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Distributive Processing by the Iron(II)/α-Ketoglutarate-Dependent Catalytic Domains of the TET Enzymes Is Consistent with Epigenetic Roles for Oxidized 5-Methylcytosine Bases

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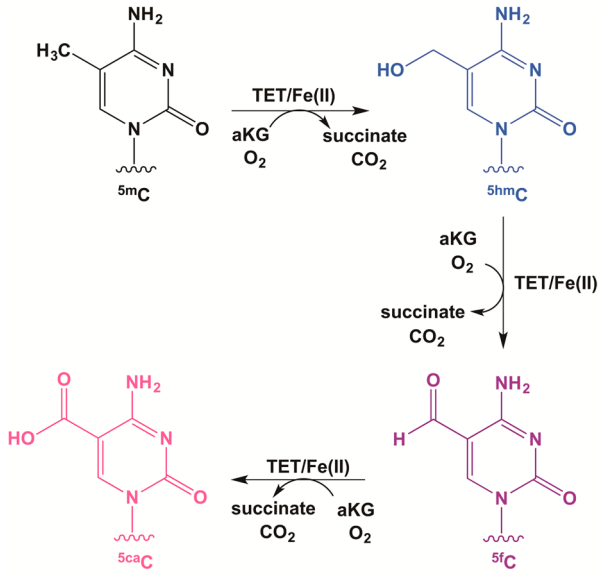
Supporting Information

ABSTRACT: The ten-eleven translocation (TET) proteins catalyze oxidation of 5-methylcytosine (^{5m}C) residues in nucleic acids to 5-hydroxymethylcytosine (^{5hm}C), 5-formylcytosine (^{5f}C), and 5-carboxycytosine (^{5ca}C). These nucleotide bases have been implicated as intermediates on the path to active demethylation, but recent reports have suggested that they might have specific regulatory roles in their own right. In this study, we present kinetic evidence showing that the catalytic domains (CDs) of TET2 and TET1 from mouse and their homologue from *Naegleria gruberi*, the full-length protein NgTET1, are distributive in both chemical and physical senses, as they carry out successive oxidations of a single ^{5m}C and multiple ^{5m}C residues along a polymethylated DNA substrate. We present data showing that the enzyme neither retains ^{5hm}C/^{5f}C intermediates of preceding oxidations nor slides along a DNA substrate (without releasing it) to process an adjacent ^{5m}C residue. These findings contradict a recent report by Crawford et al. (*J. Am. Chem. Soc.* **2016**, *138*, 730) claiming that oxidation of ^{5m}C by CD of mouse TET2 is chemically processive (iterative). We further elaborate that this distributive mechanism is maintained for TETs in two evolutionarily distant homologues and posit that this mode of function allows the introduction of ^{5m}C forms as epigenetic markers along the DNA.

Methylation of C5-cytosine in DNA is a primary epigenetic marker that controls numerous important biological processes. Mammalian ten-eleven translocation (TET) proteins are iron(II)- and α-ketoglutarate-dependent (Fe/αKG) oxygenases that transform this methylated base (^{5m}C) to 5-hydroxymethylcytosine (^{5hm}C), 5-formylcytosine (^{5f}C), and 5-carboxycytosine (^{5ca}C) in three consecutive reactions.[1−7] In each step, substrate oxidation is coupled to conversion of αKG to succinate and CO_2 (Scheme 1).[8,9] Results from studies such as those demonstrating *in vitro* recognition and excision of ^{5f}C and ^{5ca}C by thymine-DNA glycosidase (TDG) and increased levels of the two modifications in mouse embryonic stem cells lacking TDG supported the notion that the oxidized species might merely be intermediates in the active reversion of the ^{5m}C marker.[10−12] However, recent data revealing the association of ^{5hm}C, ^{5f}C, and ^{5ca}C with regulatory elements have led to the hypothesis that they might have their own roles in gene regulation.[11,13,14] This

Scheme 1. Oxidation Reaction of ^{5m}C by the TET Enzymes



is supported by enrichment-based and single-base resolution sequencing studies, which show ^{5hm}C, ^{5f}C, and ^{5ca}C to be enriched at various promoter, enhancer, and exon sites.[11,15−27] Two recent studies by Bachman and co-workers showed that ^{5hm}C and ^{5f}C are both stable modifications in mouse DNA and are most abundant in the adult brain.[28,29] Levels of ^{5m}C-oxidized forms in various tissues did not correlate with the levels of their oxidation precursors.[28,30,31] Both sets of observations imply that the three-step oxidation of ^{5m}C to ^{5ca}C does not occur on a genome-wide level but is probably limited to specific sequences and directed by specific cellular signals. All in all, the above observations evoke a role for ^{5m}C modifications in gene regulation.

