Structures of Human ALKBH5 Demethylase Reveal a Unique Binding Mode for Specific Single-stranded N6-Methyladenosine RNA Demethylation

Received for publication, January 15, 2014, and in revised form, April 24, 2014. Published, JBC Papers in Press, April 28, 2014, DOI 10.1074/jbc.M114.550350

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Background: ALKBH5 catalyzes demethylation of m6A single-stranded RNA (ssRNA).

Results: ALKBH5 structures reveal the structural basis of its substrate selectivity and inhibition by citrate.

Conclusion: ALKBH5 specifically binds to and demethylates m6A ssDNA/ssRNA. Citrate is a modest inhibitor of ALKBH5.

Significance: This study provides insights into the molecular mechanism of ALKBH5 as an m6A ssRNA demethylase and will facilitate the design of selective inhibitors.

N6-Methyladenosine (m6A) is the most prevalent internal RNA modification in eukaryotes. ALKBH5 belongs to the AlkB family of dioxygenases and has been shown to specifically demethylate m6A in single-stranded RNA. Here we report crystal structures of ALKBH5 in the presence of either its cofactors or the ALKBH5 inhibitor citrate. Catalytic assays demonstrate that the ALKBH5 catalytic domain can demethylate both single-stranded RNA and single-stranded DNA. We identify the TCA cycle intermediate citrate as a modest inhibitor of ALKBH5 (IC50 ~ 488 μM). The structural analysis reveals that a loop region of ALKBH5 is immobilized by a disulfide bond that apparently excludes the binding of dsDNA to ALKBH5. We identify the m6A binding pocket of ALKBH5 and the key residues involved in m6A recognition using mutagenesis and ITC binding experiments.

In higher eukaryotic organisms, more than 100 distinct modifications have been identified in cellular mRNA, tRNA, and rRNA (1, 2). Among these modifications, N6-methylated adenosine (m6A) is the most prevalent internal modification in mRNA; m6A has been identified in many eukaryotes and viruses (3, 4). There is increasing evidence that m6A is associated with mRNA metabolism by affecting, for example, the stability of nascent mRNA, the rate of transcription, and mRNA splicing (5). Based on a recently developed high throughput sequence analysis, m6A is not distributed randomly but is enriched near stop codons and in coding sequences (6).

Emerging studies on regulatory roles of mRNA modifications have identified m6A “writers” (m6A methyltransferases), m6A “erasers” (m6A demethylases), and m6A “readers” (m6A specific binding domains). METTL3 was the first identified functional m6A RNA methyltransferase and contains both a S-adenosylmethionine binding motif and the key catalytic residues for methylation (7). In 2011 and 2013, He and co-workers (8, 9) reported that FTO and ALKBH5 can act as specific mRNA m6A demethylases, suggesting that the m6A modification is dynamic in vivo. They also demonstrated that METTL14 displays m6A methylation activity and forms a stable complex with METTL3 to methylate mammalian nuclear RNA (10). Very recently, the YTH domain containing proteins YTHDF1–3 were shown to specifically recognize m6A containing single-stranded RNA (6, 11).

Both the FTO and ALKBH5 RNA demethylases belong to the AlkB subfamily of the Fe(II)/2-oxoglutarate (2OG) dioxygenase superfamily. Members from the 2OG dioxygenase superfamily act on diverse substrates involved in the regulation of protein biosynthesis. For example, some members of the Jumonji C (JmjC) subfamily are histone demethylases (12, 13), and the TET subfamily of dioxygenases catalyze 5-methylcytosine oxidation (14, 15). Although various 2OG oxygenases act on different substrates, they share a common distorted double-stranded β-helix (DSBH) fold and conserved, though not identical, 2OG and ferrous ion binding sites, suggesting a common evolutionary origin (16–19).

