Atomic mutagenesis of N^6-methyladenosine reveals distinct recognition modes by human m⁶A reader and eraser proteins

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ABSTRACT: N^6-methyladenosine (m⁶A) is an important modified nucleoside in cellular RNA associated with multiple cellular processes and implicated in diseases. The enzymes associated with the dynamic installation and removal of m⁶A are heavily investigated targets for drug research, which requires detailed knowledge of the recognition modes of m⁶A by proteins. Here we use atomic mutagenesis of m⁶A to systematically investigate the mechanisms of the two human m⁶A demethylase enzymes FTO and ALKBH5 and the binding modes of YTH reader proteins YTHDF2/DC1/DC2. Atomic mutagenesis refers to atom-specific changes that are introduced by chemical synthesis, such as replacement of nitrogen by carbon atoms. Synthetic RNA oligonucleotides containing site-specifically incorporated 1-deaza-, 3-deaza-, and 7-deaza-m⁶A nucleosides were prepared by solid-phase synthesis and their RNA binding and demethylation by recombinant proteins were evaluated. We found distinct differences in substrate recognition and transformation and revealed structural preferences for enzymatic activity. The deaza m⁶A analogues introduced in this work will be useful probes for other proteins in m⁶A research.

Introduction

N^6-methyladenosine (m⁶A) is an important modified nucleoside found at internal positions in various types of RNAs, predominantly in eukaryotic mRNAs and also in non-coding RNAs.[1-6] The methyl group is enzymatically installed by S-adenosylmethionine (SAM)-dependent methyltransferases, such as the multi-component METTL3/14 m⁶A writer complex, which targets adenosines in a subset of consensus DRACH (D=A/G/U, R=A/G, H=A/C/U) sequence motifs at certain locations within RNA polymerase II transcripts.[7-8] Additionally, more structure- or sequence-specific m⁶A writers have been characterized, including ZCCHC4,[9] METTL16,[10-11] METTL5,[12] PCIF1/CAPAM.[13] The methyl group attached to the exocyclic amino group at position 6 of adenosine can be located in *syn* or *anti* orientation with respect to the N1 in the purine ring (Figure 1A). While the *syn* orientation is energetically preferred in the free nucleosides and in single-stranded RNA, formation of a standard Watson-Crick base pair requires the methyl group to rotate into the *anti* conformation.[14-16] Thus, m⁶A influences local structures and base-pairing kinetics, which in turn affect global RNA folding, structures and associated functions. Multiple studies have shown that m⁶A modulates the fate of mRNAs by influencing their stability, translation efficiency, and splicing through direct or indirect interaction with proteins.[2-3, 17-18] A group of direct m⁶A reader proteins each contain the YTH domain, which forms a hydrophobic binding pocket for m⁶A. Well-established human m⁶A readers include the proteins YTHDF1/DF2/DF3 and YTHDC1/DC2.[18] Although these YTH-domain containing proteins seem to bind m⁶A-containing RNA via similar mechanisms, they cause different downstream effects.[19-20] Interestingly, m⁶A can be reverted to adenosine through oxidative demethylation by the human m⁶A demethylase enzymes FTO and ALKBH5.[4] Both enzymes belong to the family of non-heme iron(II)- and α-ketoglutarate-dependent dioxygenases but remove the N^6-methyl group via distinct reaction pathways: while demethylation by ALKBH5 directly yields adenosine accompanied by release of formaldehyde,[21-22] the oxidized intermediates N^6-hydroxymethyladenosine (hm⁶A) and N^6-formyladenosine (f⁶A) have been observed in the FTO-mediated pathway (Figure 1A).[23-24] While ALKBH5 has been observed to remove only internal m⁶A, preferably in DRACH motifs, FTO has also been shown to act on m⁶Am, m¹A, m³U and m³T.[25-28] The dysregulation of m⁶A and associated writer, reader and eraser proteins has been connected to tumor development and disease. Therefore, m⁶A-modifying proteins have gained increasing attention as targets for drug development.[29] The design of chemical inhibitors requires knowledge of specific interactions between RNA and proteins. Such insights can be derived from crystal structures in combination with molecular dynamics simulations,[29-30] and are supported by biochemical data obtained with mutated proteins, in which key functional amino acids in the active site are exchanged, e.g. by replacement with alanine. Introducing smaller changes by exchanging single functional groups via incorporation of artificial amino acids is more challenging (e.g. via stop-codon suppression). On the other hand, single atom exchanges on the nucleoside level can be introduced into synthetic oligonucleotides by solid-phase synthesis. Atomic mutagenesis of nucleosides is a very powerful strategy for biochemical investigations and

has been successfully used for the detailed analyses of ribozyme mechanisms,[31-34] including the ribosome,[35-36] but has barely been used for modified nucleosides or the analysis of RNA-protein interactions.

