Oxidative demethylation of 3-methylthymine and 3-methyluracil in single-stranded DNA and RNA by mouse and human FTO

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Abstract

The human obesity susceptibility gene, *FTO*, encodes a protein that is homologous to the DNA repair AlkB protein. The AlkB family proteins utilize iron(II), α-ketoglutarate (α-KG) and dioxygen to perform oxidative repair of alkylated nucleobases in DNA and RNA. We demonstrate here the oxidative demethylation of 3-methylthymine (3-meT) in single-stranded DNA (ssDNA) and 3-methyluracil (3-meU) in single-stranded RNA (ssRNA) by recombinant human FTO protein *in vitro*. Both human and mouse FTO proteins preferentially repair 3-meT in ssDNA over other base lesions tested. They showed negligible activities against 3-meT in double-stranded DNA (dsDNA). In addition, these two proteins can catalyze the demethylation of 3-meU in ssRNA with a slightly higher efficiency over that of 3-meT in ssDNA, suggesting that methylated RNAs are the preferred substrates for FTO.

Keywords

DNA/RNA repair; FTO; oxidative demethylation

1. Introduction

Alkylating agents from the environment or formed inside cells can attack N- or O-atoms of nucleobases, which can lead to formation of alkylation base damages that require prompt repair [1]. Almost all living organisms have evolved various machineries to recognize and process these modifications. Among them, direct removal of alkyl adducts from the damaged bases is one of the most efficient repair strategies [2]. The AlkB protein family performs this type of direct repair by utilizing an oxidative demethylation mechanism (Fig. S1).

The *E. coli* AlkB protein belongs to a superfamily of α-KG- and Fe^{2+}-dependent dioxygenases [2–9]. Its homologues are found in viruses, bacteria, and eukaryotes. Although eight human homologues (ABH1–8) were identified before 2007 [10,11], only ABH2 and ABH3 were confirmed to have a similar repair function to AlkB [4,5,12]. In particular, ABH2 was found

to be primarily responsible for repairing 1-meA base lesions in genomic DNA while the exact role of ABH3 remains unclear [13]. ABH2 prefers dsDNA substrates over ssDNA ones; however, both hABH3 and AlkB are more active with ssDNA and ssRNA substrates [4,14]. Only recently, the mechanisms underlying the substrate preferences of these proteins were elucidated through X-ray structural studies of the AlkB-dsDNA and ABH2-dsDNA complexes [15]. The ABH2 protein adopts a commonly observed base flipping mechanism with a finger residue which intercalates inside the DNA duplex to fill the gap left by the flipped base [15]. The AlkB protein, however, squeezes the DNA duplex to eliminate the gap left by base flipping. This distortion imposed by AlkB on DNA explains its preference to flexible ssDNA over relatively rigid duplex DNA [15].

All three of these proteins exhibited the highest activities against 1-methyladenine (1-meA) and 3-methylcytosine (3-meC) [4–7], but with lower activities they can also process 1-methylguanine (1-meG) and 3-methylthymine (3-meT) [16,17]. In addition, AlkB, ABH2, and ABH3 have been shown to repair exocyclic DNA base lesions: $1,N^6$-ethenoadenine (εA) and (or) $3,N^4$-ethenocytosine (εC) [18–20].

In 2007, it was a great surprise that a human obesity-linked gene, *FTO,* was found to encode a functional homologue of AlkB [21,22]. The *FTO* gene is highly expressed in the hypothalamus part of the brain, and a defect of the *FTO* gene has been linked to an increase of body fat [23–25]. This gene was found to be only present in vertebrates and marine algae [26]. The FTO protein possesses a homologous sequence to the AlkB family proteins, and the purified recombinant mouse FTO protein can oxidatively demethylate 3-meT in ssDNA in the presence of iron(II), α-ketoglutarate (α-KG), and dioxygen [21]. This discovery has assigned FTO as a nucleic acid demethylase that may work on DNA or RNA and also has raised several very interesting questions: i) what is the biochemical activity of the human FTO? ii) is 3-meU in ssRNA a substrate for FTO proteins? iii) how can the DNA/RNA demethylation function of FTO be linked to obesity? Perhaps, the methylation reversed by human FTO is a signal for gene regulation rather than merely for DNA/RNA damage. To help investigate the functional role of FTO, we present here the first study for the biochemical activity of recombinant human FTO protein *in vitro*. We also include our evaluation of the demethylation of 3-meU in ssRNA mediated by both mouse and human FTO proteins.

