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

Guifang Jia[a,b,1,2], Cai-Guang Yang[a,1], Shangdong Yang[a], Xing Jian[a], Chengqi Yi[a], Zhiqiang Zhou[b], Chuan He[a,*]

[a] *Department of Chemistry, The University of Chicago, 929 East 57th Street, Chicago, IL 60637, USA*
[b] *Department of Applied Chemistry, China Agricultural University, Beijing 100094, China*

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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 *Escherichia coli* AlkB protein belongs to a superfamily of α-ketoglutarate (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-methyladenine (1-meA) base lesions in genomic DNA while the exact role of ABH3 remains unclear [13]. ABH2 prefers double-stranded DNA (dsDNA) substrates over single-stranded DNA (ssDNA) ones; however, both hABH3 and AlkB are more active with ssDNA and single-stranded RNA (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-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 (mFTO) protein can oxidatively demethylate 3-meT in ssDNA in the presence of iron(II), 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 (hFTO)? (ii) Is 3-methyluracil (3-meU) in ssRNA a substrate for FTO

*Corresponding author. Fax: +1 773 702 0805.
E-mail address: chuanhe@uchicago.edu (C. He).

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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; HPLC, high performance liquid chromatography

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proteins? (iii) How can the DNA/RNA demethylation function of FTO be linked to obesity? Perhaps, the methylation reversed by hFTO 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 hFTO protein in vitro. We also include our evaluation of the demethylation of 3-meU in ssRNA mediated by both mFTO and hFTO proteins.

2. Materials and methods

2.1. Construction, expression and purification of mFTO and hFTO

The cDNA sequences encoding full length mFTO (Image ID: 4237261) and hFTO (GenBank 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 isopropyl-β-D-thiogalactopyranoside (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), $(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$ (283 μM), α-KG (300 μM), L-ascorbic acid (2 mM), bovine serium albumin (BSA, 50 μg ml^{-1}) and 50 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (pH 6.0). This was incubated for 12 h at 16 °C. The reaction was quenched by addition of ethylene diamine tetraacetic acid (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 DpnII cleavage sequence. The sequence was [5′-TAGACATTGCCATTCTCGATAG*G* (*replaced by 1-meG*) *A* (*replaced by 1-meA or εA*) *TC*CGGTCAAACCTAGACGAATTCCA-3′ complementary to 5′-TGGAATTCGTCTAGGTTTGACCG*GATC*CTATCGAGAATGGCAATGTCTA-3′]. The reactions were run at both 37 °C for 1 h 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, 15-mer 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 high performance liquid chromatography (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% trifluoroacetic acid (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.

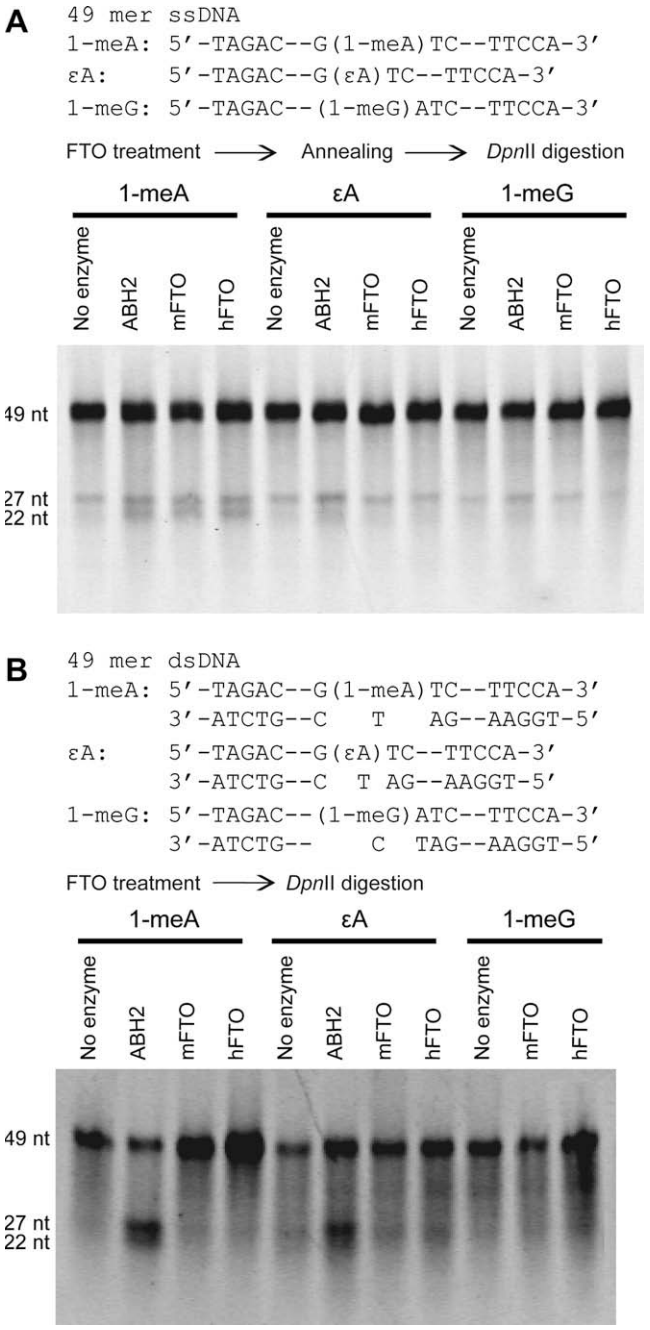

Fig. 1. A restriction enzyme digestion assay for repair of 1-meA, εA and 1-meG by ABH2, mFTO and hFTO. (A) A 49-mer ssDNA (0.2 nmol) with 1-meA, εA, or 1-meG incorporated into a GATC sequence (can be recognized and cleaved by DpnII) was used for the assay. The modified dsDNA probe was resistant to DpnII cleavage. Repair of the base lesion led to cleavage of the 49-mer dsDNA into two fragments of 27 and 22 bp, 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.