Here we introduce atomic mutagenesis of m⁶A to analyze the contributions of hydrogen bonding and electrostatic interactions between m⁶A-modified RNA and reader as well as eraser proteins. We synthesized a series of RNA oligonucleotides containing atomic mutants of m⁶A to examine RNA binding, enzyme activity and product distribution. The endocyclic nitrogen atoms at position 1, 3 and 7 of the purine ring were individually replaced by a CH group, yielding 1-deaza, 3-deaza and 7-deaza-N^6-methyladenosine, respectively (Figure 1B). This study revealed insights into the sensitivity of reader and eraser proteins to atomic mutagenesis of their substrates. Interestingly, we found distinct differences between the dioxygenases FTO and ALKBH5, and also among individual, yet apparently similar, YTH domains. Our results also suggest c⁷m⁶A as a structurally neutral probe for m⁶A readers that is more resistant to demethylation than native m⁶A.
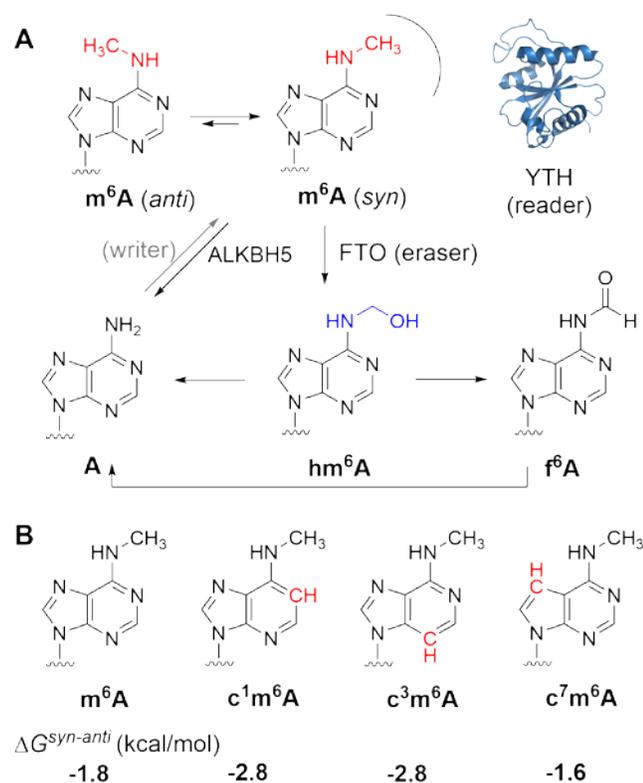
Figure 1. A) N^6-methyladenosine (m⁶A) in Watson-Crick-competent *anti* orientation and protein-binding-competent *syn* conformation is installed by writer enzymes; m⁶A is removed by eraser enzymes (ALKBH5, FTO), and interacts with reader proteins (containing a YTH domain). B) m⁶A and the three deaza m⁶A mutants used in this study. The predicted free energy preference for the *syn* conformation is indicated.

Results and Discussion

Synthesis and Characterization. The m⁶A nucleoside (**1a**) was obtained by nucleophilic substitution of 6-chloropurine riboside with methylamine as previously described.[37-38] Similarly, three deaza variants of m⁶A, c¹m⁶A (**1b**), c³m⁶A