2. Experimental procedures

2.1 Construction, expression and purification of mFTO and hFTO

The cDNA sequences encoding full length mFTO (Image ID: 4237261) and hFTO (Genebank Accession No.: NP_001073901.1) were subcloned into pET28a to generate a His-tagged fusion protein. The plasmids were transformed into *E. coli* BL21 Star (DE3) and bacteria were grown on LB-agar plates containing 50 mg/L of kanamycin. Overnight precultures, which were grown aerobically at 37°C with a shaking speed of 190 rpm, were used to inoculate 1 L LB medium with 50 mg/L kanamycin and grown at 37°C and 250 rpm until OD_{600} reached ~1.0. Then the bacterial cells were induced by IPTG (0.5 mM) at 15°C and grown overnight at 15°C and 250 rpm. The cells were harvested by centrifugation, frozen by liquid nitrogen, and stored at −80° C. All subsequent steps were performed at 4°C. The cell pellets were resuspended in buffer A (50 mM imidazole, 300 mM NaCl, 50 mM sodium phosphate, pH 8.0), sonicated on ice, and centrifuged at 12,000 rpm for 22 min. The filtered supernatant was purified by Ni-NTA chromatography (GE Healthcare). The fractions collected from the column were further purified with a gel filtration column (GE Healthcare). The fractions were analyzed through denaturing SDS-PAGE.

2.2 Repair of methylated DNA and RNA by mFTO and hFTO

The typical reaction mixture (100 μL) contained DNA/RNA (1 nmol), FTO (0.05–0.5 nmol), $(NH_4)_2Fe_2(SO_4)_2 \cdot 6H_2O$ (283 μM), α-KG (300 μM), L-ascorbic acid (2 mM), bovine serium albumin (BSA, 50 μg mL^{-1}), and 50 mM MES buffer (pH 6.0). This was incubated for 12 h at 16°C. The reaction was quenched by addition of EDTA to 5 mM.

2.3 Restriction enzyme digestion assay

A previously published procedure [13,15] was followed to evaluate repair of a 49-mer oligonucleotide containing 1-meA, 1-meG, and εA in a *Dpn*II cleavage sequence. The sequence was [5′-TAGACATTGCCATTCTCGATAG*G(replaced by 1-meG)A(replaced by 1-meA orεA)TC*CGGTCAAACCTAGACGAATTCCA-3′ complementary to 5′-TGGAATTCGTCTAGGTTTGACCG*GATC*CTATCGAGAATGGCAATGTCTA-3′]. The reactions were run at both 37°C and 16°C for 12 h with 0.2 nmol FTO and 0.2 nmol substrates. For ssDNA, substrates were annealed to the complementary strand for the digestion assay after incubation with the FTO protein and cofactors.

2.4 DNA/RNA digestion and HPLC assay

After the repair reaction, ssDNA/ssRNA was digested into nucleosides with nuclease P_1 (Sigma, N8630) and alkaline phosphatase (Sigma, P4252), based on a previous procedure [27]. The digestion solution was analyzed in an isocratic HPLC system equipped with a C18 separation column (150×4.6 mm) equilibrated with buffer A (HPLC grade aqueous solution containing 50 mM ammonium acetate) and buffer B (50 mM ammonium acetate, 50% of acetonitrile, 50% water and 0.1% TFA) at 95:5 (v/v) ratio with a flow rate of 1 mL min^{-1} at room temperature. The detection wavelength was set at 266 nm (for 3-meT) or 261 nm (for 3-meU).

2.5 Kinetics of mFTO and hFTO

To determine K_m and K_{cat} values for the repair reactions, initial rates were obtained by keeping the enzyme concentration constant and varying the substrate concentration. Reactions were adjusted to assure that less than 20% of the substrate was expended. All reactions were performed at 20°C in triplicate and analyzed by Origin 8.0 with the Michaelis-Menten equation.