Despite mounting *in vivo* evidence that the TET enzymes and their reaction products are important in epigenetic regulation, the kinetic behavior of the enzymes upon encountering their ^{5m}C substrate in genomic DNA is poorly understood. Two specific issues that have not been satisfactorily addressed are the extent to which the enzymes are programmed to suppress the accumulation of partially oxidized intermediates (^{5hm}C and ^{5f}C) by ensuring further oxidation and their

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propensity to act processively (or distributively) in oxidations of multiple methylation sites within a given substrate molecule. Two recent studies have provided conflicting conclusions endeavoring to address the first issue. Xu et al.[32] followed the extent of ^{5f}C formation on symmetrically methylated CpG oligonucleotides using a pyrene-based fluorescence probe specific to ^{5f}C. The observation of dominant levels of symmetric ^{5f}C among total ^{5f}C sites led to the conclusion that the catalytic domain (CD) of mouse TET1 (mTET1$_{CD}$) behaves distributively and releases the oxidation product of each turnover rather than proceeding to the next oxidation reaction until ^{5m}C has been converted to ^{5ca}C. Conversely, Crawford et al.[33] followed the *in vitro* reaction of mTET2 CD (mTET2$_{CD}$) using isotopically labeled ^{5m}C, ^{5hm}C, ^{5f}C, and ^{5ca}C and concluded that mTET2$_{CD}$ remains bound through ^{5ca}C formation. In the present study, we show that a truncated form of mTET2$_{CD}$ (mTET2$_{CD\Delta}$: mTET2$_{CD}$(1042−1912Δ1378−1746)), mTET1$_{CD}$, and the full-length protein of their evolutionarily distant homologue NgTET1 (NgTET1$_{FL}$) from *Naegleria gruberi* all act distributively, favoring release of partially oxidized ^{5hm}C and ^{5f}C bases. We thus confirm the aforementioned observation of distributive chemistry for mTET1$_{CD}$ and provide evidence for the conservation of chemically distributive behavior in this class of enzymes. Additionally, we show that dissociation of the TET enzymes from their DNA substrates after each turnover ensures physically (site-to-site) distributive catalytic behavior in all three cases examined. These *in vitro* observations support the notion that TETs in the cell are not merely involved in active demethylation of DNA but also generate three distinct epigenetic markers along the genome to confer additional dimensions to the dynamic regulation of cellular processes.

To determine whether the TET enzymes promote or suppress accumulation of oxidized intermediates, we monitored the multi-turnover kinetics of mTET2$_{CD\Delta}$, mTET1$_{CD}$, and NgTET1$_{FL}$ on duplex DNA substrates with a single ^{5m}C site (Figure 1, Supporting Information (SI) Methods, and Table S1). The traces reveal significant accumulation of both ^{5hm}C and ^{5f}C on the path to ^{5ca}C with mTET2$_{CD\Delta}$ (Figure 1A) and NgTET1$_{FL}$ (Figure 1C) (Table S2). The accumulation of intermediate products to concentrations greater than the input enzyme concentration for mTET2$_{CD\Delta}$ (Figure 1A) and NgTET1$_{FL}$ (Figure 1C) indicates that these enzymes must release the oxidized products rather than catalyze sequential oxidations of the same substrate without release of intermediates. On the basis of this evidence, we propose that mTET2$_{CD\Delta}$ and NgTET1$_{FL}$ affect their three sequential oxidation reactions in a distributive manner and that the distribution of the formed products at a specific time point of the reaction is dependent on the corresponding substrate abundance and the kinetics of that particular oxidation step. The mode of function of mTET1$_{CD}$ was not clear under these experimental conditions because of the low observed turnover rate (Figure 1B).