(**1c**) and c⁷m⁶A (**1d**) were prepared from suitable 6-chloro-substituted nucleoside analogs by substitution with methylamine. The required precursors were obtained by ribosylation of the respective nucleobase analogs under Silyl-Hilbert-Johnson conditions. The 1-deaza and 3-deaza purine analogs were obtained from 2-amino-4-chloropyridine (for **1b**) or 4-amino-2-chloropyridine (for **1c**), while 6-chloro-7-iodo-7-deazapurine was used for **1d** (Figure 2). The non-methylated nucleosides (c¹A, c³A, c⁷A) were also prepared; details of the synthetic steps are given in the Supporting Information. The choice of the respective 2'-silyl protecting group was inspired by previous work on deazaadenosines.[39-40] The m⁶A nucleosides **1a-d** exhibit distinctly redshifted UV absorption spectra (Figure S1, Table S1) compared to the respective unmethylated (deaza)adenosines. Moreover, the 1-deaza and 3-deaza analogues exhibit methylation-dependent changes in fluorescence emission intensity (Figure S2). DFT-based geometry optimization predicts the *syn* orientation of the methyl group as the energetically preferred conformation (Figure 1B, Figure S3), consistent with previously reported experimental data for m⁶A.[41]
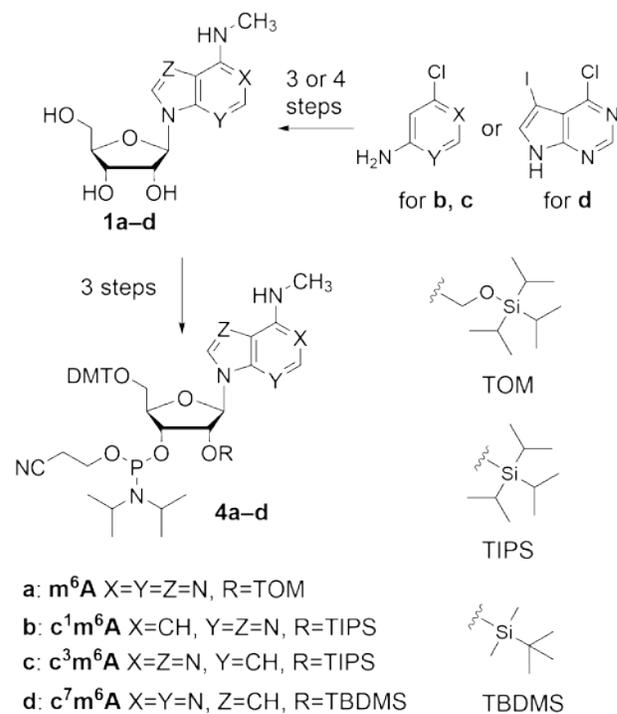
Figure 2. Synthesis of nucleosides and phosphoramidite building blocks for incorporation of m⁶A and its deaza mutants. Details of synthetic steps are in the Supporting Information. DMT = 4,4'-dimethoxytrityl, TOM = triisopropylsilyloxymethyl, TIPS = triisopropylsilyl, TBDMS = t-butyldimethylsilyl.

The free nucleosides **1a-d** were converted to the respective phosphoramidite building blocks **4a-d** in three steps, involving installation of 5'-DMT and 2'-silyl protecting groups, followed by 3' phosphitylation (Figure 2). These building blocks were used to incorporate the m⁶A variants into 12-mer oligonucleotides by solid phase synthesis using standard coupling and deprotection protocols. Each m⁶A analogue was installed in place of **X** in the sequence Hex-AACC**GGXCU**GUC (**R1**: X=m⁶A, **R2**: X=c¹m⁶A, **R3**: X=c³m⁶A,

R4: X=c^{7}m^{6}A, **R5**: X=A), thereby placing the m^{6}A mutants inside a DR**A**CH motif (underlined); RNAs were analyzed by HPLC and ESI-MS (Figure S4, Table S2); Hex denotes a hexynyl linker attached at the 5'-end for fluorescent labeling of the RNA using copper(I)-catalyzed azide-alkyne cycloaddition with fluorescein azide.

Table 1. Thermal melting of 12-mer RNA duplexes containing N^6-methylated adenosine derivatives and pK_a of free nucleosides.

RNA	A	T_m [°C]^a	ΔT_m [°C]^b	$\Delta G°$ [kcal/ mol]	pK_a^{c}
R1	m^{6}A	63.4	-2.8/0	-15.8	4.0 [42-43]
R2	c^{1}m^{6}A	53.9	-12.3/-9.5	-12.9	4.9 (4.7[44])
R3	c^{3}m^{6}A	58.7	-7.5/-4.7	-14.2	7.1 (6.8[44])
R4	c^{7}m^{6}A	62.9	-3.3/-0.5	-15.5	5.3 (5.2[44])
R5	A	66.2	0/+2.8	-16.2	3.6 [42, 45]