3. RESULTS AND DISCUSSION

The full length mFTO and hFTO were cloned, expressed, and purified (Fig. S2). A restriction endonuclease digestion assay with the use of *Dpn*II was adopted to evaluate the repair activities of both hFTO and mFTO towards 1-meA, 1-meG and εA in ssDNA or dsDNA [13,15,28]. A modified 49 mer DNA with 1-meA, 1-meG, or εA incorporated into the GATC sequence is resistant to *Dpn*II cleavage. Removal of the base lesion by FTO allows *Dpn*II to cleave the 49 mer DNA probe into two fragments, providing an assay for the repair activity (Fig. 1). As reported previously [21], we found very low activities of both proteins toward repairing 1-meA in 49 mer ssDNA (Fig. 1A). Both proteins failed to repair 1-meA in dsDNA (stoichiometric amount) after a 12 h incubation at either 16°C (Fig. 1B) or 37°C (data not shown); whereas, purified ABH2 exhibited good repair activities for 1-meA and εA in dsDNA under the same conditions (Fig. 1B). In addition, we did not observe any noticeable activity of mFTO or hFTO toward εA and 1-meG in either ssDNA or dsDNA. The 3-meT base lesion, when incorporated into the *Dpn*II cleavage sequence, could not block the enzymatic digestion of the DNA probe. Thus, a different assay was used to evaluate repair of this base lesion.

To probe 3-meT demethylation by FTO proteins, a 15 mer ssDNA [5′-CTTGTCA(3-meT)CAGCAGA-3′] with 3-meT incorporated in the middle was synthesized and purified. This

DNA was digested into nucleosides by nuclease P1 and alkaline phosphatase, and subsequently analyzed by HPLC. As shown in Fig. 2A, nucleosides dC, dG, dT, dA and 3-medT could be cleanly separated. The change in intensity of the 3-medT peak was monitored with other nucleoside peaks as internal references. The 3-meT-containing ssDNA was incubated with either mFTO or hFTO at 16°C for 12 h. After quenching the reaction, the content of 3-meT was measured by the digestion assay and HPLC analysis. The results indicated complete demethylation of 3-meT in ssDNA by both mFTO and hFTO (Fig. 2A). When a 15 mer 3-meC-containing ssDNA was subjected to the same repair assay, only ~15% of 3-meC was demethylated after 12 h under the same conditions (Fig. 2B), showing strong preference of FTO proteins toward 3-meT over 3-meC.

However, almost negligible demethylation activities (< 5% after 12 h) were observed for both proteins (Fig. 2C) when a 3-meT-containing dsDNA (obtained by annealing the 15 mer ssDNA with its complementary strand) was subjected to the same repair procedure. Thus, we conclude that recombinant mFTO and hFTO are not likely to catalyze demethylation of dsDNA substrates *in vitro* and perhaps *in vivo*.

The strong preference for 3-meT in ssDNA by the FTO proteins raised the question of whether 3-meU in ssRNA could also serve as a substrate for these proteins. We synthetically prepared 3-meU-CE phosphoramidite (Fig. S3) and incorporated it into a 15 mer ssRNA via solid state synthesis (Fig. S4). When this RNA substrate was incubated with either mFTO or hFTO, complete removal of the methyl group on 3-meU was observed in both cases (Fig. 2D). Thus, both mFTO and hFTO are capable of repairing 3-meT in ssDNA and 3-meU in ssRNA *in vitro*.

The inhibition effect of α-KG at millimolar concentrations was evaluated as noted previously [29] (Fig. S5), and 300 μM of α-KG was chosen for further studies. Next, the pH-activity profiles were estimated for the demethylation of 3-meT in ssDNA and 3-meU in ssRNA. In the case of mFTO, a decrease of the demethylation activity was observed with increasing pH for both ssDNA and ssRNA substrates (Fig. 3A and 3B). However, hFTO exhibited the highest activity at pH 6.0 for both ssDNA and ssRNA substrates (Fig. 3C and 3D). Thus, we chose pH 6.0 for detailed kinetic analysis.

The kinetic studies were performed at 20°C with varying concentrations of ssDNA or ssRNA substrates. The results are shown in Fig. 4 and summarized in Table 1. The mFTO protein showed higher activities toward both ssDNA and ssRNA substrates than hFTO. It should be noted that this result may not reflect the *in vivo* activities of these proteins because the purified recombinant mFTO appears to be more stable than the purified recombinant hFTO *in vitro*. The kinetic studies revealed that both mFTO and hFTO exhibit a 2-fold preference for 3-meU in ssRNA as the substrate over 3-meT in ssDNA. The experiments were repeated in triplicate with similar results obtained.