To clarify the mode of function of mTET1$_{CD}$ and to obtain additional evidence that mTET2$_{CD\Delta}$ and NgTET1$_{FL}$ are distributive in their sequential reactions, we performed a "pulse−chase" experiment in which the "pulse" phase involved reaction of the enzyme with an equimolar quantity of a short duplex substrate (60m$_1$ or 33m$_1$) until ∼30−70% of ^{5m}C was converted and the "chase" entailed mixing with an excess of a longer substrate (90m$_1$ or 60m$_1$) having an identical internal sequence (Figures 2 and S1 and Tables S1 and S3). The

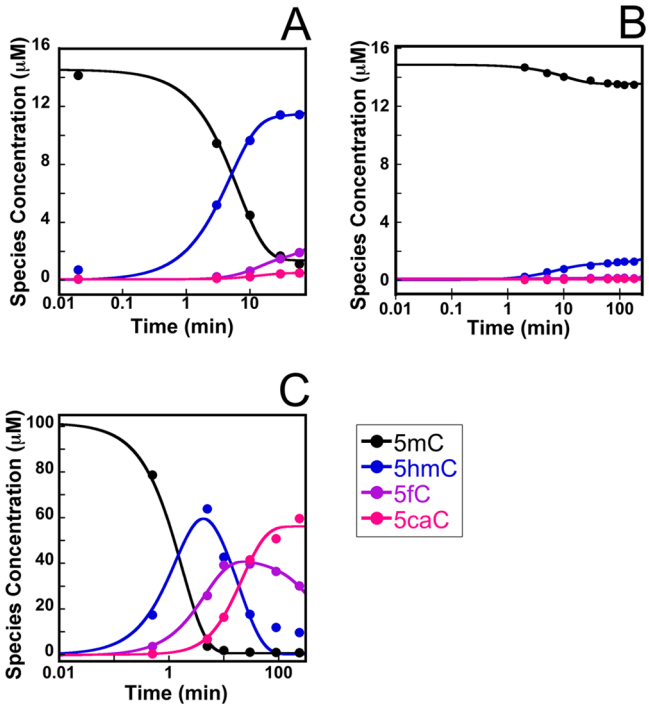

Figure 1. Simultaneous accumulation of oxidized ^{5m}C products by TETs. Kinetic time courses of (A) 3 μM mTET2$_{CD\Delta}$ with 15 μM 60m$_1$, (B) 3 μM mTET1$_{CD}$ with 15 μM 60m$_1$, and (C) 10 μM NgTET1$_{FL}$ with 100 μM 60m$_1$ are shown. Curve fits were generated as described in SI Methods. The sequence of 60m$_1$ is detailed in Table S1.

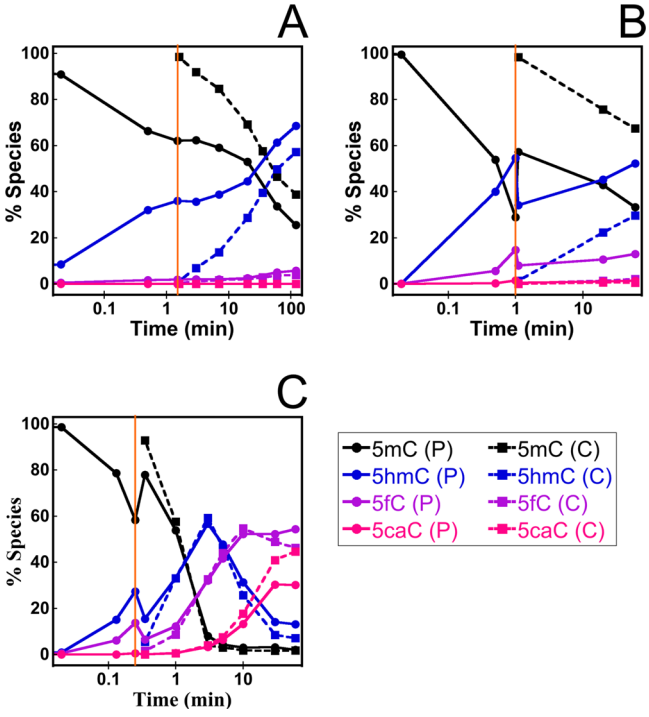

Figure 2. Distributive chemistry of TETs. (A) mTET2$_{CD\Delta}$: **P** = 60m$_1$, **C** = 90m$_1$, chase time (t_c) = 1.5 min. (B) mTET1$_{CD}$: **P** = 33m$_1$, **C** = 60m$_1$, t_c = 1.0 min. (C) NgTET1$_{FL}$: **P** = 33m$_1$, **C** = 60m$_1$, t_c = 0.25 min. [TET] = 15 μM; [**P**] = 15 μM; [**C**] = 60 μM. Solid and dashed traces depict the experimental behavior of **P** (circles) and **C** (squares), respectively. The vertical orange lines correspond to t_c. The sequences of 60m$_1$ and 33m$_1$ are detailed in Table S1.

