**BIOPHYSICS**

*N*<sup>6</sup>-methyladenosine binding induces a metal-centered rearrangement that activates the human RNA demethylase Alkbh5

Jeffrey A. Purslow<sup>1</sup>, Trang T. Nguyen<sup>1</sup>, Balabhadra Khatiwada<sup>1</sup>, Aayushi Singh<sup>1</sup>, Vincenzo Venditti<sup>1,2*</sup>

Alkbh5 catalyzes demethylation of the *N*<sup>6</sup>-methyladenosine (m<sup>6</sup>A), an epigenetic mark that controls several physiological processes including carcinogenesis and stem cell differentiation. The activity of Alkbh5 comprises two coupled reactions. The first reaction involves decarboxylation of α-ketoglutarate (αKG) and formation of a Fe<sup>4+</sup>=O species. This oxyferryl intermediate oxidizes the m<sup>6</sup>A to reestablish the canonical base. Despite coupling between the two reactions being required for the correct Alkbh5 functioning, the mechanisms linking dioxygen activation to m<sup>6</sup>A binding are not fully understood. Here, we use solution NMR to investigate the structure and dynamics of apo and holo Alkbh5. We show that binding of m<sup>6</sup>A to Alkbh5 induces a metal-centered rearrangement of αKG that increases the exposed area of the metal, making it available for binding O<sub>2</sub>. Our study reveals the molecular mechanisms underlying activation of Alkbh5, therefore opening new perspectives for the design of novel strategies to control gene expression and cancer progression.

**INTRODUCTION**

AlkB homolog 5 (Alkbh5) is an essential regulator of RNA epigenetics that controls several metabolic pathways by modulating transcription, translation, and cellular localization of RNA (1–5). Overexpression of Alkbh5 links to development of various types of cancer, including leukemia, breast, and brain cancer (6–8). Deletion of the Alkbh5 gene or inactivation of the enzyme via mutations or small-molecule inhibitors increases the sensitivity of tumors to immunotherapy (9). Therefore, Alkbh5 is actively researched to understand the fundamental mechanisms regulating gene expression and as a promising therapeutic target for anticancer treatments.

Alkbh5 belongs to the nonheme Fe<sup>2+</sup> and αKG–dependent AlkB dioxygenases and catalyzes oxidative demethylation of *N*<sup>6</sup>-methyladenosine (m<sup>6</sup>A) residues in single-stranded nucleic acids (10) with little to no activity against other modifications (11, 12). Biochemical and structural studies have led to a consensus mechanism for this family of dioxygenases that involves ordered binding of cofactor and substrate molecules and two coupled reactions (13–15). In particular, binding of the metal cofactor (Fe<sup>2+</sup>) and of the secondary substrate (αKG) precedes binding of the primary substrate (the m<sup>6</sup>A-containing oligonucleotide for Alkbh5) to the enzyme. Once the catalytic complex is fully assembled, binding of O<sub>2</sub> to Fe<sup>2+</sup> triggers rapid decarboxylation of αKG to generate carbon dioxide, succinate, and a reactive oxyferryl (Fe<sup>4+</sup>=O) intermediate that oxidizes the primary substrate to generate the unmodified nucleotide (Fig. 1A). While it is established that the highly reactive and potentially cytotoxic oxyferryl intermediate cannot form in the absence of the primary substrate (16, 17), the mechanisms coupling the binding of the primary substrate to dioxygen activation are not fully understood, and it is not clear whether all αKG dioxygenases share a common activation pathway.

In the complex formed by AlkB dioxygenases with Fe<sup>2+</sup> and αKG, the metal binding site is provided by a 2-His-1-carboxylate facial-triad motif (18) formed by the side chains of His<sup>204</sup>, His<sup>266</sup>, and Asp<sup>206</sup> in Alkbh5 (19). αKG is directly coordinated to the Fe<sup>2+</sup> center in an off-line geometry, in which the C2 carbonyl oxygen is positioned trans with respect to the carboxylate group of the facial-triad motif (Asp<sup>206</sup> in Alkbh5) (Fig. 1, A and B) (19). Binding of the primary substrate displaces the water molecule occupying the sixth coordination site, enabling the metal center to bind and activate dioxygen for catalysis (20–22). If the O<sub>2</sub> molecule binds at the position previously occupied by the water molecule (path A in Fig. 1A), the subsequently formed Fe<sup>4+</sup>=O species points away from the primary substrate (Fig. 1), and a structural rearrangement (commonly referred to as “ferryl flip”) is required for efficient catalysis. Alternatively, αKG can transition from the off-line to the in-line geometry before O<sub>2</sub> binding to the metal site (path B in Fig. 1A). Such metal-centered rearrangement allows formation of the reactive Fe<sup>4+</sup>=O adjacent to the primary substrate and ready for catalysis. Although computational studies indicate the latter mechanism (path B in Fig. 1A) to be energetically favored (23, 24), analysis of the crystal structures reported for AlkB dioxygenases in complex with their primary and secondary substrates reveals contrasting results. While the structure of Alkbh2 in complex with an m<sup>6</sup>A-containing nucleotide shows αKG in the in-line configuration compatible with path B (25), αKG adopts the off-line configuration in the complex of AlkB and FTO with their primary substrates (note that an αKG analog is used for FTO) (Fig. 1B) (16, 17, 26–28), suggesting that dioxygen activation might follow path A in these enzymes.