In summary, this study has established the DNA/RNA demethylation activity of the recombinant human FTO for the first time (Fig. 5). Both mFTO and hFTO showed no observable activity towards εA and 1-meG in ssDNA. A very low demethylation activity of 1-meA in ssDNA was observed for these two proteins. They could also catalyze demethylation of 3-meC in ssDNA, but with a much lower efficiency as compared to 3-meT in ssDNA. Both mFTO and hFTO failed to repair base lesions in dsDNA, strongly suggesting that they are involved in ssDNA or ssRNA processing.

Importantly, we showed that both recombinant hFTO and mFTO can demethylate 3-meU in ssRNA *in vitro* (Fig. 5). The FTO protein's RNA demethylation activity is slightly more efficient than the demethylation of 3-meT in ssDNA mediated by the same proteins. Considering the negligible repair of dsDNA substrates by the FTO proteins and their slight

preferences for 3-meU in ssRNA over 3-meT in ssDNA, it is attractive to suggest FTO as a RNA demethylase [21]. Perhaps it catalyzes the reverse reaction of a previously unrecognized RNA methylation and exerts gene regulation function at the RNA level. Disruption of this regulatory role of FTO may lead to the obesity phenotype linked to this protein. Of course, detailed *in vivo* experiments are required to further test this hypothesis. This current work serves as the first comprehensive evaluation of the activities of both mFTO and hFTO *in vitro*, and provides a foundation for further inquiry into the role of this very interesting nucleic acid demethylase.

Acknowledgements

Abbreviations

mFTO
mouse FTO

hFTO
human FTO

3-meT
3-methylthymine

3-meU
3-methyluracil

1-meA
1-methyladenine

1-meG
1-methylguanine

3-meC
3-methylcytosine

εA
$1,N^6$-ethenoadenine

εC
$3,N^4$-ethenocytosine

IPTG
isopropyl-β-D-thiogalactopyranoside

α-KG
α-ketoglutarate

BSA
bovine serium albumin

MES
2-(N-morpholino)ethanesulfonic acid

EDTA
ethylene diamine tetraacetic acid

TFA

trifluoroacetic acid

TLC

thin layer chromatography

NMR

nuclear magnetic resonance

MALDI-MS

matrix-assisted laser desorption-ionisation mass spectrometry

HPLC
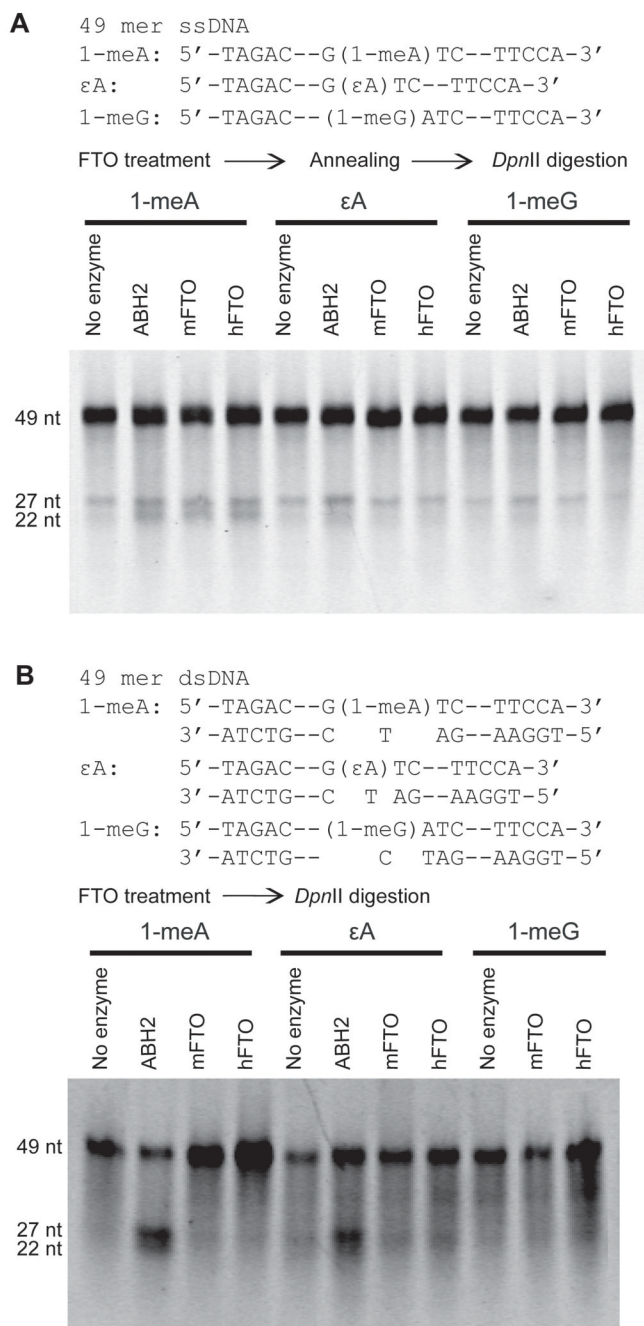
high performance liquid chromatography