^{a} Determined by absorbance at 260 nm, for 5 µM RNA duplex with complementary **R6** 5'GACAGUCCGGUU3'; (error ±0.5 °C). ^{b} Difference relative to the unmodified duplex/m^{6}A-modified duplex, respectively. ^{c}pK_a of free nucleoside (unmethylated deazaadenosine in parentheses).
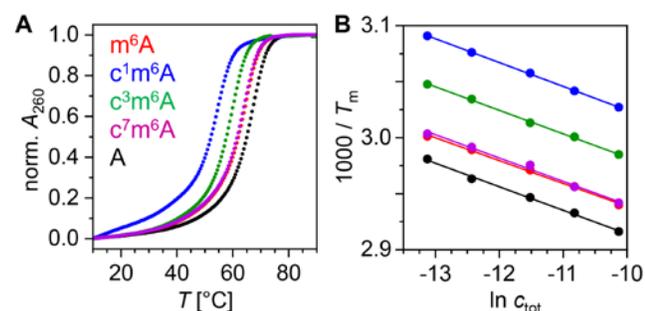
Figure 3. A) Melting curves (at 5 µM) and B) van't Hoff plots for RNA duplexes R1/R6 (m^{6}A), R2/R6 (c^{1}m^{6}A), R3/R6 (c^{3}m^{6}A), R4/R6 (c^{7}m^{6}A), R5/R6 (unmodified A), (1, 2, 5, 10, 20 µM duplex), measured at 260 nm in 100 mM NaCl, 10 mM Na-phosphate buffer pH 7.0.

The base pairing properties of m^{6}A and its deaza analogs in RNA duplexes was analyzed by thermal melting and van't Hoff analysis. The 12-mer RNAs **R1-R5** were hybridized to the complementary RNA **R6** and thermal denaturation was monitored by UV absorbance at 260 nm (Figure 3, Table S3, Figure S5). Consistent with previous reports, m^{6}A slightly destabilized the duplex by less than one kcal/mol.[15-16] Replacing the N1 atom by carbon impaired Watson-Crick base pairing and resulted in a large drop of the T_m by 9.5 °C compared to m^{6}A. Replacing the N3 atom in the minor groove resulted in a reduced T_m by 4.7 °C, while removing the N7 atom in the major groove had a negligible effect of only 0.5 °C. These melting data are consistent with the effects of deaza adenosines on RNA base pairing, where the destabilizing effect of c^{3}A was attributed to its shifted pK_a value and an altered hydration pattern in the minor groove.[45-46] To evaluate the influence of the methyl group on N^6, we

determined the pK_a of c^{3}m^{6}A (**1c**) and found it similarly shifted to neutrality (Figure S6, Table 1, Table S4). Thus, N1 of c^{3}m^{6}A is partially protonated at pH 7, reducing its Watson-Crick base pairing ability and resulting in the reduced T_m of the **R3/R6** duplex. The higher predicted energetic preference of the *syn* conformation of c^{1}m^{6}A and c^{3}m^{6}A compared to m^{6}A and c^{7}m^{6}A may also contribute to the lower T_m of **R2/R6** and **R3/R6** duplexes.

Demethylation of m^{6}A and its atomic mutants by FTO and ALKBH5
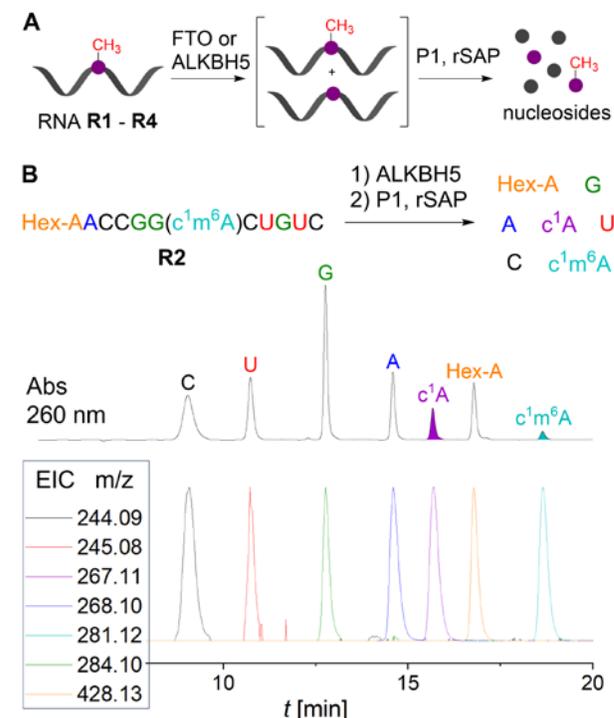
Figure 4. A) Schematic illustration of m^{6}A demethylation assay. B) Exemplary data for R2 treated with ALKBH5 for 2 min. The signals in the UV absorbance trace are assigned to the nucleoside species based on the respective extracted ion chromatograms (EIC, m/z ±0.01). The extent of demethylation is calculated based on the respective integrated peak area (cyan and purple shade), using ε^{260} of the free nucleosides.