The human AlkB family comprises nine members: ALKBH1–8 and FTO; the family is named after their Escherichia coli ortholog, AlkB (20). Although the E. coli AlkB and...
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the human AlkB family members share the stereotypical 2OG oxygenase DSBH fold and have related 2OG and iron binding sites, their substrate selectivities differ. E. coli AlkB exhibits activity toward m1A and m3C containing DNA/RNA (21, 22); ALKBH1 and ALKBH3 strongly prefer m1A- or m3C-containing ssDNA/RNA (23–25); ALKBH2 preferentially demethylates m1A or m3C containing double-stranded DNA (dsDNA) (24, 25); ALKBH4 regulates the demethylation of actin (26); and ALKBH7 is involved with the programmed cell necrosis, although a nucleic acid demethylase activity for it has yet to be reported (27); ALKBH8 was demonstrated to hydroxylate 5-methoxycarbonylmethyluridine in tRNA (28); and FTO prefers both m2T ssDNA and m6A ssRNA (9, 29). ALKBH5 is reported to catalyze N-demethylation of m6A ssRNA or ssDNA, but not other tested modified nucleotides (8, 30).

Despite the important and diverse biological functions of the AlkB family, only a few human protein structures in the AlkB family have been characterized structurally, including ALKBH2 in complex with dsDNA (31), apo-ALKBH3 (32), ALKBH8 RRMA-AlkB double domain (33), and FTO in complex with 3meT (34) and in complex with various inhibitors (35). As the first identified eukaryotic RNA demethylase, FTO catalyzes demethylation of 3meU in ssRNAs and 3meT in ssDNAs (9, 29) and has been implicated in obesity (36, 37). ALKBH5 is expressed in different tissues compared with FTO (8) and apparently exhibits a stricter substrate preference by only catalyzing demethylation of m6A containing ssRNAs and favoring the sequence of (Pu[G>A] m6AC [A/C/U]) over random sequences (8). Although a preferred substrate of ALKBH5 has been identified, the molecular basis of its selectivity for m6A, as well as for its discrimination between ssRNA and dsRNA/dsDNA, has been unclear.

Here we present two crystal structures of the ALKBH5 catalytic domain: one in a 2OG-bound form and the other in an inhibitor-bound form. Our enzymatic assay results show that the ALKBH5 catalytic domain can demethylate m6A containing ssRNA and ssDNA and that citrate is a weak ALKBH5 inhibitor. The crystal structures provide insights into how ALKBH5 specifically acts on single-stranded RNA containing m6A. By molecular modeling of the ALKBH5 substrate complex structure, we identify potential m6A binding residues, the identities of which are validated by mutagenesis and ITC binding experiments. During revision of this manuscript, two other manuscripts on human ALKBH5 structures were reported (38, 39); their structural data and conclusions are consistent with our structural results, which are presented here.

**EXPERIMENTAL PROCEDURES**

Cloning, Expression, and Purification of Recombinant ALKBH5 Catalytic Domain—The human ALKBH5 catalytic AlkB domain (residues 74–294) was subcloned into pET28a-MHL vector and expressed in a method similar to previously described (40). The cloned vector was transformed into E. coli BL21-V2R-pRARE2. Recombinant protein was produced at 16 °C as an N-terminal His-tagged protein after induction using 0.8 mM isopropyl β-d-thiogalactopyranoside (final concentration) at an A600 of 1.0. The recombinant protein was purified by HiTrap nickel column, and the His tag was cleaved overnight by the pTEV protease. The cleaved protein was applied to a second HiTrap Ni column and further purified by Superdex 75 gel filtration (GE Healthcare) and ion exchange chromatography (HiTrapTM). The desired protein was pooled and concentrated to 25 mg/ml in a buffer containing 20 mM MES, pH 6.5, 150 mM NaCl.

### Table 1

**Data collection and refinement statistics**

The data were compiled using PDB_EXTRACT (57), PHENIX (58), and IOTBX (59) software. The highest resolution shell is shown in parentheses.

