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Direct Analysis of Enzyme-Catalyzed DNA Demethylation

Anna A. Karkhanina,[†] Jasmin Mecinović,[‡] Michael U. Musheev,[†] Svetlana M. Krylova,[†] Alexander P. Petrov,[†] Kirsty S. Hewitson,[‡] Emily Flashman,[‡] Christopher J. Schofield,[‡] and Sergey N. Krylov*[,†]

Department of Chemistry, York University, Toronto, Ontario M3J 1P3, Canada, and Department of Chemistry and the Oxford Centre for Integrative Systems Biology, University of Oxford, Oxford, OX1 3TA, United Kingdom

N/O-methylation of DNA can be cytotoxic and mutagenic; therefore, enzymes that reverse DNA methylation are essential for organism survival. Several 2-oxoglutarate-dependent oxygenases and methyltransferases that remove a methyl group from a methylated DNA base have been identified. Studies of their kinetics and search for their inhibitors have been retarded by the lack of an approach to directly quantitate DNA substrates and products that differ by a single methyl group. Here, we introduce such an approach, which is based on capillary electrophoresis with laser-induced fluorescence detection. We achieved baseline separation of a fluorescently labeled 15-nucleotide-long single-base methylated DNA substrate from its demethylated product, followed by its quantitative detection. We then used this approach to study the kinetics of AlkB-catalyzed DNA demethylation and screen a number of potential inhibitors of this reaction. Ten new inhibitors, which can be used as templates in developing therapies targeting AlkB-like enzymes, were identified. Our approach will be applicable for *in vitro* kinetic studies of known DNA demethylating and methylating enzymes and in the discovery of new ones.

C-Methylation of DNA in the form of 5-methylcytosine is a well-established mechanism for the regulation of gene expression. In contrast, N/O-methylation of DNA (or RNA) bases has been largely considered as a mutagenic modification, and various mechanisms for its reversal (N/O-demethylation) have been identified.[1,2] These demethylation mechanisms are important for the protection of genomic DNA against damage by alkylation agents. Because of the apparently low frequency of N/O-methylation in genomic DNA,[3] relatively long oligonucleotides with a single methylated base are biologically relevant DNA substrates for in vitro studies on demethylation. The use of such substrates faces the challenge of quantitatively distinguishing them from their products, which differ only by a single methyl group. It is worthwhile to mention that bisulfite sequencing, a standard procedure to monitor 5-methylcytosine, is not applicable to the analysis of N/O-methylation. To date, there has been no direct method to distinguish the intact substrate from the product, and it appears that the current methodological paradigm in the field assumes that such a method is very difficult to develop. Therefore, enzyme-catalyzed DNA demethylation of biologically relevant substrates has been studied *in vitro* only indirectly via detection of reaction coproducts.[4−7] Coproduct formation, however, does not necessarily represent quantitative demethylation.[6] Furthermore, the formation of coproducts depends on the demethylation mechanism of a particular enzyme; therefore, monitoring coproduct turnover is not applicable to the discovery of new demethylases operating by unknown mechanisms.[2,8,9] Here, we reveal a new method that uses biologically relevant substrates for *in vitro* study and discovery of DNA demethylating enzymes by direct and quantitative detection of the demethylated product. In our method, the fluorescently labeled single-base methylated substrate and the demethylated product are separated by capillary electrophoresis (CE) and then quantitatively detected with laser-induced fluorescence (LIF) detection. This simple method enables direct analysis of DNA-demethylating and DNA-methylating enzyme activities via the separation of intact product from substrate. The method will be useful for the discovery and study of DNA-demethylating and DNA-methylating enzymes and the development of small molecules that inhibit and activate these enzymes.

As an experimental model in this proof-of-principle work, we employed DNA demethylation by AlkB from *E. coli*, an enzyme of the 2-oxoglutarate oxygenase subfamily, which is conserved from bacteria to humans. AlkB is an Fe(II)- and 2-oxoglutarate (2OG)-dependent oxygenase, which catalyzes DNA demethylation in response to damage by S_N2 alkylating agents.[12] AlkB homologue inhibitors could also be used in conjunction with alkylating agents to enhance chemotherapeutic treatment and reduce cytotoxic side effects.[6] AlkB

* To whom correspondence should be addressed. E-mail: skrylov@yorku.ca.
† York University.
‡ University of Oxford.