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version, at doi:XXXX.

A
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49 mer ssDNA
1-meA: 5'-TAGAC--G(1-meA)TC--TTCCA-3'
εA:    5'-TAGAC--G(εA)TC--TTCCA-3'
1-meG: 5'-TAGAC--(1-meG)ATC--TTCCA-3'
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FTO treatment ⟶ Annealing ⟶ *Dpn*II digestion

B
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49 mer dsDNA
1-meA: 5'-TAGAC--G(1-meA)TC--TTCCA-3'
       3'-ATCTG--C    T  AG--AAGGT-5'
εA:    5'-TAGAC--G(εA)TC--TTCCA-3'
       3'-ATCTG--C   T AG--AAGGT-5'
1-meG: 5'-TAGAC--(1-meG)ATC--TTCCA-3'
       3'-ATCTG--      C  TAG--AAGGT-5'
```

FTO treatment ⟶ *Dpn*II digestion

Fig. 1.
A restriction enzyme digestion assay for repair of 1-meA, εA, and 1-meG by ABH2, mFTO, and hFTO. (A) A 49 mer ssDNA (0.2 nmol) with 1-meA, εA, or 1-meG incorporated into a GATC sequence (can be recognized and cleaved by *Dpn*II) was used for the assay. The modified dsDNA probe is resistant to *Dpn*II cleavage. Repair of the base lesion led to cleavage of the 49 mer dsDNA into two fragments of 27 and 22 base pairs, which was analyzed by a denaturing DNA gel. Both mFTO (0.2 nmol) and hFTO (0.2 nmol) exhibited very low activities toward 1-meA in ssDNA (12 h at 16°C and pH 6.0). Repair of εA and 1-meG by mFTO and hFTO were not observed. (B) No activities toward 1-meA, εA, and 1-meG in dsDNA were

observed for either mFTO or hFTO under the same assay conditions. ABH2 showed good activities to repair 1-meA and εA in dsDNA in control experiments.
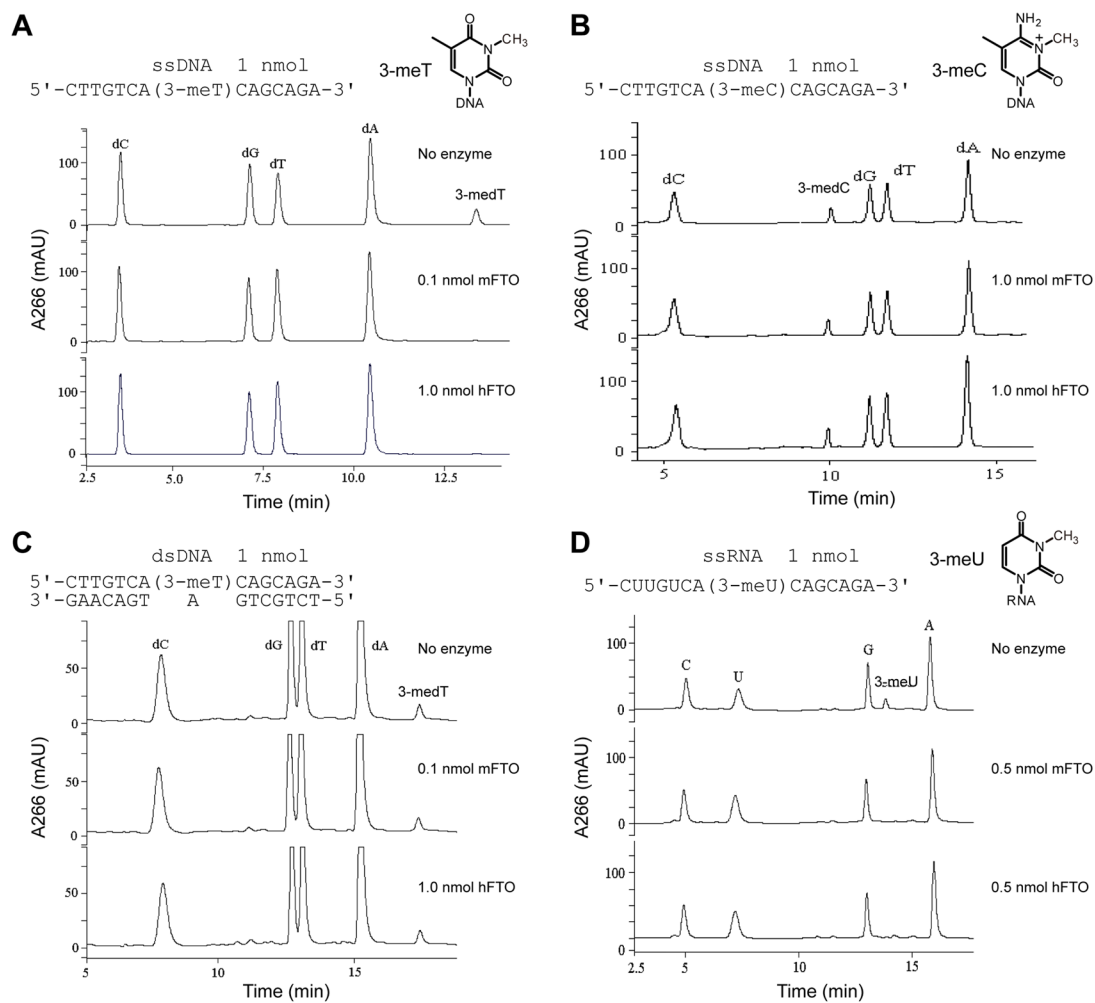
Fig. 2.
HPLC chromatograms of digested nucleosides from 3-meT-containing DNA (1 nmol), 3-meC-containing DNA (1 nmol) and 3-meU-containing RNA (1 nmol). (A) Complete demethylation of 3-meT in a 15 mer ssDNA by mFTO and hFTO. All reactions were run for 12 h at 