Here, we investigate the structure and dynamics of the apo and holo human Alkbh5 by solution nuclear magnetic resonance (NMR) and molecular dynamics (MD) simulations. We show that addition of the secondary and primary substrates introduces a progressive quenching of picosecond-nanosecond (ps-ns) and microsecond-millisecond (ms-ms) conformational dynamics, confirming that modulation of intramolecular flexibility plays an important role in regulating the sequential binding of substrate and cofactor molecules to AlkB enzymes (29, 30). We show that binding of the primary...
substrate introduces a rearrangement within the αKG binding site that is consistent with the conformational transition from the off-line to the in-line configuration. Such conformational switch, which is not triggered by binding of oligonucleotides containing the unmodified A or other methyl modifications, effectively exposes the metal center, making it available for binding O₂. Our study reveals the molecular mechanism of Alkbh5 activation and highlights the ability of solution NMR spectroscopy to detect and characterize structural heterogeneity at protein active sites, which is critical for understanding functional regulation of complex enzymes (31–33).

RESULTS
Identification of an NMR-friendly divalent cation
As all other members of the AlkB dioxygenase family, Alkbh5 requires Fe²⁺ to bind the primary and secondary substrate (13–15). However, other divalent cations such as Mn²⁺ and Co²⁺ are commonly used in structural and thermodynamic studies on AlkB enzymes (16, 17, 19, 26–28). These metals preserve the octahedral geometry typical of Fe²⁺, are more stable in solution, and do not activate dioxygen for catalysis, therefore facilitating structural investigation of the holoh enzyme. Nonetheless, Fe²⁺, Mn²⁺, and Co²⁺ are not ideal metals for NMR studies on AlkB dioxygenases, as their paramagnetism results in extensive line broadening and complete loss of the NMR signals from the protein active site. To overcome this limitation, we have evaluated the ability of diamagnetic cations such as Zn²⁺, Cd²⁺, and Mg²⁺ to stabilize the binding of αKG and an m⁶A-containing oligonucleotide to Alkbh5 by isothermal titration calorimetry (ITC) and pull-down experiments, respectively. As a reference, the same experiments were also acquired in the presence of Mn²⁺, which was shown to bind to AlkB enzymes with the same geometry as the physiological Fe²⁺ (28).

Analysis of the ITC data returned equilibrium dissociation constants (Kₒ) for the Alkbh5-αKG complex of 5.9 ± 0.4, 4.0 ± 0.4, or 2.4 ± 0.3 μM when the metal cofactor is Mn²⁺, Zn²⁺, or Cd²⁺, respectively (Fig. 2A and Table 1). No binding was detected in the presence of Mg²⁺ (Fig. 2A). Results of the pull-down assay (Fig. 2B) indicate that Alkbh5 binds the primary substrate in the presence of either Mn²⁺ or Zn²⁺ (lanes 2 and 4 in Fig. 2B) but fails to recognize the m⁶A-containing nucleotide when Cd²⁺ or Mg²⁺ are used as the metal cofactors (lanes 7 and 9 in Fig. 2B), indicating that the latter two cations are not suitable diamagnetic analogs of Fe²⁺ for NMR studies on Alkbh5. The enzyme is not able to recognize the primary substrate in the absence of αKG, as evidenced by the lack of a protein band in the pull-down assay run in the presence of Mn²⁺ and Zn²⁺ without αKG (lanes 1 and 3 in Fig. 2B). This observation is consistent with the sequential binding model in which binding of the metal cofactor and αKG precedes binding of the primary substrate to the enzyme (13–15).

In summary, the data reported here indicate that replacing Mn²⁺ with Zn²⁺ does not alter substantially the ability of Alkbh5 to bind its primary and secondary substrate and that the sequential binding of αKG and m⁶A, which is a hallmark of AlkB enzymes, is fully retained by Alkbh5 in the presence of Zn²⁺. In addition, in the presence of Zn²⁺, Alkbh5 is able to recognize m⁶A-containing oligonucleotides over oligonucleotides incorporating the canonical A or other methylated bases (see below). Therefore, Zn²⁺ is a suitable diamagnetic cation for investigating the holoh form of Alkbh5 by solution NMR spectroscopy. Notably, Zn²⁺ has been previously used as a diamagnetic analog of Fe²⁺ for structural and thermodynamics studies on the bacterial enzyme AlkB and its complexes (29).

Effect of the primary and secondary substrates on the NMR spectra of Alkbh5
We have recently reported the assignment of the ¹H–¹⁵N and ¹H–¹³C methyl transverse relaxation–optimized spectroscopy (TROSY) spectra of the apo form of human Alkbh5 at pH 7.4 (34, 35). In the present work, solution NMR data are measured at pH 6.0 to increase the solubility of the metal cofactor. Backbone resonance assignments for apo Alkbh5 and for its complexes with αKG and αKG/m⁶A were
Table 1. Equilibrium dissociation constants and thermodynamic parameters for the Alkbh5-αKG complex.