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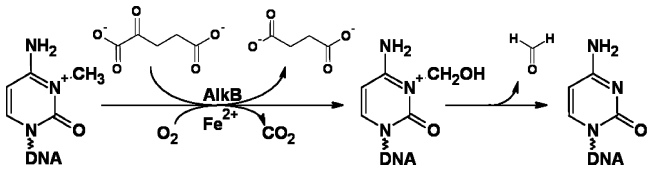

Figure 1. Scheme for AlkB-catalyzed demethylation of DNA.

oxidizes the methyl group in 1-methyladenine (1-meA) or 3-methylcytosine (3-meC) to a hydroxymethyl group, which fragments resulting in formaldehyde and the normal base, A or C, respectively (Figure 1).[10,11] Here, for the first time, the kinetics of the AlkB-catalyzed DNA demethylation was studied by directly measuring the formation of the demethylated DNA product from a 15-nt-long 1-base methylated substrate. We identified new AlkB inhibitors that can be used as chemical starting points in the development of selective inhibitors of human AlkB homologues and the fat-mass-and-obesity protein that was shown recently to be a nucleic acid demethylase.[13,14]

The developed method will help to establish a new methodological paradigm in the area of *in vitro* studies of DNA modifications: direct analysis of DNA-demethylating and DNA-methylating enzyme activities is possible through physical separation of the intact product from the intact substrate.

EXPERIMENTAL SECTION

Materials. Synthetic fluorescently labeled DNA substrate (5′-TTCmTTTTTTTTTTTT-3′-fluorescein), and product (5′-TTCTTTTTTTTTTTT-3′-fluorescein) were synthesized by AT-Dbio (University of Southampton, U.K.). AlkB protein was purified according to the published procedure.[6] The potential AlkB inhibitors tested were purchased from Sigma-Aldrich or synthesized by reported routes.[15] All other chemicals were purchased from Sigma-Aldrich (Toronto, ON). An uncoated fused-silica capillary was purchased from Polymicro (Phoenix, AZ). All solutions were made using Milli-Q-quality deionized water filtered through a 0.22 μm filter (Millipore, Nepean, ON).

Instrumentation. An uncoated fused silica capillary with a length of 50 cm (40 cm to the detection window) and inner and outer diameters of 75 and 365 μm, respectively, was used in all experiments. The capillary was mounted on a capillary electrophoresis (CE) instrument (P/ACE MDQ, Beckman Coulter, Fullerton, CA) with a temperature control to keep the capillary at 15 °C. A sample was introduced into the capillary by a pressure pulse of 0.5 psi × 5 s. The reaction product (P) and unreacted substrate (S) were separated by CE using a 20 mM Borax, 60 mM SDS run buffer at pH 8.0 and quantitated with laser-induced fluorescence (LIF) detection (fluorescence excitation at 488 nm and fluorescence detection at 520 nm).

Measuring Enzyme Kinetics. The demethylation reaction was initiated by the addition of AlkB protein at a final concentration of 5 nM to a mixture containing 50 mM Tris-HCl at pH 7.5, 4 mM L-ascorbic acid, 160 μM 2-oxoglutarate, 80 μM $(NH_4)_2SO_4 \cdot FeSO_4 \cdot 6H_2O$, 5′-TTCmTTTTTTTTTTTT-3′-fluorescein, and 2154 units of catalase as the final concentrations. The final volume of the reaction mixture was 200 μL. Fifteen microliters of the reaction mixture was taken at different times since the reaction initiation and added to an EDTA solution (5 mM final concentration) to stop the reaction. The initial reaction rate (nM/min) was measured as a slope to the initial (linear) part of the "product versus reaction time" curve. The initial reaction rate was determined for varying concentrations of the methylated DNA substrate; K_m and k_{cat} values were calculated by fitting the Michaelis−Menten curve using GraFit 6.0.6 software. Measurements were done in duplicate for every substrate concentration; the presented values are averages with standard deviations as errors.

IC_{50} Determination. In the inhibition assays, the conditions were the same as those used for the kinetic measurements. The concentration of the methylated DNA substrate was 50 nM, and the concentrations of the inhibitors were varied. The reaction was stopped 2.5 min after the initiation by the addition of EDTA at a final concentration of 5 mM. The demethylated DNA product formed was separated from the unreacted substrate by CE and quantified with LIF. Measurements were conducted in duplicate. The relative activity of AlkB at different inhibitor concentrations was plotted against the inhibitor concentration in an Excel worksheet, and the IC_{50} values were calculated as the concentration of an inhibitor at half of the enzyme activity.

