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Kinetic studies of *Escherichia coli* AlkB using a new fluorescence-based assay for DNA demethylation

Todd W. Roy and A. S. Bhagwat*

Department of Chemistry, Wayne State University, Detroit, MI 48202, USA

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ABSTRACT

The *Escherichia coli* AlkB protein catalyzes the direct reversal of alkylation damage to DNA; primarily 1-methyladenine (1mA) and 3-methylcytosine (3mC) lesions created by endogenous or environmental alkylating agents. AlkB is a member of the non-heme iron (II) α-ketoglutarate-dependent dioxygenase superfamily, which removes the alkyl group through oxidation eliminating a methyl group as formaldehyde. We have developed a fluorescence-based assay for the dealkylation activity of this family of enzymes. It uses formaldehyde dehydrogenase to convert formaldehyde to formic acid and monitors the creation of an NADH analog using fluorescence. This assay is a great improvement over the existing assays for DNA demethylation in that it is continuous, rapid and does not require radioactively labeled material. It may also be used to study other demethylation reactions including demethylation of histones. We used it to determine the kinetic constants for AlkB and found them to be somewhat different than previously reported values. The results show that AlkB demethylates 1mA and 3mC with comparable efficiencies and has only a modest preference for a single-stranded DNA substrate over its double-stranded DNA counterpart.

INTRODUCTION

DNA can be damaged by many endogenous and exogenous agents leading to mutations, disease or death. A major source of damage to DNA occurs from reaction with alkylating agents, endogenous sources of which include lipid peroxidation and exogenous sources include atmospheric halocarbons. These agents cause damage through nucleophilic additions to the nucleic acid bases and the phosphodiester backbone of DNA (1). Cells contain multiple mechanisms for the repair of alkylation damage to DNA, including removal and replacement of the damaged nucleotides, and direct reversal of the damage (1–3). The importance of these repair activities is demonstrated by their conservation from bacteria to mammals.

The AlkB protein of *Escherichia coli*, and its orthologs in other organisms, catalyzes the removal of alkyl groups from DNA through oxidation in the form of aldehydes and its major substrates are 1-methyladenine (1mA) and 3-methylcytosine (3mC). Additionally, it also repairs 3-methylthymine, 1-methylguanine, 1,N^6-ethenoadenine and 3,N^4-etheno cytosine (1,4–6). These lesions occur preferentially in single-stranded (SS) DNA due to the availability of affected atoms (N1 of purines and N3 of pyrimidines) to participate in S_N2 type additions. In duplex DNA, these atoms are involved in Watson–Crick base pairing and are inaccessible. AlkB is a member of the non-heme iron (II) α-ketoglutarate-dependent dioxygenase superfamily, which reverses alkylation damage to DNA through oxidation of the attached alkyl group creating a hydroxyalkyl intermediate that is unstable and decomposes to an oxidized alkylgroup (an aldehyde) and the regenerated base (5,7,8). The general scheme for this reaction is shown in Figure 1.

Some of the previous assays developed to characterize the activity of AlkB class of DNA dealkylating enzymes have relied on gel electrophoresis of radiolabeled DNA, or analysis of non-radioactive DNA using HPLC or mass spectrometry (5,6,8–12). These methods are discontinuous, time-consuming, require special sample preparation and waste handling, and in some cases require highly specialized equipment. These assays have been used to determine some kinetic constants for AlkB and its orthologs (10,12–14).

To facilitate studies of this important class of DNA repair enzymes, we developed a much simpler assay for DNA demethylation. This assay relies on the production of a fluorophore coupled to the repair of alkylation lesions in DNA by AlkB and is a significant modification of an assay developed to detect formaldehyde (15). Demethylation of DNA by AlkB creates one formaldehyde molecule for every methyl group oxidized. Formaldehyde can then be oxidized to formate by formaldehyde dehydrogenase (FDH) and in the process

*To whom correspondence should be addressed. Tel: +1 313 577 2549; Fax: +1 313 577 8822; Email: axb@chem.wayne.edu