| Divalent cation | $K_d$ (µM) | $\Delta H$ (cal mol$^{-1}$) | $\Delta S$ (cal mol$^{-1}$ K$^{-1}$) |
|----------------|------------|-----------------|-------------------------------|
| Mn$^{2+}$      | 5.9 ± 0.4  | −10.7 ± 0.2     | −11.9 ± 0.8                   |
| Zn$^{2+}$      | 4.0 ± 0.4  | −13.1 ± 0.3     | −19.4 ± 1.1                   |
| Cd$^{2+}$      | 2.4 ± 0.3  | −20.4 ± 0.5     | −42.0 ± 1.8                   |

As expected, analysis of the $^1$H-$^15$N TROSY spectra indicates that addition of a saturating amount of Zn$^{2+}$ and αKG to ~0.5 mM Alkbh5 (see Materials and Methods) induces chemical shift perturbations ($\Delta_{H/N}$) within the jelly-roll motif containing the binding site for αKG and the metal cofactor (Fig. 3, A and C). Addition of the primary substrate results in large $\Delta_{H/N}$ values upon addition of the m$^6$A-containing oligonucleotide (Fig. 3B). This observation implies that the exchange rate between the free and DNA-bound states of Alkbh5 is similar to the value of $\Delta_{H/N}$ measured (in rad s$^{-1}$) for these peaks. However, NMR signals whose chemical shift is affected to a lesser extent by the DNA are still visible in the presence of the unmodified oligonucleotide (Fig. 3E). For such signals, the binding kinetics are on the fast time scale regime, and their chemical shift can be monitored to report on saturation of the enzyme-DNA complex during the NMR titration experiment (Fig. 3E) (39). As expected, the unmodified DNA has a lower affinity for Alkbh5 than its methylated analog, and a 6:1 DNA-to-protein ratio is required to nearly saturate binding (Fig. 3E). Only one set of NMR peaks is observed for the jelly-roll motif and α-helix 3, indicating that the αKG binding site is present in one single configuration when Alkbh5 is bound to the unmodified DNA. The latter conformation will be referred to as state A. The alternative configuration, accessible by the αKG binding site when Alkbh5 is bound to the methylated oligonucleotide, will be referred to as state B. Assignment of the $^1$H-$^15$N TROSY peaks of states A and B was performed by comparison of the NMR spectra measured for the Alkbh5 complexes with methylated and unmethylated DNA, as shown in Fig. 3E. It is also important to notice that the presence of the unmodified DNA causes line broadening beyond detection level at residues Phe$^{134}$,
Fig. 3. Solution NMR of human Alkbh5. (A) $\Delta_{HN}$'s induced by a saturating concentration of Zn$^{2+}$ and $\alpha$KG on the $^1$H-$^15$N TROSY spectrum of Alkbh5 are plotted versus the residue index. (B) The $\Delta_{HN}$'s measured upon addition of 1.2 equivalents of 5′-GG(m$^6$A)CT-3′ (black circles) or 6 equivalents of 5′-GGACT-3′ (red circles) to Alkbh5-$\alpha$KG are plotted versus the residue index. Flips 1, 2, and 3 are highlighted by gray boxes. (C) $\Delta_{HN}$'s induced by addition of Zn$^{2+}$ and $\alpha$KG to Alkbh5 are plotted on the structure of Alkbh5. (D) $\Delta_{HN}$'s induced by addition of 5′-GG(m$^3$A)CT-3′ to Alkbh5-$\alpha$KG are plotted on the structure of Alkbh5. The relationship between $\Delta_{HN}$ and sphere color and size is depicted by the color bar. (E) Selected region of the $^1$H-$^15$N TROSY spectrum showing the splitting of some NMR resonances into two peaks upon addition of 5′-GG(m$^6$A)CT-3′. The spectrum acquired in the absence of DNA (but in the presence of Zn$^{2+}$ and $\alpha$KG) is colored pink. The spectrum measured at a saturating concentration of 5′-GG(m$^6$A)CT-3′ is colored red. Spectra acquired at 1 mM and 3 mM 5′-GG(m$^6$A)CT-3′ are colored cyan and blue, respectively. The NMR peaks assigned to states A and B are labeled on the structure of Alkbh5. 

In addition, while binding of Alkbh5 to a 5-mer DNA containing the m$^6$A residue in the sequence.

An m$^6$A-induced metal-centered transition

Analysis of the solution NMR data acquired on the Alkbh5-$\alpha$KG/m$^6$A complex indicates that the $\alpha$KG binding site exists in two equally...
populated states (referred to as states A and B) with different $^{1}H-^{15}N$ chemical shifts. The two states share identical Cα and Cβ chemical shifts, suggesting that they do not originate from a transition in the backbone structure but are rather the result of a conformational change involving αKG and the surrounding side chains. Here, we hypothesize that states A and B correspond to the off-line and in-line configurations of αKG observed in the crystal structures of AlkB and Alkbh2, respectively, bound to their primary and secondary substrates.