Nondenaturing Electrospray Ionization Mass Spectrometry (ESI-MS). All 16 tested inhibitors were screened for binding with the AlkB by nondenaturing ESI-MS. AlkB was desalted using a Bio-Spin 6 Column (Bio-Rad, Hemel Hempstead, U.K.) in 15 mM ammonium acetate (pH 7.5). The stock solution was diluted with the same buffer to a final concentration of 100 μM. $FeSO_4 \cdot 7H_2O$ was dissolved in 20 mM HCl at a concentration of 100 mM. This was then diluted with Milli-Q water to give final working concentrations of 500 μM. The protein was mixed with Fe(II) and an inhibitor to give final concentrations of 15 μM AlkB, 75 μM Fe(II), and 15 μM inhibitor. The solution was then incubated for 30 min at room temperature prior to ESI-MS analysis. Competitive binding experiments were then performed to rank the 11 inhibitors that formed the strongest complexes with AlkB. For competition experiments, the protein was mixed with Fe(II) and two inhibitors to give final concentrations of 15 μM AlkB, 75 μM Fe(II), and 15 μM of each inhibitor. The solution was then incubated for 30 min at room temperature

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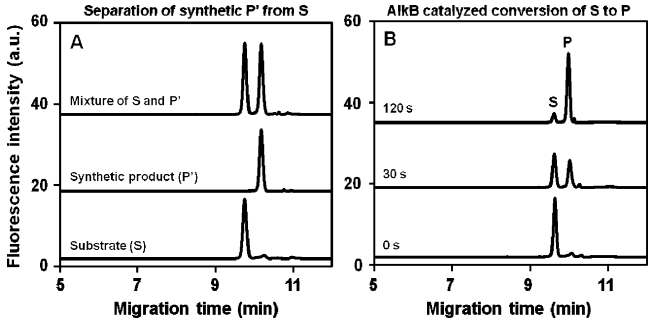

Figure 2. CE separation of a single-base-methylated, 15-nt-long, and fluorescently labeled ssDNA substrate (S) from synthetic demethylated DNA product (P′) (panel A) and from demethylated product (P), which is formed from the substrate after 0, 30, and 120 s of the AlkB-catalyzed reaction (panel B).

prior to ESI-MS analysis. Data were acquired on a Q-TOF mass spectrometer (Q-TOF micro, Micromass, Altrincham, U.K.) interfaced with a NanoMate (Advion Biosciences, Ithaca, NY) with a chip voltage of 1.70 kV and a delivery pressure 0.25 psi (1 psi = 6.81 kPa). The sample cone voltage was typically 80 V with a source temperature of 40 °C and with an acquisition/scan time of 10 s/1 s. Calibration and sample acquisition were performed in the positive ion mode in the range of 500−5000 m/z. The pressure at the interface between the atmospheric source and the high vacuum region was fixed at 6.60 mbar. External instrument calibration was achieved using sodium iodide. Data were processed with the MassLynx 4.0 (Waters).

RESULTS AND DISCUSSION

The choice of substrate for our analyses was based on the following considerations: (i) the use of single-stranded (ss) rather than double-stranded DNA because it is favored by AlkB;[15] (ii) the use of a sequence with nonpairing bases in order to minimize secondary structure and hybrid formation; (iii) the use of a single-methylated base because on average, in a healthy organism, only one modified nucleotide is found per 10^6−10^8 DNA bases;[16] (iv) the choice of DNA length of 15-nt (sufficient for enzymatic activity but short enough to allow the challenging separation of the substrate from the product); and (v) the labeling of DNA with fluorescein at the 3′ end to make the substrate and product detectable. To minimize possible interference of the dye with enzymatic activity, we shifted the methylated base closer to the 5′ end. The substrate 5′-TTC$_m$TTTTTTTTTTT-fluorescein-3′ (where C$_m$ is 3-methylated cytosine) was used in this work as it satisfied the five considerations.

Slab gel electrophoresis is a typical approach for separation of DNA; however, our attempts to separate the product from the substrate by this method failed due to the lack of difference in the lengths and conformations of the product and substrate (data not shown). To our surprise, we were able to separate this substrate from the product by gel-free CE (Figure 2A). Because peak areas in CE are directly proportional to the quantities of the corresponding species, our method can be used for quantitative analyses (Figure 2B).

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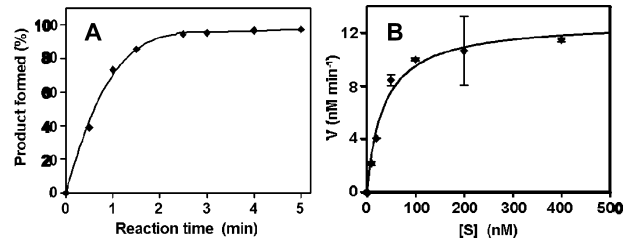
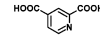
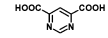
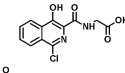
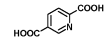


Figure 3. Kinetics of AlkB-catalyzed DNA demethylation studied using the CE-based method. Panel A shows the time dependence of the formation of the demethylated product. Panel B depicts the Michaelis−Menten plot. [S] is the initial concentration of the substrate, and V is the initial reaction rate.