| Data | ALKBH5 (74–294) with 2OG and Mn2⁺ | ALKBH5 (74–294) with citrate |
|------|----------------------------------|-------------------------------|
| PDB code | 4OCT | 4O61 |
| Space group | P2₁ | P2₁ |
| Unit cell a, b, c (Å) | 46.93, 57.23, 78.41 | 50.47, 57.17, 78.64 |
| α, β, γ (°) | 90.00, 101.07, 90.00 | 90.00, 102.23, 90.00 |
| Resolution (outer shell, Å) | 38.47–2.28 (2.36–2.28) | 38.43–1.90 (1.94–1.90) |
| Unique observed HKLs | 18,808 (16,48) | 34,700 (22,37) |
| Completeness (%) | 99.9 (100.0) | 100.0 (100.0) |
| Friedel redundancy | 7.3 (7.1) | 7.2 (7.1) |
| R_sym | 0.127 (1.058) | 0.129 (0.983) |
| Mean (I/σ(I)) | 13.3 (2.1) | 13.7 (2.2) |
| Refinement data resolution | 30.00–2.28 | 30.00–1.90 |
| HKLs used/free | 17,856/934 | 32,850/1810 |
| Rcryst/Rfree (%) | 0.236/0.284 | 0.174/0.230 |
| Number of atoms/average B (Å²) | 3325/45.5 | 3763/24.0 |
| Protein | 3257/45.6 | 3355/23.3 |
| Inhibitor, cofactors, etc. | 22/46.1 | 26/41.2 |
| Root mean square deviation bonds (Å)/angles (°) | 0.015/1.4 | 0.016/1.5 |
| Residues inRamachandran plot of residues in favored region/allowed region, no outliers (%) | 96.8/3.2 | 96.9/3.1 |

* Data analysis with HKL-3000 suites.
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The ALKBH5 was crystallized with citrate in a buffer containing ammonium citrate, 20% PEG 3350. Before flash-freezing crystals in liquid nitrogen, crystals were soaked in a cryoprotectant consisting mother liquor plus 12% glycerol.

Structure Determination—Diffraction data were collected using an FR-E rotating copper anode with an R-axis-IV-HTC detector (FR-E, Rigaku Corp.) under cooling (Cryostream, Oxford Cryosystems) at 100 K. Diffraction images were reduced to intensities with XDS (41) and merged with AIMLESS (42). Merged intensities were converted to structure factor amplitudes with TRUNCATE (43). Free reflections were selected in thin resolution shells with the program SFTOOLS (B. Hazes). A crystal structure of the citrate complex was solved using the program PHASER (44) with a search model identified by the BALBES server (45) and based on coordinates from PDB entry 3H8R (46). Map improvement with ARP/WARP (47) and PARROT (48) was followed by iterations of automated model building in BUCCANEER (49) and further phase improvement with PARROT. Additional automated model building was performed with ARP/WARP (50). The structure of the cofactor complex was solved by direct placement of preliminary protein coordinates from the citrate complex structure into the nearly isomorphous unit cell of the cofactor complex, followed by rigid body refinement of the protein chains. Both complex atomic models were further refined using iterations of manual rebuilding with COOT (51), restrained refinement with REFMAC (52), and model geometry validation with MOLPROBITY (53). Electron density at the metal binding

3350. The ALKBH5 was crystallized with citrate in a buffer containing ammonium citrate, 20% PEG 3350. Before flash-freezing crystals in liquid nitrogen, crystals were soaked in a cryoprotectant consisting mother liquor plus 12% glycerol.

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A

\[
\begin{align*}
&\text{N6-methyladenine (m6A)} \\
\text{ALKBH5} \quad \text{Fe (II)} \\
&\begin{align*}
2\text{OG} \\
\text{O}_2 \\
\text{Succinate} \\
\text{CO}_2 \\
\text{Adenine (A)}
\end{align*}
\end{align*}
\]