To investigate the stability of these configurations in Alkbh5, 1.2-μs MD simulations of the Alkbh5-αKG and Alkbh5-αKG/m$^{6}$A complexes were run with Fe$^{2+}$ as the metal center and αKG either in the off-line or in the in-line conformation. The starting structure for Alkbh5-αKG/m$^{6}$A in the off-line geometry was prepared by modeling the 5-mer oligonucleotide used for solution NMR (see above) into the crystal structure of the Alkbh5-αKG complex [Protein Data Bank (PDB) code: 4NRO]. The in-line geometry of αKG was modeled on the basis of the crystal structure of Alkbh2 in complex with αKG and the primary substrate (PDB code: 3RZJ) (more details on the modeling and equilibration of the starting structures are provided in Materials and Methods). Analysis of the MD simulations on the Alkbh5-αKG/m$^{6}$A complex indicates that the metal coordination geometry of αKG is stable in both trajectories, with the dihedral angle formed by Nε1 of H204 and O1, C1, and C2 of αKG fluctuating around 44 ± 8° and −50 ± 9° for the off-line and in-line geometry, respectively (Fig. 4, A and B). On the other hand, only the off-line configuration provides a stable metal coordination geometry in the MD simulations of the Alkbh5-αKG complex (Fig. 4, A and B), suggesting that the methylated oligonucleotide is absolutely required to provide structural stabilization to the in-line form of αKG.

Analysis of the MD simulations on the Alkbh5-αKG/m$^{6}$A complex also reveals that in-line αKG is more conformationally restrained than off-line αKG. The C1-C2-C3-C4 dihedral angle of αKG experiences larger fluctuations when off-line (Fig. 4, A and C), indicating that the in-line configuration results in a more efficient packing of the jelly-roll motif around the αKG molecule compared to the off-line configuration. The observation that the jelly-roll motif has different structural properties when αKG is off-line or in-line agrees well with our NMR data, indicating that the residues displaying different $^{1}H-^{15}N$ TROSY signals for states A and B are spread throughout the jelly-roll motif (Fig. 3F).

Overall, the results of the MD study support our hypothesis that the two states identified by NMR correspond to the off-line and in-line geometries of αKG. In particular, state A, which is the state adopted by αKG in the presence of unmodified DNA, corresponds to the off-line configuration observed in the crystal structure of Alkbh5-αKG. State B, which is only accessible in the presence of the m$^{6}$A-containing nucleotide, corresponds to the in-line configuration.

**Effect of the primary and secondary substrates on the conformational dynamics of Alkbh5**

We have recently shown that the active site and the N-terminal region of apo Alkbh5 experience conformational dynamics on both the ps-ns and μs-ms time scale (34). Here, the effect of the primary and secondary ligands on the Alkbh5 conformational dynamics is investigated by means of $^{15}N$ NMR relaxation measurements.

In particular, residue-specific $^{15}N$ longitudinal ($R_L$) and transverse ($R_T$) relaxation rates were obtained at 800 MHz and 25°C by acquisition of TROSY-detected $R_L$ and $R_T$ experiments (40) on $^{1}$H-$^{15}$N-labeled Alkbh5. Analysis of the $^{15}N-R_L/R_T$ ratios is a convenient method to investigate ps-ns dynamics in proteins. For a rigid protein, where global tumbling is the only significant contribution to the ps-ns dynamics, the $R_L/R_T$ values are expected to be constant throughout the amino acid sequence and proportional to the rotational correlation time ($\tau_c$) (41). On the other hand, the presence of flexible structural elements within the protein (such as long and flexible loops) that increase locally the ps-ns dynamics experienced by the backbone amide groups is revealed by a local shift of the $R_L/R_T$ ratios toward lower than average values (41). The measured $R_L/R_T$ ratios are graphed as a function of the residue index in Fig. 5A and plotted as a gradient on the structure of Alkbh5 in Fig. 5B. The data show a comparable trend for the apo enzyme and for the Alkbh5-αKG and Alkbh5-αKG/m$^{6}$A complexes, with mean ratios of 40 ± 12, 36 ± 10, and 37 ± 12, respectively. These values translate to $\tau_c$ ~ 15 ns that is consistent with the $\tau_c$ (~16 ns) predicted at 25°C for a globular protein of the size of Alkbh5 using the Stokes’ law. For all three Alkbh5 states, the N-terminal region (residues 67 to 74) and flip 2 show lower than average $R_L/R_T$ values, indicative of enhanced local motion in addition to overall molecular tumbling (Fig. 5, A and B). Noteworthy, the flip 2 region produces average $R_L/R_T$ of 16 ± 4, 15 ± 5, and 26 ± 8 for the apo, αKG-bound, and αKG/m$^{6}$A-bound states, respectively. Such local increase in $R_L/R_T$ observed for flip 2 upon addition of nucleic acid suggests that DNA binding reduces the flexibility of this long loop, which is consistent with the direct participation of flip 2 in DNA binding revealed by NMR chemical shift perturbation (Fig. 3A) and mutagenesis experiments (19, 38). In summary, analysis of the $R_L/R_T$ data presented here indicates that binding of the metal and αKG has a negligible influence on the ps-ns dynamics of the Alkbh5 backbone, while DNA
binding results in rigidification of flip 2. It should also be observed that, in apo and αKG-bound Alkbh5, a lower than average $R_{ex}$ value is measured for Gly198, which is located on a marginal turn of the jelly-roll motif (Fig. 5, A and B). However, this NMR correlation was not assigned in the spectrum of the Alkbh5-αKG/m^6A complex, and the effects of nucleic acid binding on the ps-ns dynamics of this residue are unknown.