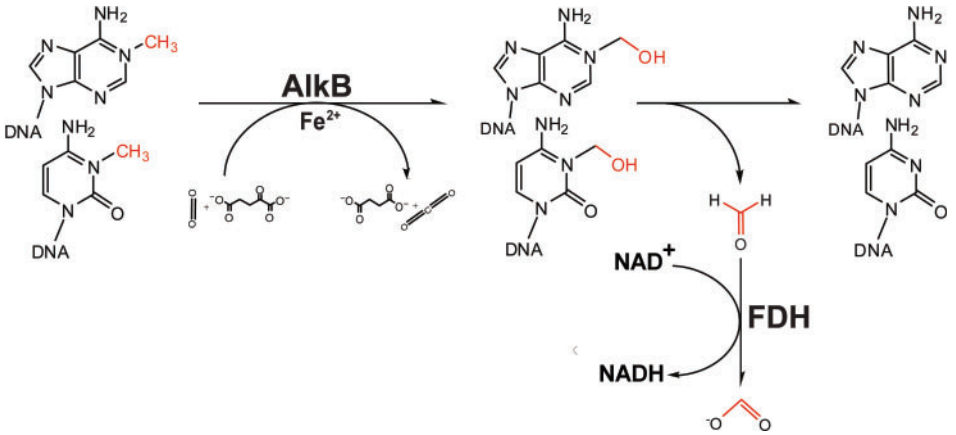

Figure 1. Oxidative demethylation of DNA by AlkB coupled with FDH reaction. The demethylation#of 1mA or 3mC in DNA by AlkB and production of formaldehyde are shown schematically. Also shown are the hydroxylated base intermediates and the conversion of formaldehyde to formate by FDH through a coupled reaction. In the process, coenzyme NAD^+ is converted to NADH.

coenzyme NAD^+ becomes reduced to NADH. Lizcano *et al.* (15) monitored NADH production using its absorption at 340 nm, a wavelength at which NAD^+ does not show significant absorption (16). This property has been used previously to detect production of formaldehyde in the AlkB reaction, but has not been used for quantitative studies (14). We have made two important changes to this assay, replacing NAD^+ with a more stable analog and using the fluorescence property of the reduced cofactor for quantifying the reaction. These and other changes have led to signal stability and greater sensitivity allowing us to use this assay for studies of enzyme kinetics. We have used this assay to determine the *E. coli* AlkB substrate preferences and kinetic constants for four different DNA substrates.

MATERIALS AND METHODS

Strains and genomic DNA

HK82 [thr-1 araC14 leuB6(Am) DE(gpt-proA)62 lacY1 tsx-33 qsr′-0 glnV44 galK LAM- Rac-0 hisG4(Oc) rfbC1 mgl-51 rpoS396(Am) rpsL31(strR) kdgK51 xylA5 mtl-1 argE3(Oc) thi-1 nalA alkB22] was a kind gift from M. Sekiguchi. BH249 (=HK82λDE3) was constructed using the λDE3 Lysogenization Kit from Novagen (Madison, WI, USA). *Escherichia coli* strain BL21(DE3) was obtained from Novagen (Madison, WI, USA) *Psuedomonas putida* KT2440 genomic DNA was obtained from American Type Culture Collection (Manassas, VA, USA).

Chemicals and oligonucleotides

Methylated Substrates 5′-CGTCGXATTCTAGAGCCC C-3′, where X is 1-methyl-dA or 5′-CGTCGAATTXTA GAGCCCC-3′ where X is 3-methyl-dC, were synthesized by ChemGenes Corp (Wilmington, MA, USA). 3-Acetylpyridine adenine dinucleotide ($APAD^+$), α-keto-glutaratic acid, L-ascorbic acid and HEPES were purchased from Sigma–Aldrich Co. (St Louis, MO, USA). Formaldehyde solution (16%) was obtained from Electron Microscopy Sciences (Hatfield, PA, USA).

Expression and purification of *E. coli* AlkB

The *alkB*$^+$ gene was amplified from the *E. coli* B strain BL21 genomic DNA using PCR with the primers 5′-GGT GGTGAATTCATGTTGGATCTGTTTGCC-3′ and 5′-G GTGGTCTCGAGTTATTCTTTTTTACCTGC-3′. The amplification product was cloned into pGEX-4T-3 (Amersham) as an EcoRI–XhoI fragment to create the GST-AlkB fusion plasmid pGAlkB. The plasmids pGAlkB, and pET28a+ were digested with restriction enzymes BamHI and XhoI. The fragment containing the *alkB*$^+$ gene was extracted and purified from agarose gel with the Agarose Gel-Out Kit (EURx Ltd., Gdańsk, Poland). The DNA fragment was then ligated into the digested pET28a+ vector and transformed into *E. coli* strain DH10B. Transformants were selected on LB plates with kanamycin. The resulting plasmid was designated pETAlkB and *alkB* gene was verified by DNA sequencing.