B

C

D

\[
\begin{align*}
\text{WT} \\
\text{Kd} = 9 \text{ µM} \\
\text{R130A} \\
\text{Kd} = 15 \text{ µM} \\
\text{Y139A} \\
\text{Kd} = 14 \text{ µM}
\end{align*}
\]
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The asymmetric unit contains two ALKBH5 molecules with a backbone root mean square deviation of 0.1 Å. The dimer interhelix motif and pack against the long helix α2; together, these three helices buttress the seven-stranded β-sheet (Fig. 1B). On the basis of the search results from the Dali Server, the fold of ALKBH5 is most similar to those of ALKBH2, ALKBH3, and ALKBH8, with z scores of 18.3, 17.3, and 17.8, respectively. For the highest score hit, ALKBH2, the root mean square deviation between ALKBH5 and ALKBH2 is 2.5 Å, although the sequence identity between the catalytic domains of ALKBH5 and ALKBH2 is only 19%. Structure-based sequence alignment of ALKBH1–8, FTO, and E. coli AlkB suggests that the DSBH core fold (corresponding to β4–β11 of ALKBH5) is well conserved (Fig. 1A). ALKBH5 Binds to 2OG and Mn$^{2+}$—Like with other ALKBH proteins, ALKBH5 requires binding of the cosubstrate 2OG and ferrous iron for catalysis (Fig. 2A) (8). In our crystallization trial, we replaced the Fe(II) ion with Mn(II) to obtain a catalytically inactive form of ALKBH5 (Fig. 2B) (20). In the 2OG-Mn(II) complex ALKBH5 structure, the Mn(II) ion is complexed by NE2 of His-264, a carboxylate oxygen of Asp-206, NE2 of His-266, and the C1-carboxylate and C2-carbonyl groups of 2OG, respectively (Fig. 2C). Based on the sequence alignment of the ALKB family members, His-204, Asp-206, and His-266 are absolutely conserved across the family (Fig. 1A). Binding of 2OG is also apparently stabilized by electrostatic and hydrogen bonds with the ALKBH5 active site. One oxygen of the C1-carboxylate in 2OG is positioned to interact electrostatically with the side chain of Arg-283 in addition to chelating Mn, whereas the other oxygen of the 2OG C-1 carboxylate is recognized by the side chain of Asn-193 via electrostatic interaction. The 2OG C-5 carboxylate forms a salt bridge with Arg-277 and a hydrogen bond with the side chain of Tyr-195 (Fig. 2C). Both Arg-277 and Arg-283 are well conserved in the AlkB family (Fig. 1A). Hence, ALKBH5, like other AlkB family members, binds to 2OG and the metal ion in a conserved manner.

ALKBH5 Can Catalyze the Demethylation of m$^6$A ssRNA, as Well as ssDNA—To investigate whether the crystallized ALKBH5 catalytic domain is active, we performed catalytic assays; we found that in the presence of Fe(II) and 2OG, the ALKBH5 construct demethylates m$^6$A in both ssRNA and ssDNA (Fig. 3, A and B). In contrast, ALKBH5 was not observed...
FIGURE 3. Enzyme assays for the ALKBH5 catalytic domain wild type enzyme and variants. A, MALDI spectra showing the demethylation status (−14 Da) of 20 μM of 5-mer ssRNA (5′-GGm6ACU-3′) (1580 Da). From top to bottom, the panels show: without enzyme, in the presence of wild type ALKBH5, R130A, Y139A, and E153A, relative to wild type are shown. The activity of E153A is similar to that of wild type; whereas the R130A and Y139A variants are not active within the limit of detection (<5%). The error bars were derived from standard deviations of triplicate experiments (n = 3). B, ALKBH5 also catalyzes the ssDNA: dGdG(m6A)dCdT. The activity of ALKBH5 toward ssRNA was set to 100% for comparison. The activity of ALKBH5 toward ssDNA was 168 ± 39%. No activity was detected for ALKBH5 when Fe(II) was replaced by Mn(II). C, Kd values of ALKBH5 (wild type or variant) binding to modified ssDNA/ssRNA as determined by ITC.
to display any activity toward the same substrates when Fe(II) was replaced by Mn(II), consistent with a previous report (Fig. 3B) (20). ALKBH5 displayed 1.5-fold higher activity with modified ssDNA compared with modified ssRNA under our standard assay conditions (Fig. 3B). ITC binding assays of ALKBH5 to GG(m6A)CU RNA and dGdG(m6A)dCdT DNA indicated that the m6A ssDNA binds to ALKBH5 with similar strength to ssRNA (33 μM versus 37 μM, Fig. 3C). Taken together, the results reveal that the ALKBH5 catalytic domain (residues 74–294) is active and can demethylate ssDNA and ssRNA with similar activity; note, however, that m6A ssDNA may not be a physiologically relevant ALKBH5 substrate.

