cells, which has direct implications for the increased activity of JMJD2C in cancer versus normal cells. Future studies will focus on probing the effect of αKG concentration on the activity of HDMs in vivo, as well as the implication of αKG concentration variation in the epigenetic control of cancer versus normal cells.

## ■ ASSOCIATED CONTENT

### Supporting Information

Protein expression and purification, and enzyme kinetic data for FDH, $O_2$ electrode, and MALDI-TOF MS assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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### Notes

The authors declare no competing financial interest.

## ■ ADDITIONAL NOTES

[a]The higher $K_m$ (peptide) and lower $k_{cat}$ values obtained through the FDH coupled assay are likely due to the less-than-optimal activity of FDH at high peptide concentrations, (see the Supporting Information).

[b]αKG shows a weak, mixed-mode nonlinear inhibition of JMJD2C with respect to the peptide substrate (Figures S4 and S6 of the Supporting Information).

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