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different substrate lengths were implemented as a means of distinguishing between the "pulse" substrate (**P**) and the "chase" substrate (**C**), and the percentages of modified cytosines of both substrate(s) and product(s) were determined at various points of the reaction, as described in SI Methods and Table S3. As noted above, if the TET enzymes were to act in a chemically processive manner upon binding the ^{5m}C of **P**, then the ^{5hm}C formed should remain bound while succinate and CO_2 are released. Rebinding of αKG and addition of O_2 would initiate the next oxidation step to form ^{5f}C. The same would be the case for ^{5f}C oxidation. Only upon formation of ^{5ca}C would base release be favored, permitting binding of a new ^{5m}C, with concentration favoring binding of **C** in this next event. Therefore, in a processive mechanism, minimal quantities of oxidized products derived from the ^{5m}C in **C** should be formed under these experimental conditions. Figure 2 shows that for all three enzymes studied, ^{5m}C of both substrates is substantially consumed before ^{5ca}C becomes prevalent, resulting in similar kinetics for the formation of oxidized ^{5m}C products from the two substrates. Furthermore, the consumption and formation of the ^{5m}C species from **P** in Figure 2 are delayed relative to those of the corresponding species in the control experiment in which **C** was omitted from the "chase" solution (Figure S2 and Table S4), indicating that the enzyme activity is distributed between the **P** and **C** substrates rather than acting solely on **P** until the one-carbon unit appended to C5 has been fully oxidized.

Even with knowledge that TETs must release the methyl-oxidized base at the end of each turnover, the question remains whether the DNA molecule must be fully released for the enzyme to process an adjacent C5-methyl on the same strand or whether the enzyme can slide along the DNA to engage a new oxidation target. To address the question of site-to-site (physical) processivity, we designed a distinct "pulse−chase" experiment with two substrates, $60m_{12}$ (60 bp) and $90m_{12}$ (90 bp), each bearing 12 ^{5m}C sites on one of the two DNA strands with one to seven bases between sites (Table S1). The reaction was initiated (the "pulse" phase) with equimolar $60m_{12}$ and chased with excess $90m_{12}$ (Figure 3 and Table S5). If TET were to act in a physically processive manner, then the decay of ^{5m}C in $60m_{12}$ and the concomitant formation of ^{5hm}C should largely precede oxidation of the ^{5m}C in $90m_{12}$. In addition, with its chemically distributive behavior, the presence of multiple targets along the **P** substrate should, in a physically processive mechanism, engender a lag in the formation of ^{5f}C and ^{5ca}C species. Conversely, if TET were to act in a distributive mode, oxidation of ^{5m}C on $60m_{12}$ and $90m_{12}$ would be expected to occur concomitantly, and processing of **C** should proceed without a pronounced lag phase. The results in Figure 3 are clearly more in line with the expectations of a physically distributive reaction for all three TET enzymes. The data imply that after a single turnover, the enzyme releases the oxidized product and dissociates from the DNA to search in what we assume to be a random three-dimensional manner for its next target substrate. Further proof is provided by a comparison of the kinetics of ^{5m}C consumption in $60m_{12}$ after a second mix with the $90m_{12}$ **C** substrate (Figure 3) to that after a control mock-chase mix with buffer (Figure S3 and Table S6). A detectable slowing of the consumption of $60m_{12}$ ^{5m}C and formation of $60m_{12}$ ^{5hm}C, ^{5f}C, and ^{5ca}C is seen in Figure 3 compared with Figure S3, which implies competition by $90m_{12}$. Additionally, the crystal structures of human TET2 (hTET2)[34] and NgTET1[35] show that the architecture of the active site