**Crystal Structure of ALKBH5 in Complex with the Inhibitor Citrate**—We also crystallized the ALKBH5 catalytic domain in a crystallization condition containing 0.2M ammonium citrate and determined the structure at a 1.9 Å resolution. Interestingly, a citrate molecule, instead of 2OG and Mn²⁺, was observed in the active site of ALKBH5, apparently competing out both cofactors under the crystallization conditions. The C5-carboxylate of the citrate molecule is positioned to hydrogen bond with the side chains of Lys-132 and Asn-193; the C-1 and C-6 carboxylates of the citrate are positioned to hydrogen bond with Ser-217 and Arg-283, respectively; and the citrate C-3 carbonyl oxygen is positioned to hydrogen bond with N2 of His-266 (Fig. 4A). Most of the residues involved in the citrate binding are involved in binding 2OG and Mn(II) as observed in the ALKBH5–2OG/Mn(II) structure (Fig. 2C).

Recently, Aik et al. (35) reported that citrate can act as a modest inhibitor of the human ALKBH enzyme FTO. In the FTO-citrate complex structure, the citrate molecule binds to FTO in a different manner, i.e. the citrate molecule replaces 2OG, but active site metal ion is still present (Fig. 4B).
formed the ITC experiments to measure the binding affinity of ALKBH5 to citrate ($K_d$, 50 μM), which was found to be ~5-fold weaker than that of the $\text{2OG/Mn}^2$ binding to ALKBH5 (9 μM) (Fig. 4C). The IC$_{50}$ of citrate for ALKBH5 was measured at 488 μM, which is comparable to that for human FTO (300 μM) under standard assay conditions (Fig. 4D) (35). Therefore, although citrate is observed to adopt different binding modes in the FTO and ALKBH5 crystal structures, it can act as an inhibitor for both human m$^6$A demethylases.

**Structural Elements of ALKBH5 Determining Its Specificity toward Single-stranded m$^6$A**—It has been reported that ALKBH5 specifically demethylates m$^6$A single-stranded RNA. In contrast, ALKBH2 is known to just modify double-stranded DNA. To investigate the substrate specificity of ALKBH5, we superimposed the catalytic domains of ALKBH5 and ALKBH2. Despite the structural similarities between the two 2OG oxygenases, significant differences exist in the corresponding nucleic acid binding regions of ALKBH2 and ALKBH5 (Fig. 5). First, the positively charged motif of ALKBH2, Arg-241 to Lys-243, is not conserved in ALKBH5 (Fig. 1A). The RKK motif of ALKBH2, located in a loop corresponding to the loop linking β10 and β11 (Fig. 1A), is critical in recognizing the backbone of the complementary DNA strand in the ALKBH2-dsDNA complex (Fig. 5B). Second, in ALKBH2 two short β strands of (β3-β4) contact the major groove of dsDNA, and Phe-102 in the loop between β3 and β4 points to the major groove and stacks with the A-T base pair; These structural features are missing in the corresponding region of ALKBH5 (Fig. 5C). Last but not least, a long loop connecting β7 and β8 in ALKBH2, which contacts the backbones of dsDNA, deviates from the corresponding loop (amino acids 229–243) of ALKBH5. The loop of ALKBH5 (amino acids 229–243) would cause a steric clash with the complementary strand nucleic acids. Furthermore, the conformation of the analogous loop in ALKBH5 is apparently “anchored” to β10 because of the presence of a disulfide bond between Cys-230 (loop) and Cys-267 (β10) (Fig. 5D). These two cysteine residues are well conserved across all ALKBH5 orthologs, but not in other members of the AlkB family (Figs. 5C and 5D).
A and 6). Collectively, these structural features differentiate ALKBH2 and ALKBH5 and rationalize the preference of ALKBH5 for single-stranded over double-stranded nucleic acids.