Next, we used the oligonucleotides **R1–R4** to investigate the influence of m^{6}A atomic mutagenesis on the enzymatic activity of the human m^{6}A demethylases FTO and ALKBH5. **R1–R4** were incubated with recombinantly expressed and purified enzymes (see supporting information for details) in a buffer containing Fe(II) and α-ketoglutarate (α-KG) at 25 °C.[47] After digestion using Nuclease P1 and recombinant Shrimp Alkaline Phosphatase (rSAP), the obtained nucleoside mixtures were subsequently analyzed by HPLC-MS (Figure 4). The nucleosides were identified based on the ESI mass spectra and quantified based on the UV absorption at 260 nm, using the experimentally determined extinction coefficients of the respective nucleosides.
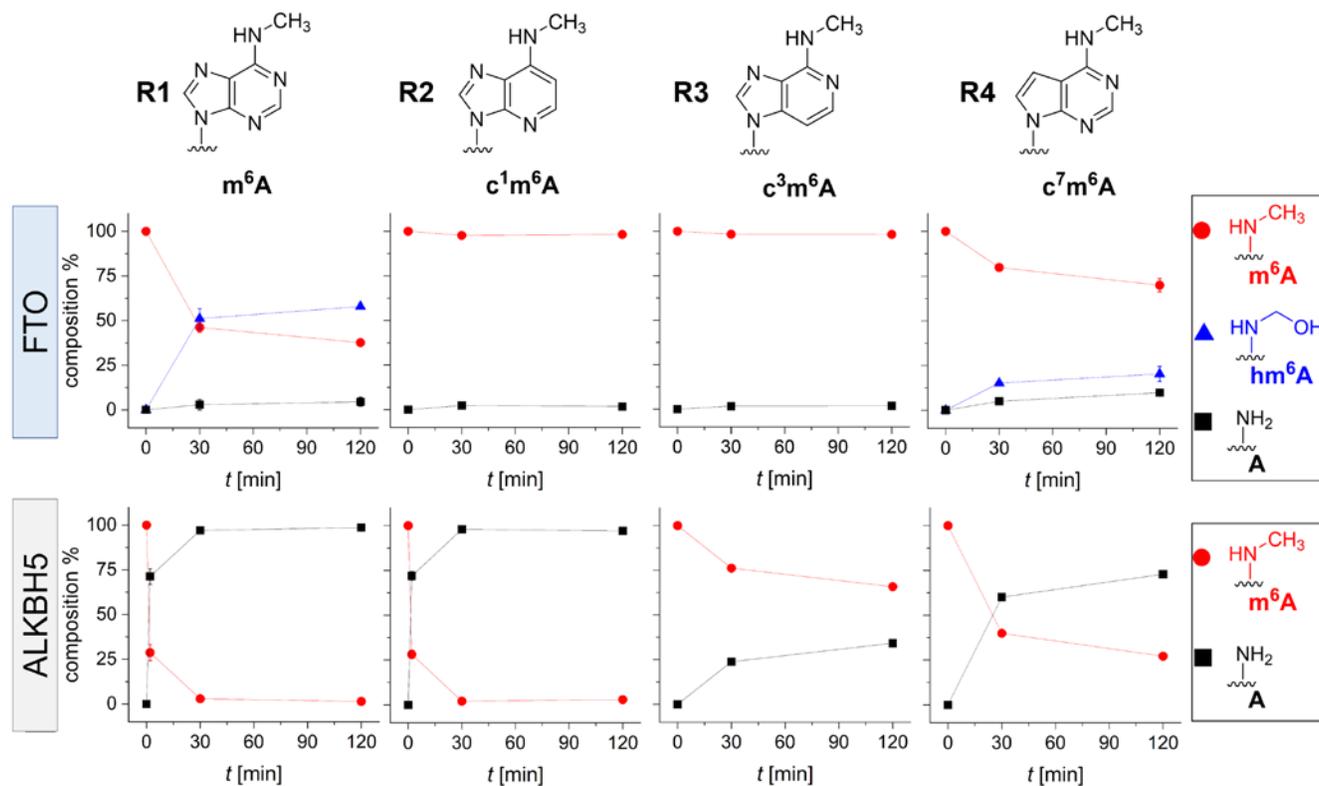
Figure 5. Product distribution obtained from oxidative demethylation of m6A analogs with FTO or with ALKBH5. All data points were collected as duplicates and are represented as mean ± s.e.m.

Details of the analysis are described in the Supplementary Information. The product distribution was analyzed at various time points, and the data are presented in Figure 5. Raw data for two independent replicates are shown in Figures S7-S10 and Tables S5-S8.