pETAlkB was introduced into *E. coli* strain BL21(DE3) and cells were grown in LB broth at 37°C to an OD_{600} of 0.5. $Fe(NH_4)_2(SO4)_2$ was added to a concentration of 10 μM and the transcription of the fusion gene was induced by adding IPTG to the growth media to 1 mM. Cells were grown for another 3 h and harvested. The cell pellet was resuspended in 20 ml of 1× Ni/NTA Bind buffer [50 mM HEPES pH 8.0, 10 μM $Fe(NH_4)_2(SO4)_2$, 300 mM NaCl and 5 μM imidazole] and frozen at –70°C. The suspension was thawed on ice and sonicated for 10 pulses of 10 s duration separated by 1 min. The resulting lysate was centrifuged for 20 min at 12 000*g* in an Eppendorf 5810R centrifuge to pellet cell debris. The supernatant was then passed over a 500 μl bed volume of Ni/NTA His-Bind resin (Novagen, Madison, WI, USA). The bound protein was washed with three 2 ml volumes of wash buffer [50 mM HEPES pH 8.0, 10 μM $Fe(NH_4)_2(SO4)_2$, 300 mM NaCl and 20 μM imidazole] and eluted with four 500 μl volumes of elution buffer[50 mM HEPES pH 8.0, 10 μM $Fe(NH_4)_2(SO4)_2$,300 mM NaCl and 250 μM imidazole]. The fractions eluted were combined and dialyzed with a 30 kDa MWCO Amicon Ultra dialysis spin column at 4°C with SP–Sepharose running buffer [50 mM HEPES pH 7.3, 10 μM $Fe(NH_4)_2(SO4)_2$ and 2 mM DTT]. The concentrated

protein solution was adjusted to a volume of 2 ml in SP–Sepharose running buffer and applied to an 8 ml SP–Sepharose cation exchange affinity column using an Amersham AKTA FPLC system. Proteins were eluted using a 0–1 M gradient of NaCl and collected in 1 ml fractions. The protein fractions were then analyzed by 15% SDS–PAGE eletrophoresis to identify the fractions containing AlkB. Fractions found to contain AlkB were concentrated and dialyzed with a 30 kDa MWCO Amicon Ultra dialysis spin column at 4°C against storage buffer [100 mM HEPES, pH 8, 20 μM $Fe(NH_4)_2(SO4)_2$, 4 mM EDTA and 6 mM DTT], glycerol was added to 50% v/v, aliquoted into 1 μl aliquots and stored at –70°C. Analysis of 5 μg of the final AlkB concentrate using 12% Tris–Tricine SDS–PAGE followed by staining with Coomassie blue showed to a single protein band (Supplementary Data, Figure S1).

Expression and purification of *P. putida* FDH

The *P. putida* FDH gene was PCR amplified from strain KT2440 genomic DNA (ATCC, Manassas, VA, USA) using the primers 5′-GGGGCATATGTCTGGCAATCG TGGAGTGG-3′ and 5′-GGGGCTCGAGTTACGCCG CACCCCACATTTTGTGC-3′. It was then cloned into pET28a + (Novagen) as an EcoRI–XhoI fragment to create the IPTG-inducible poly(His)–FDH fusion plasmid pETFDH.

The plasmid containing His-Tag FDH fusion, pETFDH was introduced into *E. coli* strain BH249. With the exception of cell culture incubation at 25°C, overproduction and purification over a Ni/NTA affinity column was performed in the same manner as described for AlkB. The eluent fractions from the Ni/NTA column were combined and dialyzed by a 30 kDa MWCO Amicon Ultra dialysis spin column at 4°C using 20 mM Tris buffer, pH 6.5. The concentrated protein solution was resuspended to a volume of 2 ml in 20 mM Tris pH 6.5 and applied to an 8 ml Q–Sepharose anion exchange affinity column on an Amersham AKTA FPLC system. Proteins were eluted using a 0–1 M gradient of NaCl and collected in 1 ml fractions. The protein fractions were then analyzed by 15% SDS–PAGE. The fractions found to contain FDH protein were concentrated and dialyzed with a 30 kDa MWCO Amicon Ultra dialysis spin column at 4°C against storage buffer (100 mM HEPES, pH 8, 4 mM EDTA and 6 mM DTT), glycerol was added to 50% v/v, aliquoted into 12 μl aliquots and stored at –70°C. Analysis of 5 μg of the final FDH concentrate using a 12% Tris–Tricine SDS–PAGE followed by staining with Coomassie blue gave rise to a single protein band (Supplementary Data, Figure S1).