The μs-ms time scale dynamics in Alkbh5 were investigated by measuring the exchange contribution to the $^{15}$N-$R_{ex}$ ($R_{ex}$) by Carr-Purcell-Meiboom-Gill relaxation dispersion NMR experiments at 800 MHz and 25°C. Backbone amides sensing conformational dynamics on the μs-ms time scale produce $R_{ex}$ values significantly larger than 0 s$^{-1}$ and can be easily identified by plotting the experimental $R_{ex}$ values versus the residue index (Fig. 5C). In the apo enzyme, large ($>10$ s$^{-1}$) $R_{ex}$ values are measured for several amino acids located within flip 1, flip 2, the αKG binding site, and the C-terminal fragment (residues 284 to 292) (Fig. 5, C and D). Addition of Zn^2+ and αKG results in a generalized reduction of $R_{ex}$ across the protein (Fig. 5, C and F), indicating that binding of the metal cofactor and of the secondary substrate induces a rigidification of the enzyme structure. Complete quenching of $R_{ex}$ is observed for several amino acids located within the Ala^190-Ile^210 and Ile^268-Leu^282 fragments that form part of the αKG binding site and provide the platform for DNA binding to Alkbh5 (Fig. 5, C, F, and G). Last, binding of the methylated nucleic acid results in further stabilization of the Alkbh5 structure, as evidenced by the near-complete quenching of the $R_{ex}$ contributions throughout the entire protein (Fig. 5, C and G). In summary, analysis of the $^{15}$N relaxation dispersion experiments...
indicates that binding of the secondary and primary substrate to Alkbh5 results in a progressive stabilization of the enzyme structure into a well-defined fold, which is competent for catalysis.

**Effect of the primary and secondary substrates on the structure of Alkbh5**

Analysis of the NMR data indicates that addition of αKG does not alter the 13C chemical shifts of the Alkbh5 backbone and that binding of m6A is associated with slight changes in the Ca secondary chemical shifts for residues located within flips 2 and 3 and for residue 203 from the m6A binding site (Fig. 6A). These data suggest that the interactions with the primary and secondary substrate generate minor and localized changes in the backbone of Alkbh5. To further characterize structural changes associated with substrate binding, we collected backbone amide 1D_NH RDC data for the apo Alkbh5 and for the Alkbh5-αKG and Alkbh5-αKG/m6A complexes. Singular value decomposition (SVD) fitting of the full set of 1D_NH RDCs to the x-ray coordinates of the Alkbh5-αKG complex (PDB: 2NRO) yielded R-factors of 48% for all the investigated samples (Fig. 6B and Table 2). As previously shown for apo Alkbh5 at pH 7.4 (34), the poor correlation between experimental and back-calculated RDC data originates from the high degree of conformational disorder within the active site that is not properly captured by the static crystallographic structure. Repeating the SVD fits on a subset of experimental RDCs from which all data coming from the active site residues are removed substantially improves the agreement between solution NMR data and crystal structure, returning R-factors of 20, 20, and 21% for apo Alkbh5, Alkbh5-αKG, and Alkbh5-αKG/m6A, respectively (Fig. 6B and Table 2). The fact that the RDCs originating from the jelly-roll motif and the three α helices of apo Alkbh5, Alkbh5-αKG, and Alkbh5-αKG/m6A can be fit using the same crystallographic model confirms that binding of the primary and secondary substrate does not alter the backbone fold and relative orientation of these rigid structural elements.

To visualize the structural changes in the nucleotide recognition loops associated with substrate binding, we have calculated structural ensembles for the apo Alkbh5 and for the Alkbh5-αKG and Alkbh5-αKG/m6A complexes by coupling the experimental 1D_NH RDC data with accelerated MD (aMD) simulations. This protocol has been proven successful in generating MD-derived structural ensembles of dynamical proteins that satisfy solution NMR data (34, 43). Analysis of the changes in R-factor as a function of the ensemble sizes indicates that 13-, 18-, and 13-member ensembles are required to fully satisfy the experimental RDC data measured for apo Alkbh5 (R-factor ~ 26%), Alkbh5-αKG (R-factor ~ 25%), and Alkbh5-αKG/m6A (R-factor ~ 26%), respectively (fig. S4 and Table 2). The modeled structural ensembles are visualized in Fig. 6E and confirm that addition of αKG and m6A causes a progressive quenching of protein conformational dynamics. Analysis of the average structures calculated from the conformational ensembles indicates that

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**Fig. 6. Solution structure of apo and holo Alkbh5.** (A) The secondary Ca chemical shifts (CS) measured for Alkbh5-αKG (blue circles) and Alkbh5-αKG/m6A (red circles) are plotted versus the Ca secondary chemical shifts measured for apo Alkbh5 (top). Ca atoms that show a significant change in secondary chemical shift upon addition of m6A are plotted as red spheres on Alkbh5 (bottom). (B) The RDCs back-calculated from the Alkbh5 structure are plotted versus the experimental data. SVD fitting of the full set of RDCs against the x-ray structure is shown as blue circles. SVD fitting performed discarding all data from the active site is shown as red circles. RDCs back-calculated from the x-ray structure (red spheres). Blue spheres indicate the RDCs removed from the SVD analysis. The lower number of analyzed RDCs for Alkbh5-αKG/m6A reflects the lower quality of the NMR spectrum measured in the presence of methylated oligonucleotide (fig. S3). (D) The average structures of the conformational ensembles are used to calculate the Ca displacement of Alkbh5-αKG (red line) and Alkbh5-αKG/m6A (green line) from the structure of the apo Alkbh5. The Ca displacement of the apo Alkbh5 average structure from the x-ray structure is shown as black line. (E) Conformational ensembles generated for apo Alkbh5 (left), Alkbh5-αKG (middle), and Alkbh5-αKG/m6A (right). Cartoons are colored according to the β-factor.
and \( \alpha \)KG originate from a conformational change that involves \( \alpha \)KG signals (Fig. 3) caused within the jelly-roll motif by addition of \( \alpha \)KG and Alkbh5-