Treatment of **R1** with FTO or with ALKBH5 resulted in removal of the methyl group from m6A, as expected.[4,21-24] While FTO converted m6A to the oxidized intermediate hm6A and unmethylated A (58% and 4.5%, respectively, after 2 h), hm6A was not detected in the reaction with ALKBH5, which resulted in 71% demethylated product already after 2 min and nearly quantitative conversion after 30 min. These results are consistent with a previous report showing higher *in vitro* activity of ALKBH5 than FTO.[21]

Interestingly, ALKBH5 and FTO showed remarkably different results for demethylation of the atomic mutants. FTO did not tolerate the removal of either N1 or N3. The enzyme was essentially inactive on **R2** (c1m6A) and **R3** (c3m6A). Introduction of c7m6A into the oligonucleotide (**R4**) also decreased the demethylation efficiency of FTO (50% less conversion after 2 h compared to m6A); besides formation of unmethylated c7A (9.8% after 2 h), accumulation of the oxidized intermediate c7hm6A (20.3%) was observed.

ALKBH5 displayed markedly higher tolerance toward atomic mutagenesis of the endocyclic nitrogen atoms than FTO. Most notably, **R2** (c1m6A) was demethylated just as efficiently (ca. 72% demethylation after 2 min) as the native m6A substrate **R1**. This presents a major difference compared to FTO, which showed no activity on the c1m6A

substrate. RNAs containing c3m6A (**R3**) and c7m6A (**R4**) substrates were also accepted by ALKBH5, although demethylation occurred less efficiently (34% and 73% demethylation after 2 h, respectively), and oxidized intermediates were also not observed.

A previous study by Toh *et al.* traced the lack of oxidized m6A intermediates during ALKBH5-mediated demethylation back to a covalent intermediate in the enzymatic demethylation mechanism.[21] The authors visualized the covalent intermediate as a supershifted band in an electrophoretic mobility shift assay. We reproduced this result and found an analogous covalent ALKBH5-RNA adduct in the case of c7m6A demethylation (Figure S11). Of note, stabilization of the covalent adduct is achieved by pre-oxidation of the methylated RNA substrate by FTO (see Supplementary Information for experimental details). As c1m6A and c3m6A are not substrates of FTO, stabilization of **R2**-ALKBH5 and **R3**-ALKBH5 covalent adducts was not possible.

To examine if m6A atomic mutagenesis impairs recognition (binding) of the substrate oligonucleotides rather than enzymatic activity, we performed a competitive demethylation assay in which FTO or ALKBH5 were challenged with either only **R1**, or with a mixture of **R1** with either **R2**, **R3** or **R4**. In this experiment, m6A demethylation would be reduced compared to the **R1**-only sample if the present competitor RNA is bound by the demethylase. Indeed, for both enzymes we observed reduced m6A demethylation in the presence of **R2**–**R4** (Figures S12,13, Table S9), in each case to a similar extent. However, addition of **R5** had a

comparable effect, suggesting that FTO and ALKBH5 bind DRACH motifs regardless of the methylation status or the absence or presence of N1/N3/N7. Thus, these qualitative data support the interpretation of the demethylation results in Figure 5 as effects on catalysis rather than binding.

Overall, our systematic demethylation study provides comprehensive information about the molecular interactions responsible for accommodation of the m6A substrate inside the catalytic centers of FTO and ALKBH5. These data are largely consistent with available crystal structures (Figure 6).[22, 48] Hydrogen-bond interactions of N1 and N^6 of m6A with charged amino acid residues in the FTO catalytic site (R96 and E234) are crucial for the alignment of the N^6-methyl group with the catalytic Fe(IV)-oxo center. Disruption of these interactions, as for the 1-deaza substrate, results in misorientation of the nucleobase and thus loss of catalytic activity. N7, in contrast, is not involved in stabilizing interactions and is therefore not crucial for m6A demethylation. Interestingly, the crystal structure of the FTO-m6A complex[48] does not indicate substrate-enzyme interactions involving the N3 of m6A, while atomic mutagenesis of this position caused a complete loss of enzymatic activity. We rationalize this observation with the protonation state of c3m6A due to its elevated pK_a of 7.1, suggesting that electrostatic repulsion between a protonated N1 and the cationic side chain of R96 leads to misalignment of the c3m6A nucleobase in the catalytic site. Inside the ALKBH5 active site,[22] by contrast, N1 of the m6A substrate is not involved in electrostatic interactions; hence, the enzyme tolerates c1m6A as a substrate, and also maintains activity for demethylation of c3m6A . In addition, hydrogen bonding between N7 and the K132 side chain appears to contribute to correct substrate alignment and supports demethylation.
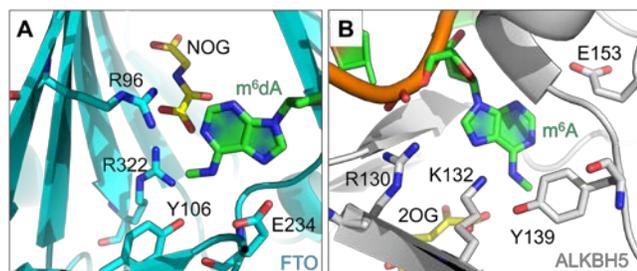
Figure 6. View into the active site of A) FTO (pdb 5zmd) and B) ALKBH5 (pdb 7wkv).