Table 1. IC_{50} Values of Compounds Tested for Inhibition of AlkB Using the New Method[a]

No	Structure	IC_{50} (μM)
1		35 ± 5
2		58 ± 7
3*		64 ± 8
4*		75 ± 2
5*		84 ± 4
6*		121 ± 5
7*		126 ± 1
8*		140 ± 18
9		149 ± 18
10*		191 ± 24
11*		193 ± 1
12*		260 ± 6
13*		479 ± 62
14*		>> 1,000
15*		No inhibition for 1,000
16*		No inhibition for 1,000

[a] The * indicates compounds never previously tested for AlkB.

To validate our CE-based method, we used it to study the kinetics of AlkB-catalyzed DNA demethylation. The time dependence of product formation followed classical Michaelis−Menten kinetics (Figure 3A). Under our experimental conditions, the substrate was completely converted into the product within three minutes as illustrated in Figure 3B. The values of K_m and k_{cat} determined by fitting the curve in Figure 3B were 35.3 ± 6.6 nM and 2.6 ± 0.3 min^{-1}, respectively.

To investigate the utility of our method for the identification of demethylase inhibitors, we then measured IC_{50} values for 3 known and 13 potential 2OG analogue inhibitors of AlkB (Table 1). Consistent with the prior report employing a radioactive 2OG turnover assay,[6] we found that **1**, **2**, and **9**

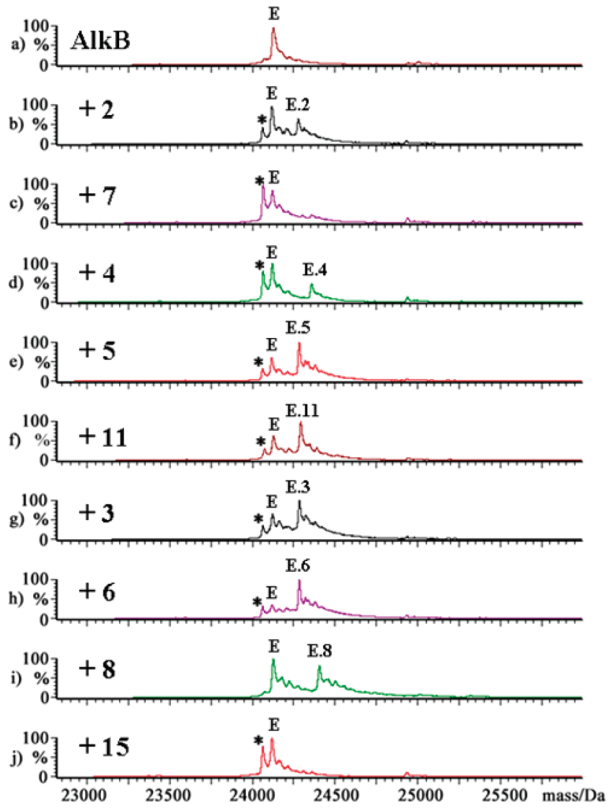


Figure 4. Nondenaturing ESI-MS data of AlkB in complex with inhibitors after 30 min incubation at room temperature. Spectra represent (a) AlkB·Fe(II) (labeled E) in the presence of compound (b) **2**, (c) **7**, (d) **4**, (e) **5**, (f) **11**, (g) **3**, (h) **6**, (i) **8**, and (j) **15**. The * represents apo-AlkB [AlkB without Fe(II)].