These conclusions support the hypothesis that the changes in \( \mu \)-ms dynamics (Fig. 4) and the appearance of two sets of 1 H-15 N TROSY

Overall, the data presented here indicate that addition of the primary and secondary substrate is coupled with small changes in the fold of the Alkbh5 backbone, mostly localized within flips 2 and 3. These conclusions support the hypothesis that the changes in \( \mu \)-ms dynamics (Fig. 4) and the appearance of two sets of \( ^1\)H-\( ^1\)N TROSY signals (Fig. 3) caused within the jelly-roll motif by addition of \( \alpha \)KG and m\( ^6 \)A originate from a conformational change that involves \( \alpha \)KG and the surrounding side chains.

**DISCUSSION**

Structural heterogeneity at the active site is emerging as a ubiquitous phenomenon governing enzymatic activity. Enzymes are dynamic nanomachines that can adopt many conformational states with different catalytic abilities (31–33). For several enzymes, modulation of structural disorder at the active site upon cofactor/substrate binding was shown to control the thermodynamic balance between active and inactive states and to provide an important source of functional regulation (31, 44, 45). In this work, we investigate the molecular mechanisms governing activation of the human RNA demethylase Alkbh5, an oncoprotein that is a promising target for the development of anticancer drugs, by solution NMR spectroscopy and MD simulations.

By using NMR relaxation experiments, we have shown that the active site of apo Alkbh5 is highly disordered and undergoes conformational dynamics on both the ps-ns and \( \mu \)-ms time scale (Fig. 5). We found that \( \alpha \)KG binding to the enzyme does not affect the ps-ns dynamics of the protein backbone (Fig. 5A) but results in quenching of the \( \mu \)-ms motions of Alkbh5 (Fig. 5C). In particular, the \( \mu \)-ms dynamics in the Ile\( ^{266} \)-Leu\( ^{282} \) fragment are completely suppressed by \( \alpha \)KG (Fig. 5E). As this segment establishes several contacts with the oligonucleotide in the Alkbh5-\( \alpha \)KG/m\( ^6 \)A complex (Fig. 5F), our data suggest that its structural stabilization in the \( \alpha \)KG-bound form promotes optimal docking of m\( ^6 \)A onto Alkbh5 and is responsible for orchestrating sequential binding of the secondary and primary substrate to the enzyme. Suppression \( \mu \)-ms dynamics upon ligand binding was also shown to promote formation of inter-domain interactions in bacterial Enzyme I (46), suggesting that ligand-induced suppression of conformational dynamics is a common mechanism that mediates signal transduction in biological systems.

Binding of the primary substrate to Alkbh5 completely abolishes \( \mu \)-ms time scale motions within the active site of the enzyme (Fig. 5, C and F). However, NMR analysis of the Alkbh5-\( \alpha \)KG/m\( ^6 \)A complex reveals that the \( \alpha \)KG binding site exists as two equally populated species that are in slow exchange on the NMR chemical shift time scale (\( \geq s \)) and result in two sets of \( ^1\)H-\( ^1\)N TROSY peaks (Fig. 3, E and F). Analysis of 1.2-\( \mu \)s MD simulations acquired on the Alkbh5-\( \alpha \)KG and Alkbh5-\( \alpha \)KG/m\( ^6 \)A complexes (Fig. 4) indicates that the two states detected by NMR correspond to the off-line and in-line geometries of \( \alpha \)KG (Fig. 1B). Oligonucleotides that do not contain an m\( ^6 \)A modification are not able to generate the in-line configuration of \( \alpha \)KG (Fig. 3E and Fig. S2) and are not demethylated by Alkbh5 (Fig. 7A), suggesting that the off-line to in-line structural transition might be required for activation of Alkbh5. Notably, the transition from the off-line to the in-line configuration of \( \alpha \)KG effectively increases the solvent-accessible surface area (SASA) of the metal center from 0.0 to 2.5 Å\(^2\) in the Alkbh5-\( \alpha \)KG complex and from 0.0 to 1.3 Å\(^2\) in the Alkbh5-\( \alpha \)KG/m\( ^6 \)A complex (Fig. 7B).

From these data, one can conclude that the off-line configuration is required to keep the metal center in the inactive +2 oxidation state.
when the primary substrate is not present in the active site. On the contrary, binding of m^6^A to Alkbh5 promotes transition of αKG to the in-line geometry, which exposes the metal, making it available to coordinate and activate O_2 for catalysis (Fig. 1A, path B).

The observation of two active site configurations with different catalytic abilities calls to mind previous works on the DNA polymerase in which transition to a protein conformation with altered essential motions was observed to trigger catalysis (47, 48). However, this does not seem to be the case for Alkbh5 as (i) principal components analysis of the MD simulations on the Alkbh5-αKG/m^6^A complex (fig. S8) does not highlight obvious changes in conformational dynamics at the active site upon transition of αKG to the in-line configuration, and (ii) the NMR signals assigned to Alkbh5 bound to in-line or off-line αKG display similar relaxation parameters.