**Structural Modeling Indicates an m6A-specific Binding Pocket**—Three enzyme-substrate complex structures of AlkB family members have been solved, i.e. for the *E. coli* AlkB protein in complex with m1A ssDNA (20, 31), ALKBH2 in complex with m1A dsDNA (31), and FTO in complex with m3T (34). In all three structures, the substrates occupy a conserved binding pocket close to the cofactors and share some common features. The methyl groups of the modified bases are positioned close to the active site metal and 2OG C-1 carboxylate binding sites (and thus presumably the dioxygen binding site). The modified nucleobases are sandwiched between the rings of the first of the iron-binding histidines (corresponding to His-204 of ALKBH5) and one of the active site assigned “aromatic cage” residues (Trp, Phe, or Tyr, corresponding to Tyr-141 of ALKBH5). In addition, the modified bases are also specifically recognized by corresponding residues via hydrogen bonds; for example, N6 of m1A forms hydrogen bond with Asp-135 of AlkB, N6 of m1A forms two hydrogen bonds with Tyr-122 and Glu-175 of AlkB, and O2 of m3T forms two hydrogen bonds with Arg-96 of FTO (Fig. 7, A–C).

On the basis of these complex structures, we modeled an ALKBH5 substrate complex structure with the metal, 2OG, and m6A (prime substrate binding succeeds that of 2OG in the consensus mechanism for 2OG catalysis, with oxygen binding last). The model predicts that ALKBH5 can accommodate m6A in a manner similar to that in which other AlkB family proteins are observed to bind modified nucleic acids. Namely, m6A is positioned in a positively charged pocket formed by Arg-130, Tyr-139, Arg-277, and Arg-283 (Fig. 7, D and E). Specifically, the methyl group of m6A is positioned close to the C-1 carboxylate of 2OG, and the side chain of Tyr-139. In contrast to the three ALKBH/FTO substrate complexes mentioned above, m6A in the modeled ALKBH5 structure is not predicted to be sandwiched between two aromatic residues. Instead, Arg-130 may stack with the m6A base via cation-π interactions or interact with the RNA backbone via electrostatic interactions. Furthermore, m6A packs against the ring of His-204 (Fig. 7E). Of note, Arg-130 and Tyr-139 are conserved in ALKBH5 orthologs in other organisms (Fig. 6). In both ALKBH5 structures, we observed that the backbone of another active site aromatic residue, Tyr-141, is invisible in current substrate-free structures. However, we could not exclude the possibility that Tyr-141 may become ordered when binding to m6A, and it is possible that this aromatic residue may also stack with m6A.

To evaluate the roles of the conserved residues in the putative m6A binding pocket, we made three variants, R130A, Y139A, and E153A. Glu-153 is not predicted to be directly involved in m6A binding and was used as a control. The enzyme assay results showed that neither R130A nor Y139A displays detectable activity toward m6A RNA. In contrast, E153A displays activity similar to wild type ALKBH5 (Fig. 3A). In support of the enzyme assay results, ITC binding results reveal that neither R130A nor Y139A binds to the 5-mer m6A RNA within the limits of detections (Fig. 3C). On the other hand, we found that both these variants bind to 2OG and Mn(II), albeit a little more weakly than does the wild type ALKBH5 (2-fold; Fig. 2D). Hence, both enzymatic assay and the ITC binding data broadly support the modeled structure and proposed m6A pocket.

**DISCUSSION**

Two recently identified m6A RNA demethylases, FTO and ALKBH5, dynamically regulate the profile of m6A *in vivo* (8, 34). Our results define a crystal structure of human ALKBH5 in

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**FIGURE 6. Sequence alignment of ALKBH5 orthologs.** Hs, *Homo sapiens*; Ga, *Gallus gallus*; Xs, *Xenopus*; Dr, *Danio rerio*; Hv, *Hydra vulgaris*. Metal binding residues are in green. The two cysteines that form the conserved disulfide bond are in orange. Arg-130, Tyr-139, Arg-277, and Arg-283 are marked.
the presence of 2OG and Mn$^{2+}$. ALKBH5 adopts an extended DSBH fold similar to other AlkB family members; cofactor/cosubstrate binding residues are conserved not only in its orthologs, but also in all other AlkB family members (Figs. 1 and 6). Additionally, we were able to crystallize ALKBH5 in complex with a modest inhibitor citrate. Surprisingly, the citrate binds to ALKBH5 in a different manner from that observed in FTO. In the FTO-citrate complex, the citrate replaces 2OG,
and the metal ion is still intact. In the ALKBH5-citrate complex, the citrate excludes both the metal ion and the 2OG cosubstrate (Fig. 4, A and B). The different modes of inhibition caused by a TCA cycle intermediate, such as citrate, may be pathophysio-
logically relevant because inhibition of some 2OG oxygenases as caused by up-regulation of TCA cycle is proposed to be pro-
carcinogenic (54, 55).