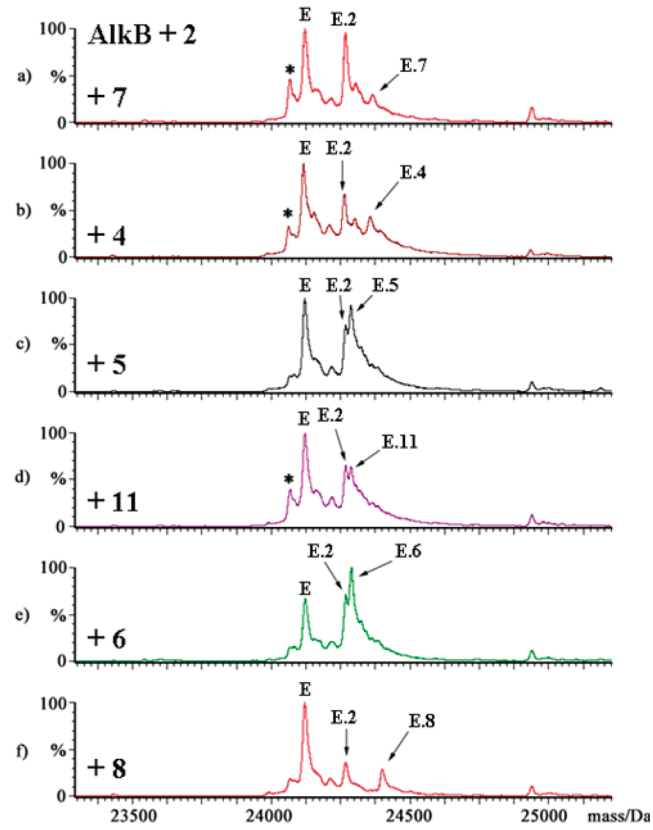


Figure 5. Nondenaturing ESI-MS data on AlkB·Fe(II) (labeled E) showing the binding competition of NOG (**2**) and an equimolar amount of (a) **7**, (b) **4**, (c) **5**, (d) **11**, (e) **6**, and (f) **8**. The * represents apo-AlkB [AlkB without Fe(II)].

were AlkB inhibitors. We also identified new inhibitors (**3**−**8** and **10**−**13**), six of which (**3**−**8**) were as potent as or more potent than those previously reported (**1**, **2**, and **9**). In agreement with the previously published results, the *S*-stereoisomer of N-oxalylglycine (**1**) showed better inhibiting potency than **9** (*R*-stereoisomer). The substitution of the methyl group on **1** with a benzyl moiety with the same stereochemistry decreased the inhibiting potency of **4**. As expected, **7**, which is the *R*-stereoisomer of **4**, had a significantly lower IC_{50} value, confirming the importance of *S*-stereochemistry within this group of AlkB inhibitors.

Because some of the tested inhibitors can chelate Fe(II) in solution as well as at the AlkB active site, in the case of **4**, **5**, **6**, **8**, **10**, and **14**, assays were carried out at variable Fe(II) concentrations. We found that inhibition by these compounds was unaffected by an increase of Fe(II) concentration (data not shown). The results indicated that the mode of inhibition by these compounds is not, at least not predominantly, due to Fe(II) chelation in solution.

To validate the binding of the inhibitors to AlkB, we used nondenaturing electrospray ionization mass spectrometry (ESI-MS).[17] Representative spectra for some of the inhibitors are presented in Figure 4. Competitive binding experiments were then performed to rank the 11 inhibitors that formed the strongest complexes with AlkB. Representative spectra for some of the competitive binding experiments are presented in Figure 5. Compounds **12**−**16**, which showed the lowest inhibition activity by the CE-based assay, were also identified as weak AlkB binders by ESI-MS. The remaining 11 compounds (**1**−**11**), which exhibited inhibition activity, bound to AlkB stronger than **12**−**16**. Competitive binding experiments were then performed to rank the AlkB-binding abilities of the inhibitors. Pairs of inhibitors were mixed with AlkB and allowed to compete with each other for binding to the AlkB active site, generating a rank order of binding. This order (**5** = **6** > **2** = **11** > **8** > **3** > **10** > **9** = **1** = **4** > **14**) correlated reasonably well with the ranked order of inhibition (Table 1). The imperfect correlation is likely in part due to the difference in conditions employed by the CE and ESI-MS methods.

To conclude, we outline the major advantages of the new quantitative method for DNA demethylating enzymes. The method allows direct quantitation of DNA product and substrate differing by a single methyl group. Advantageously, it does not require radioactive labeling or a coupled enzymatic reaction to analyze coproduct formation. The volume of the reaction mixture needed for a single assay is in the submicroliter scale, making the method highly economical. If combined with the recently revealed "inject-mix-react-separate-and-quantitate"

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approach,[18,19] the method will consume only nanoliter volumes of reactants per assay. This method is highly sensitive; the minimum amount of product that can be detected depends on the fluorescent label and detector used and can be as low as 100 molecules.[20] The method is also fast; separation takes only 10 min. Moreover, it does not depend on mechanism-specific coproducts, making it applicable to the discovery of new demethylating or methylating enzymes. Finally, the method is not limited to N/O DNA methylation and should be applicable to other DNA and RNA modifications.

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