Binding of m6A mutants by YTH domain reader proteins

Next, we investigated recognition of the m6A atomic mutants by human YTH domain-containing m6A reader proteins. We used recombinantly expressed and purified YTHDF2[380-579], YTHDC1[344-509] and YTHDC2[1277-1430] (in the following referred to as YTHDF2, YTHDC1 and YTHDC2) and the fluorescently labeled substrate oligonucleotides **R1-FAM – R5-FAM** to determine the protein–RNA dissociation constants (K_D) via fluorescence anisotropy (Figure 7, Table 2).

In agreement with previous reports, all three reader proteins bound the native m6A substrate **R1** with affinities in the micromolar to sub-micromolar range, and strongly preferred the methylated RNA **R1** over the unmethylated RNA

R5.[49-50] Introduction of the c1m6A mutation resulted in reduced binding affinities of **R2** by at least 50-fold compared to **R1** for YTHDF2 and YTHDC1; for the weakest m6A binder YTHDC2, c1m6A led to complete loss of binding. Interestingly, the c3m6A substrate was well tolerated by YTHDF2 (K_D = 0.10 μM, only 3-fold increase compared to m6A), whereas YTHDC1 and YTHDC2 did not bind **R3**. Moreover, all three YTH domain readers displayed high tolerance toward atomic mutagenesis of N7: **R4** with c7m6A showed only ca. 3-fold increased K_D values for each of the three proteins.
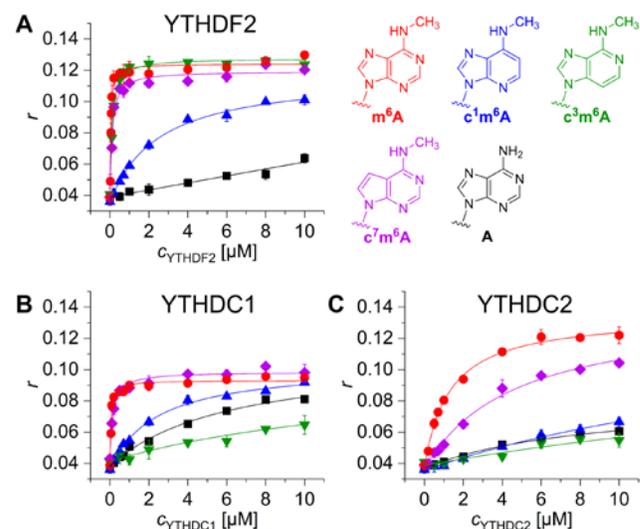
Figure 7. Binding isotherms of the (deaza)-m6A RNAs by reader proteins YTHDF2, YTHDC1 and YTHDC2, as determined by fluorescence anisotropy measurements via titration of 5'-FAM-labeled RNAs **R1**-**R5** (20 nM) with increasing concentrations of protein. All data points were collected as triplicates and are presented as mean ± s.e.m.

Table 2: Dissociation constants of YTH protein-RNA complexes determined by fluorescence anisotropy.

RNA		K_D [μM] YTHDF2	K_D [μM] YTHDC1	K_D [μM] YTHDC2
R1	m6A	0.035 ± 0.007	0.05 ± 0.01	1.3 ± 0.2
R2	c1m6A	2.5 ± 0.3	2.4 ± 0.3	n. d.
R3	c3m6A	0.10 ± 0.02	n. d.	n. d.
R4	c7m6A	0.10 ± 0.02	0.15 ± 0.03	4.6 ± 1.0
R5	A	n. d.	6.2 ± 1.5	n. d.