Determination of FDH activity

Purified FDH (26 pmol) and 10 μmol formaldehyde were incubated in reaction buffer [20 mM HEPES, pH 8, 200 μM α-ketoglutarate, 2 mM L-ascorbic acid, 20 μM $Fe(NH_4)_2(SO_4)_2$, 1 mM NAD^+ and 100 μg/ml BSA] at 22°C in a final volume of 100 μl. The production of NADH was monitored for 10 min by measuring absorbance at 340 nm using a Beckman DU 530 UV/Vis spectrophotometer. One unit of FDH activity is defined as 1.0 μmol formaldehyde oxidized per minute under the reaction conditions described above.

Formaldehyde standard curve

A standard curve of formaldehyde concentration versus intensity of APADH fluorescence was prepared by the following method. Various concentrations of formaldehyde (1–20 μM) were incubated with 1 U of FDH, $APAD^+$ (1 mM) in AlkB reaction buffer [20 mM HEPES, pH 8, 200 μM α-ketoglutarate, 2 mM L-ascorbic acid, 20 μM $Fe(NH_4)_2(SO_4)_2$ and 100 μg/ml BSA] in a final volume of 120 μl. The incubations were monitored continuously for the production of APADH with a Varian Eclipse spectrofluorometer (excitation at 363 nm; emission at 482 nm) for 10 min at 25°C. Peak intensity values were plotted against formaldehyde concentration to create the standard plot (Supplementary Data, Figure S2).

Determination of AlkB steady-state kinetics

Double-stranded (DS) methylated substrates were generated by incubating the SS oligonucleotides containing the methyl lesions with 1.5-fold molar excess of the unmethylated complementary oligonucleotide at 37°C for 30 min in annealing buffer (50 mM HEPES, pH 8.0, 10 mM EDTA).

AlkB (final concentration 14 nM) was incubated with increasing concentration of substrate (2.1–42 μM) in the AlkB reaction buffer containing 0.3 U FDH and $APAD^+$ (final concentration 1 mM) in 120 μl. All the components of the reaction except substrate DNAs were mixed together and incubated at 25°C for 5 min. Reactions were initiated with the addition of DNA and were monitored continuously for the production of APADH with a Cary Varian Eclipse spectrofluorometer (excitation at 363 nm; emission at 482 nm). Typically, four reactions were monitored concurrently for 25 min, each set containing one blank (no DNA) and three cuvettes containing identical DNA concentration (i.e. triplicates). Initial reaction velocities for the rate of formaldehyde production were determined using the formaldehyde standard curve (Figure S2). The data was analyzed using the software Prism (GraphPad, San Diego, CA, USA) and K_m and k_{cat} values were determined by non-linear regression curve fitting of the data to the Michaelis–Menten equation.

RESULTS AND DISCUSSION

Development of assay for demethylation

To detect formaldehyde released during the demethylation of DNA, we converted it to formate using FDH. During this conversion NAD^+ is converted to NADH, and the latter molecule is usually detected based on its UV absorbance (17). In our experiments, we used $APAD^+$ instead of NAD^+ as the cofactor for FDH because of its higher redox potential leading to greater stability of the reduced form at various pHs and a higher extinction coefficient which contributes to greater sensitivity (18).