16°C and pH 6.0. (B) Both mFTO and hFTO gave ~15% demethylation of 3-meC in 15 mer ssDNA; 100% repair of 3-meT was observed for both enzymes under the same conditions as shown in (A). (C) Negligible repair of 3-meT in dsDNA was observed for both mFTO and hFTO under the same conditions. (D) Complete demethylation of 3-meU in a 15 mer ssRNA by mFTO and hFTO under the same conditions.
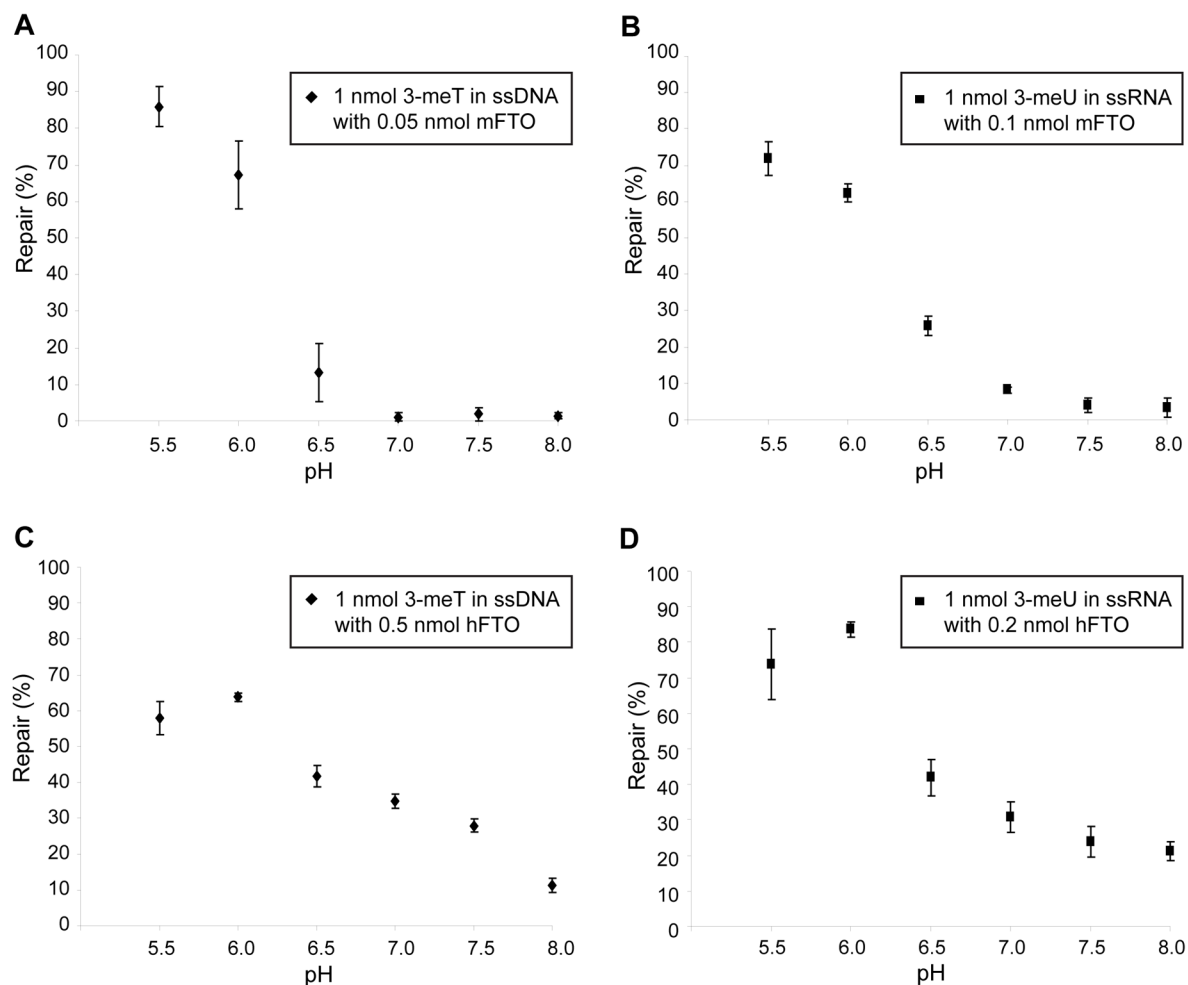
Fig. 3.
The pH-activity profiles for demethylation reactions of 3-meT in ssDNA and 3-meU in ssRNA
by mFTO and hFTO. All reactions were run in triplicate at 16°C for 12 h. (A) Demethylation
of 3-meT in the 15 mer ssDNA (1 nmol) by mFTO (0.05 nmol). (B) Demethylation of 3-meU
in the 15 mer ssRNA (1 nmol) by mFTO (0.1 nmol). (C) Demethylation of 3-meT in the 15
mer ssDNA (1 nmol) by hFTO (0.5 nmol). (D) Demethylation of 3-meU in the 15 mer ssRNA
(1 nmol) by hFTO (0.2 nmol).
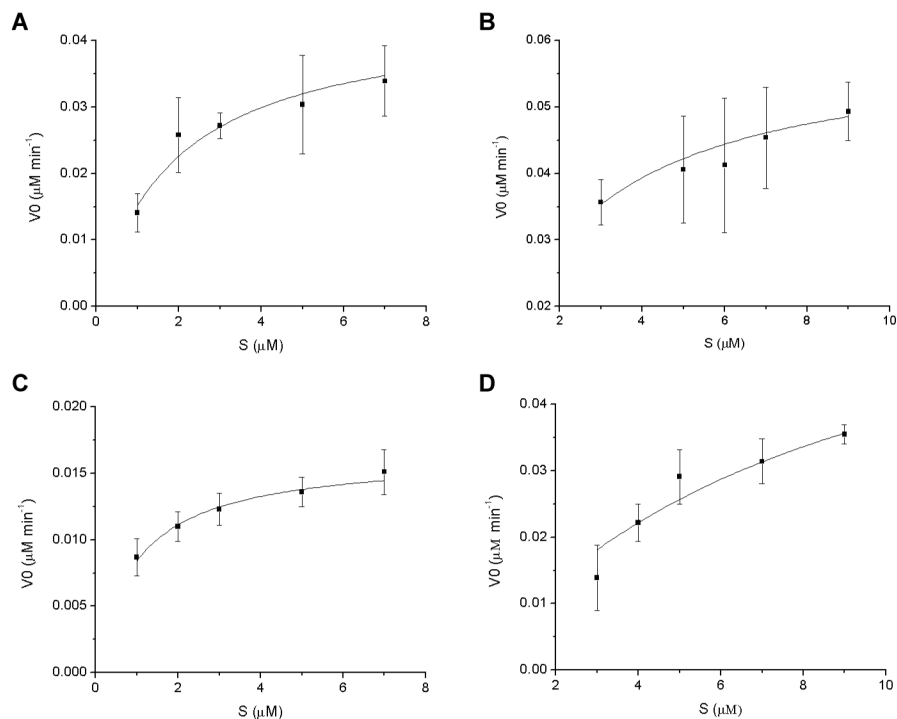