K_D values are given as mean ± s.e.m. of triplicates. In case of negligible binding at 10 μM protein, K_D values were not determined (n. d.)
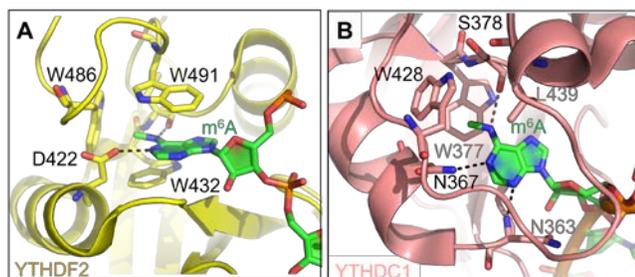
Figure 8. View into the binding site of A) YTHDF2 (pdb 7z26) and B) YTHDC1 (pdb 4r31).

These experimental observations correlate with previously reported crystal structures of m^6A bound to YTHDF2 and YTHDC1 (Figure 8).[30, 50] These structures show the m^6A nucleobase located in an aromatic cage, which is stabilized by π-π-stacking with tryptophan side chains located parallel to the purine heterocycle (W432 and W491 in DF2, and W377 in DC1), and a methyl-π interaction with a tryptophan side chain that is located orthogonal to the N^6-methyl group (W486 in DF2 and W428 in DC1). The RNA-protein complexes are further stabilized by hydrogen bonding interactions of the N1 nitrogen of m^6A with a carboxyl group (D422) in YTHDF2, or with an amide (N367) in YTHDC1. The loss of these hydrogen bonds by using c^1m^6A is likely responsible for the drastically reduced affinity of **R2**. In case of the c^3m^6A mutant in **R3**, the elevated pK_a value and the concomitant partial N1 protonation likely prevents a stabilizing interaction with N367 in YTHDC1 (and in analogy with N1300 in YTHDC2). In contrast, YTHDF2 easily accommodates c^3m^6A, which is likely supported by an electrostatic attraction between aspartate and the positive N1 site, which cannot be achieved with the asparagine at the corresponding site in YTHDC1/2.

By contrast, N7 of m^6A is not involved in any direct interactions with amino acids in any of the YTH domains, and atomic mutagenesis of this site is therefore well tolerated. Together with the results from UV melting showing that c^7m^6A has very similar thermodynamic effects as m^6A, and the fact that it is much more slowly demethylated than m^6A, our data suggest c^7m^6A as a useful tool for further m^6A research.

Conclusion

We have synthesized a series of deaza m^6A analogues and used them to systematically probe structural features for the recognition of m^6A by eraser and reader proteins. Individually replacing the nitrogen atom at position 1, 3 or 7 by a CH group resulted in the m^6A analogues c^1m^6A, c^3m^6A and c^7m^6A. Upon incorporation into oligonucleotides, the destabilizing effect on Watson-Crick base paring increased in the order $m^6A \sim c^7m^6A < c^3m^6A < c^1m^6A$.

HPLC-MS-based demethylation studies revealed that the enzymatic demethylation efficiency of human m^6A dioxygenases FTO and ALKBH5 was markedly affected by m^6A atomic mutagenesis. In the case of FTO-mediated demethylation, we identified N1 as a key structural feature that is engaged in electrostatic interactions with the enzyme active site to ensure proper alignment of the exocyclic substituent within the catalytic core. ALKBH5, by contrast, showed

tolerance toward all m^6A atomic mutants, although 7-deaza and 3-deaza mutations led to reduced demethylation efficiency. While FTO-mediated demethylation involves release of oxidized N^6-hydroxymethyl intermediates, ALKBH5 directly converts its substrates to their respective unmethylated products. Our experimental results support recent reports that this mechanistic discrepancy is based on a covalent demethylation mechanism of ALKBH5.

Further fluorescence spectroscopy-based investigations revealed that also m^6A recognition by its reader proteins YTHDF2, YTHDC1 and YTHDC2 relies on electrostatic interactions involving N1 in addition to the exocyclic methylamino group. Absence or (for YTHDC1 and YTHDC2) protonation of N1 led to drastically reduced binding affinities. Overall, our study provided valuable insights into the structural requirements for substrate recognition by m^6A reader proteins and demethylation events mediated by m^6A demethylases. The identification of crucial structural features and interactions may aid future research on these proteins as drug targets. Furthermore, the building blocks disclosed in this study may serve as useful probes for the investigation of other m^6A-interacting proteins that are involved in regulating m^6A-dependent cellular pathways.

ASSOCIATED CONTENT

Supporting Information. Supplementary methods, NMR spectra of the synthesized compounds, UV/Vis and fluorescence spectra of the free nucleosides, anion exchange and ESI-MS data of the synthesized oligonucleotides, HPLC-MS raw data, geometry-optimized structures. This material is available free of charge via the Internet at http://pubs.acs.org.

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