Last, it is worth noting that our NMR study on the interaction between Alkbh5 and oligonucleotides revealed that while nucleic acids that do not contain the m^6^A modification are not able to stabilize the in-line αKG configuration and activate the enzyme for catalysis, they are still capable of establishing weak interactions with the nucleotide recognition loops (flips 1, 2, and 3) (Fig. 3E and fig. S2). These weak binding events might facilitate the search for the m^6^A modification site on long nucleic acids, in a way similar to the target search process, whereby transcription factors locate their specific DNA binding site (49, 50).

In conclusion, our experiments highlight the ability of solution NMR to detect and characterize subtle changes in structure and dynamics at enzyme active sites and suggest that binding of the primary substrate to Alkbh5 is required to promote transition of αKG to the in-line configuration, which, in turn, allows the metal cofactor to bind dioxygen and activate the enzyme for catalysis.

**MATERIALS AND METHODS**

**Protein expression and purification**

All experiments were performed using a truncated version of the human Alkbh5 comprising residues 66 to 292. This selected construct has been used in crystallographic studies and was shown to retain the enzymatic activity of the full-length protein (19, 38). Alkbh5 was expressed and purified as previously described (36). The M9 medium for the U-[^3^H]-[^15^N]-labeled sample was prepared in 99.9% H_2^2O using[^15^N]NH_4Cl as the sole nitrogen source. For U-[^3^H]-[^15^N]-[^13^C]-Alkbh5, the M9 medium was prepared in 99.9% H_2^2O using[^15^N]HCl and[^13^C]glucose as the sole nitrogen and carbon sources, respectively.

**Isothermal titration calorimetry**

ITC experiments on the Alkbh5-αKG interactions were acquired using a previously described protocol for AlkB dioxygenases (29). Briefly, 1 mM divalent cation (either Mn^{2+}, Zn^{2+}, Cd^{2+}, or Mg^{2+}) and 1 mM αKG were titrated into the sample cell containing 70 μM Alkbh5. Samples were prepared in 20 mM tris-HCl (pH 7.4) and 150 mM NaCl. All experiments were performed at 25°C and 500 rpm using MicroCal iTC200 (GE Healthcare). Analysis of the data was performed using the Origin software provided with the instrument.

**Pull-down assays**

Pull-down assays were performed in a total volume of 100 μl consisting of 20 μl of the NeutrAvidin Plus UltraLink Resin (Thermo Fisher Scientific), 5 μM biotinylated DNA (5′-biotin-TEG-TTTTTTGG(m^6^A)CT-3′ (GenScript), 25 μM Alkbh5, 500 μM αKG, and 500 μM X^2+ (where X = Mn^{2+}, Zn^{2+}, Cd^{2+}, or Mg^{2+}). The solution was rocked at 4°C for 30 min and spun at 13,000g for 30 s, and the supernatant was discarded. The beads were washed three times...
with 100 μl of H2O to remove any residual unbound protein and then resuspended in 20 μl of SDS loading buffer, boiled for 10 min, spun at 13,000g for 30 s, and run (10 μl of the supernatant) on a Bolt 4 to 12% Bis-Tris Plus polyacrylamide gel (Invitrogen).

Enzymatic assays

The demethylation activity of AlkBH5 toward methylated substrates was tested as previously described (36). Briefly, a reaction mixture containing 5 μM AlkB5 and 10 μM substrate was prepared in 2 mM l-ascorbic acid, 150 μM Fe²⁺, 300 μM αKG, and 50 mM tris-HCl (pH 7.4). The reaction mixtures were incubated at 37°C. Aliquots (50 μl) were taken at 0 and 60 min of reaction time and quenched with a 1:1 ratio of 20% (v/v) formic acid. The nucleic acids were separated from the protein by passing the quenched reaction through a 0.2-μm, 0.4-ml Ultrafree-MC Centrifugal Filter column (Millipore) by centrifugation (16,000g for 5 min). The pH of the flow through, containing a mixture of the methylated and demethylated substrate, was neutralized by addition of high-performance liquid chromatography (LC)–grade NH₄OH and analyzed by LC–mass spectrometry (MS).

NMR spectroscopy

NMR samples (−0.5 mM) of the apo Alkbh5 were prepared in 20 mM MES (pH 6.0), 150 mM NaCl, 1 mM EDTA, 2 mM dithiothreitol (DTT), 1 mM protease inhibitor (EDTA-free), 50 mM arginine + glutamic acid, 0.02% (w/v) Na₃N₅, and 90% H₂O/10% D₂O (v/v). To study Alkhb5 in the αKG-bound state, 500 μl of ~0.5 mM Alkhb5 was diluted into a 10-ml 20 mM MES (pH 6.0), 150 mM NaCl, 100 μM ZnCl₂, 1 mM αKG, 1 mM protease inhibitor (EDTA-free), 50 mM arginine + glutamic acid, 0.02% (w/v) Na₃N₅, and 90% H₂O/10% D₂O (v/v) and concentrated appropriately to reach a final protein concentration of ~0.5 mM. EDTA and DTT were removed from the buffer to eliminate any potential chelation of Zn²⁺. Saturation of the enzyme with Zn²⁺ and αKG is confirmed by ¹H-¹⁵N TROSY. To study the DNA-bound state, the appropriate oligonucleotide was titrated directly into the NMR tube containing Alkhb5, Zn²⁺, and αKG.