Fig. 4.
Kinetics of demethylation reactions catalyzed by mFTO and hFTO. All reactions were run in triplicate at 20°C and pH 6.0. (A) Demethylation of 3-meT in the 15 mer ssDNA by mFTO (0.5 μM). (B) Demethylation of 3-meU in the 15 mer ssRNA by mFTO (0.3 μM). (C) Demethylation of 3-meT in the 15 mer ssDNA by hFTO (2.5 μM). (D) Demethylation of 3-meU in the 15 mer ssRNA by hFTO (0.4 μM).
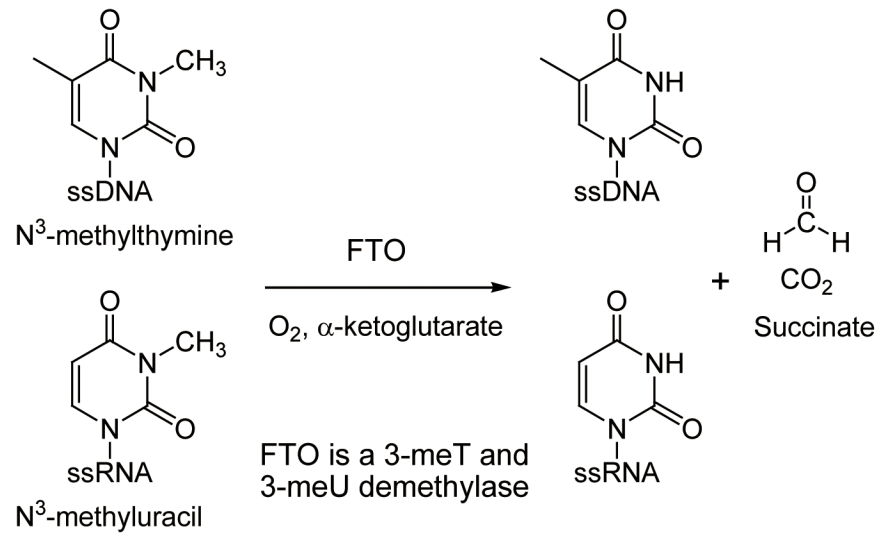
Fig. 5.
FTO can demethylate both 3-meT from ssDNA and 3-meU from ssRNA.

Table 1

Kinetic constants for 3-meT and 3-meU demethylation by mFTO and hFTO at 20°C and pH 6.0

enzyme	substrate	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1}μM^{-1})
mFTO	ssDNA (3-meT)	1.92 ± 0.43	0.089 ± 0.008	0.046 ± 0.019
	ssRNA (3-meU)	2.08 ± 0.31	0.199 ± 0.009	0.096 ± 0.029
hFTO	ssDNA (3-meT)	0.95 ± 0.12	0.007 ± 0.0002	0.007 ± 0.002
	ssRNA (3-meU)	8.51 ± 3.13	0.115 ± 0.022	0.014 ± 0.007