Although citrate is only a modest inhibitor of the ALKBH family dioxygenases, the different binding modes of citrate to FTO and ALKBH5 may be taken advantage of in the design of new types of target-specific inhibitor or chemical probes for the Fe(II)/2OG oxygenases. Although inhibition via competition with Fe(II) can be achieved of a noncatalytically active metal, e.g. inhibition of the hypoxic inducible factor 2OG-dependent prolyl hydroxylases can be achieved in isolated protein form and in vivo by Co(II) ions (including to stimulate erythropoiesis in animals) (56), such metal competition is not specific. Thus, organic compounds that compete with Fe(II) binding to 2OG oxygenase active sites could constitute a valuable new class of 2OG oxygenase inhibitors.

Although all identified AlkB family members have similar folds, they have different substrates and functions. E. coli AlkB, ALKBH1, and ALKBH3 prefer single-stranded nucleic acids, and ALKBH2 acts on double-stranded DNAs (21–25). ALKBH5 exhibits a strict preference for mA ssRNA (8, 30), whereas FTO can demethylate mA ssRNA, as well as m3T ssDNA (9, 29). To understand why ALKBH5 acts on single-stranded, but not double-stranded nucleic acids, we compared the structure of ALKBH5 with that of ALKBH2-dsDNA and found that a rigid loop in ALKBH5 will cause a steric clash with dsDNA. FTO also prefers m3T or mA in single-stranded nucleic acids and also utilizes a loop to discriminate against binding of dsDNA.

Although ALKBH5 and FTO both contain specific loops that apparently discriminate single-stranded from double-stranded nucleic acids, the underlying mechanisms differ. FTO contains a longer loop between its β5 and β6 strands, which corresponds to the loop between β4 and β5 of ALKBH5. The longer loop of FTO could cause a steric clash with the complementary strand of the nucleic acids (Fig. 1A) (34). ALKBH5 likely utilizes a different structural feature to exclude the binding of dsDNA. The loop between β7 and β8 of ALKBH5 adopts a different conformation from that observed in ALKBH2, because it is linked to the β10 strand via a disulphide bond formed between Cys-230 and Cys-267 (Fig. 5D). Hence the β7-β8 linking loop is rigidified and is proposed not to tolerate the conformational changes required to accommodate dsDNA. Furthermore, two regions of ALKBH2 that contact the backbone of the complementary strand of the dsDNA and a base pair of the dsDNA, respectively, are missing in ALKBH5 (Fig. 5, A and B). Collectively, these structural differences rationalize the preference of ALKBH5 for single-stranded over double-stranded nucleic acids.

To identify key residues of ALKBH5 involved in mA recognition (30), we modeled the mA into the ALKBH5-2OG-Mn(II) complex structure based on a few ALKBH-substrate complex structures. Analogous to the structures of E. coli AlkB, ALKBH2, and FTO complexes, mA is also predicted to occupy a pocket close to 2OG and Mn(II) in the modeled structure. In ALKBH5, the mA base is predicted to pack against His-204 and is in a pocket composed of Arg-130 and Tyr-139. The combined substitution and enzymatic assay results support the essential roles of Arg-130 and Tyr-139 in substrate recognition. Although the two variants can still bind to 2OG and meta, neither of them binds to the mA RNA substrate, nor do they display activity toward the mA RNA substrate. Although further investigations on the structure of ALKBH5 complexes are required, our biochemical and structural data not only provide insights into understanding the molecular mechanism of ssRNA demethylation by ALKBH5 but also identify citrate as a modest inhibitor for ALKBH5.

Acknowledgments—We thank Aiping Dong for reviewing the citrate complex crystal structure. We thank Michael A. McDonough for advice and helpful discussions.

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