NMR spectra were acquired on Bruker 600-, 700-, and 800-MHz spectrometers equipped with Z-shielded gradient triple resonance cryoprobes. Spectra were processed using NMRPipe and analyzed with Xplor-NIH. 

MD simulations and structure modeling

The crystal structure of Alkhb5 in complex with Mn²⁺ and αKG (PDB code: 4NRO) was used as the starting structure for all simulations on the apo enzyme and on the Alkhb5–αKG complex with the substrate in the off-line configuration. Missing residues from the x-ray structure (fragments 145 to 149) were modeled using the software Modeller. The starting structures incorporating the in-line configuration were created by replacing the off-line αKG molecule in the crystal structure of the Alkhb5–αKG complex with the in-line αKG found in the structure of Alkhb2 bound to the primary and secondary substrate (PDB code: 3RZI). The starting structures for simulations on the αKG/m₆A-bound state were created by modeling the 5-mer DNA 5'-GGG(m₆A)CT-3' into the starting structures created for the Alkhb5–αKG with the secondary substrate in the off-line or in-line configuration. Modeling was performed by enforcing the oligonucleotide backbone and the m₆A base to adopt the structure and orientation within the active site observed in the crystallographic model of the Alkhb-αKG/m₆A complex (PDB code: 4NID). Each modeled structure was equilibrated by running 1-μs simulations with weak position restraints applied to retain the starting geometry of the active site (see below).

MD simulations were run using the Amber 16 package and the Amber ff14SB force field (54). αKG was parameterized with the AM1-BCC charge model (55) and the GAFF force field (56). The initial structure was centered in a truncated octahedron, filled with TIP3P water model and neutralizing ions, and the distance between the protein atoms and the boundaries was set to 10 Å. Energy minimization of the initial structures, including 1000 steps of steepest descent and 1000 steps of conjugate gradient, was performed in three stages. First, ions and water positions were relaxed. Then, the protein (or protein complex) was allowed to relax. Last, the full system was energy-minimized. The system was equilibrated with a 1-ns run in which the temperature was gradually raised from 0 to 310 K, followed by a 5-ns run in which the temperature was held constant at 310 K. For simulations on the Alkhb5 complexes with αKG and αKG/m₆A, Fe²⁺ was used as the divalent cation and an additional 1-μs equilibration step at 310 K was performed during which the geometry of the active site (defined by a bubble of 5 Å radius centered on the αKG center of mass) was restrained to its starting coordinates by a restraining weight of 2 kcal mol⁻¹ Å⁻². The restraining potential was kept during the first half and gradually

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relaxed during the second half of the 1-μs equilibration protocol. The equilibrated system was simulated for 1.2 μs by keeping the temperature (310 K) and pressure (1 atm) constant. Periodic boundary conditions were applied, and bonds were restrained with the SHAKE algorithm. An integration step of 2 fs was used. Weak coupling to an external pressure and temperature bath was used. Particle mesh Ewald summation with a cutoff of 10 Å for long-range interactions was used to treat electrostatic interactions.

The 6-ns restrained MD simulations were run in Amber 16 using similar parameters as the unrestrained simulations. The force constant for the RDC restraints was set to 0.1 kcal mol\(^{-1}\) Hz\(^{-2}\).

**Calculation of conformational ensembles**

Conformational ensembles for Alkbh5, Alkbh5-αKG, and Alkbh5-αKG/m6A were calculated by combining Gaussian aMD (GaMD) simulations and the NMR-derived \(1^D_NH\) RDC data (see the “NMR spectroscopy” section), as recently described (34). Briefly, back-calculated RDCs from conformational ensembles were done using the following equation

\[
RDC_i = \sum_k D_k \left[ (3 \cos^2 \theta - 1) + \frac{2}{3} (\sin^2 \theta \cos 2\phi) \right] \tag{2}
\]

where \(\theta\) is the angle formed between the internuclear bond vector of the amide group of residue \(i\) and the \(z\) axis of the alignment tensor, \(\phi\) is the angle between the \(xy\) plane projection of the internuclear bond vector and the \(x\) axis, and \(D_k\) is the magnitude of the alignment tensor for ensemble member \(k\) multiplied by its fractional population in the ensemble. \(D_k, \theta, \phi\) were optimized to reduce the discrepancy between experimental and back-calculated RDCs using the MATLAB script downloadable at http://group.chem.iastate.edu/Venditti/downloads.html.

The consistency between experimental and back-calculated RDC data was evaluated in terms of \(R\)-factor

\[
R - \text{factor} = \sum_i \sqrt{(RDC_{i}^{\text{exp}} - RDC_{i}^{\text{calc}})^2 / (2RDC_{i}^{\text{exp^2}})} \tag{3}
\]

where \(RDC_{i}^{\text{exp}}\) and \(RDC_{i}^{\text{calc}}\) are the experimental and back-calculated RDC for residue \(i\), respectively. The protocol was iterated by increasing the number of clusters (and therefore the representative structures in the pool) until a stable \(R\)-factor was obtained.

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