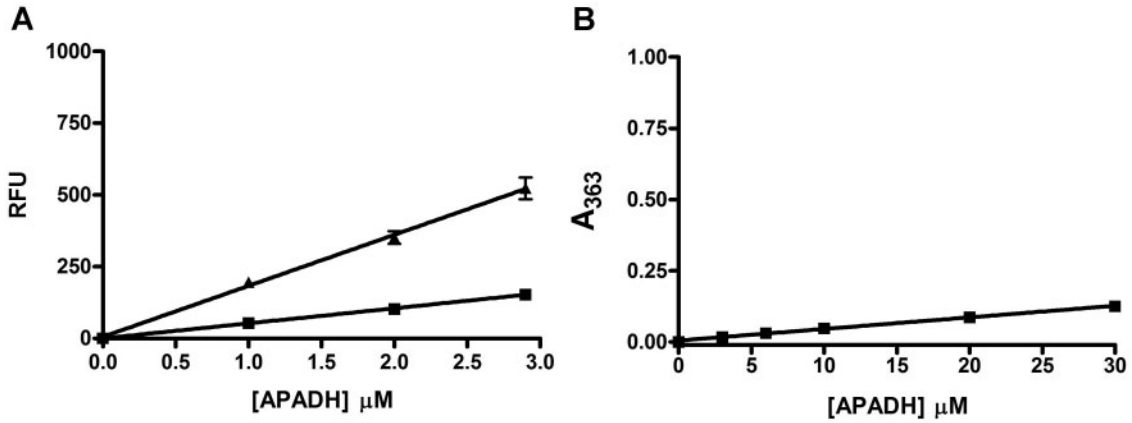

Figure 2. Absorbance and fluorescence properties of APADH. (**A**) Fluorescence intensity (arbitrary units) as a function of concentration. The ordinate is from 0 to 1000 U because this is the maximum range of the spectrophotometer. The excitation at 363 nm and emission was at 482 nm. Fluorescence intensity was determined at two different photomultiplier voltages—800 V (filled triangle); 700 V (filled square). Mean intensity and standard deviation from three independent measurements is shown. (**B**) APADH absorbance at 363 nm as a function of concentration. The ordinate is from 0 to 1.0 because this is the range in which absorbance can be meaningfully measured using most spectrophotometers.

We also chose to use fluorescence properties APADH to develop the demethylation assay because of its greater sensitivity over UV absorbance by a factor of ~30 (Figure 2). Consequently, we were able to lower the amount of methylated DNA base used for the assay from several nanomoles—for a typical UV-absorbance assay—to subnanomole amounts. This allowed us to use synthetic oligonucleotides containing methylated bases in a defined sequence context as substrates for kinetic studies. Most previous studies have used randomly methylated poly-dA (or poly-dC) or DNAs containing both 1mA and 3mC for their assays (2,9,13,14,19,20).

During some preliminary experiments, we discovered that the commonly used buffering agent, Tris, was a substrate for FDH and led to conversion of $APAD^+$ to APADH in the absence of AlkB or methylated DNA substrate in the reaction (Figure 3). This is probably because FDH also acts on some alcohols (21). Tris contains three primary alcohol groups that may be converted to acidic form by FDH. To avoid the contribution of this reaction to background fluorescence, HEPES was used as buffering agent in the reactions instead of Tris.

In coupled enzyme assays, there can be a lag period between the first and the second reaction. This is the time period before the coupling reaction converts the product of the first reaction (formaldehyde, in this case) to the final product (formate). This can affect the measurement of initial rates. However a 'rule of thumb' is that this lag period is negligible if a doubling of the coupling enzyme does not affect the rate of the overall reaction (22). This was found to be the case under the conditions used here (data not shown). In addition, if the maximal velocity of the coupling enzyme (FDH here) is much greater than the enzyme being studied, the lag time becomes inconsequential (23). In this case, the high specific activity of FDH (>250 μmol formaldehyde oxidized $min^{-1} mg^{-1}$) as compared with the specific activity for AlkB (<0.14 μmol $min^{-1} mg^{-1}$) satisfies this criterion.