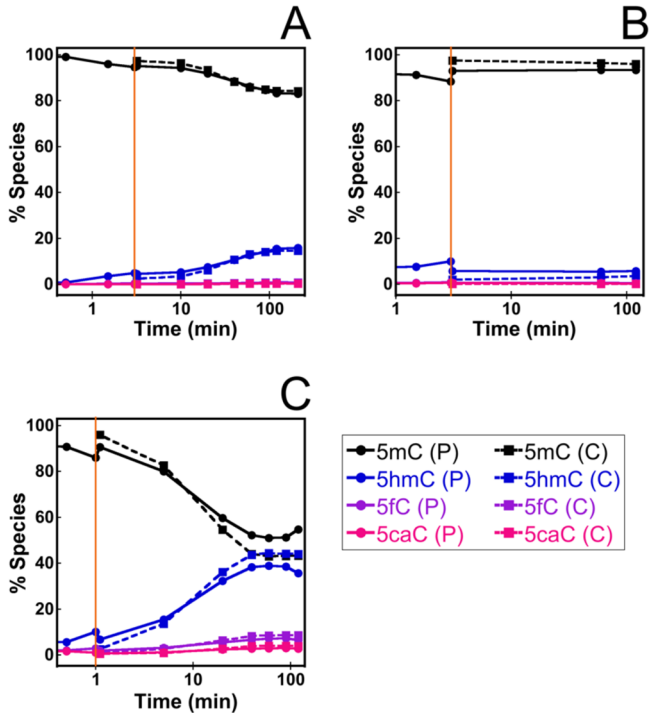

Figure 3. "Pulse−chase" reactions show a distributive physical mode for TETs. (A) $mTET2_{CD\Delta}$, t_c = 3.0 min; (B) $mTET1_{CD}$, t_c = 3.0 min; (C) $NgTET1_{FL}$, t_c = 1.0 min. For all reactions, [TET] = 15 μM, [**P**] = [$60m_{12}$] = 15 μM, and [**C**] = [$90m_{12}$] = 60 μM. Solid and dashed traces depict the experimental behavior of **P** (circles) and **C** (squares), respectively. The vertical orange lines correspond to t_c. The sequences of $60m_{12}$ and $90m_{12}$ are detailed in Table S1.

supports a chemically and physically distributive mode of function. The crystal structures show the cosubstrate, αKG, buried in the center of the active site of the enzyme, which is loosely capped by the DNA substrate bound to the surface of the enzyme, allowing for easy association and dissociation. The enzyme interacts almost exclusively with the strand bearing the oxidation target, and it makes hydrogen bonds exclusively with the CpG dinucleotide, with the ^{5m}C/^{5hm}C/^{5f}C base flipped into the active site to project toward the Fe(II) and αKG.[34−37] The disposition of the substrate and co-substrate subsites may thus dictate that partially oxidized species and DNA dissociate to allow for net replacement of succinate by αKG.

Our observations and conclusions with regard to the chemical *modus operandus* of $mTET2_{CD\Delta}$, $mTET1_{CD}$, and $NgTET1_{FL}$ align with those of Xu et al.[32] and are fundamentally discrepant with those reported by Crawford et al.[33] It is conceivable that the different forms of mTET2 used in the two studies actually behave differently. However, our data imply a chemically distributive mechanism for all three TET species studied herein (truncated CD for mTET2, intact CD for mTET1, and the full-length protein for NgTET1). This internal consistency strongly suggests that the property is intrinsic to the group of enzymes and not an artifact of any particular structural alteration that we introduced. Conversely, comparison of the activities of the protein forms used herein and by Crawford et al. (5% conversion of ^{5m}C by their $mTET2_{CD}$[33] vs >95% by our $mTET2_{CD\Delta}$; Figure 1A, Table S2, Figure S2A, and Table S4) shows that their preparations or experimental conditions might have been compromised in some way. Irrespective of the reason for the apparent discrepancy, it is

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inescapable from the data presented in this study that $mTET2_{CD\Delta}$, $mTET1_{CD}$, and $NgTET1_{FL}$ are not processive in either a chemical or physical sense under the applied conditions.

The literature indicates that ^{5m}C and its oxidized forms overlap in certain regions of the genome yet have unique distributions through other regions, suggesting that mTET chemical reactivity is dictated by specific factors such as local sequence, chromatin structure, or perhaps the participation of accessory proteins.[38,39] It seems that the *in vitro* distributive behavior of $mTET2_{CD\Delta}$ and $mTET1_{CD}$ might better rationalize how they could cause partially oxidized ^{5m}C forms to accumulate in the genome in a stable manner in different locales. As also highlighted in this report, the biochemical similarities in the modes of functionality of the CDs of mTET2 and mTET1 and their evolutionarily distant homologue NgTET1 might suggest that the distributive function of TETs is conserved during the evolution of these enzymes.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b03243.

Materials, experimental methods, and supporting figures and tables (PDF)

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Notes

The authors declare no competing financial interest.

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