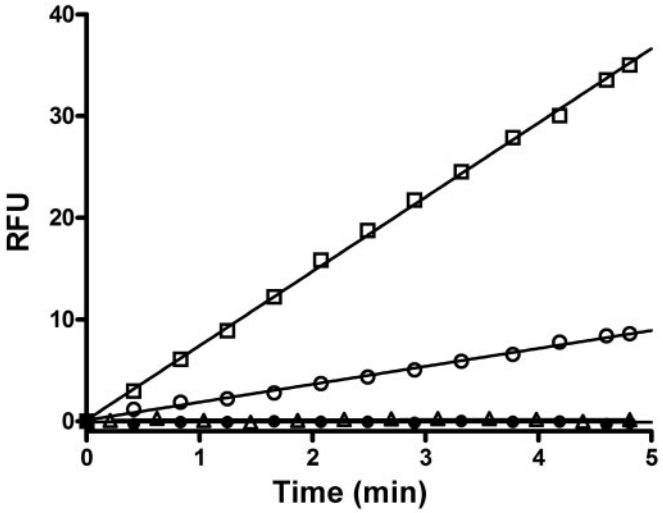

Figure 3. Tris is a substrate for FDH. Utilization of Tris as a substrate by FDH is detected by the coupled conversion of $APAD^+$ to APADH. Fluorescence intensity of resulting APADH is shown as a function of time. No AlkB or DNA was present in these reactions. Reactions with no Tris (filled circle), 0.1 mM Tris (open triangle), 1 mM Tris (open circle) and 5 mM Tris (open square) are shown.

However, we found that commercially available preparations of FDH were not homogeneous and contained material that contributed to background fluorescence even when DNA and AlkB were omitted from the reaction (data not shown). To avoid this problem, the FDH gene from *P. putida* was expressed in *E. coli* and purified to homogeneity (Supplementary Data, Figure S1). The conditions for the complete assay using purified FDH and AlkB enzymes are described in Materials and Methods section.

Steady-state kinetics for *E. coli* AlkB

Four substrates were used for studying AlkB kinetics. Two of them contained a 29-base DNA oligomer with a

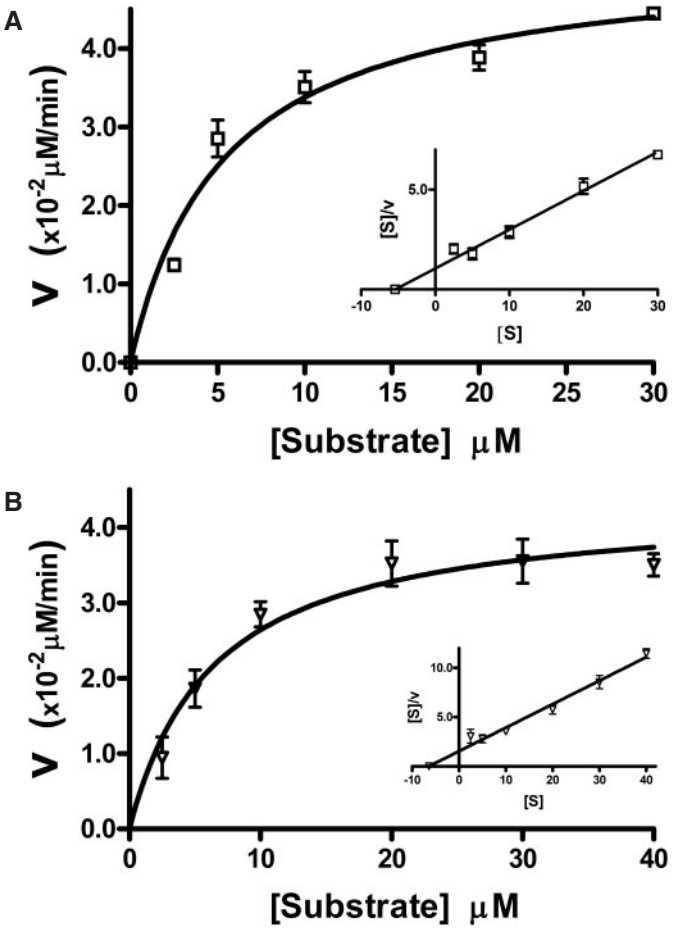

Figure 4. Kinetics of AlkB for DNA with 1mA. (**A**) Initial reaction velocities of AlkB (V) with SS DNA oligonucleotide containing a single 1mA as a function of substrate concentration [*S*]. Mean and standard deviation from three or more separate identical reactions are shown. (**B**) Similar data with DS substrate containing a single 1mA. Inset—the data re-plotted with $[S]/V$ as a function of [*S*].

single 1mA and the other two substrates had the same base sequence but contained instead one 3mC. Unmethylated complementary DNAs were hybridized to the methylated DNAs to create the DS substrates and both SS and DS versions of the methylated DNAs were used for the demethylation assays. The initial velocities of APADH production from three or more independent reactions for five or six concentrations of substrates are shown in Figure 4 and Supplementary Figure S3.

The results were considered reproducible as indicated by the small standard deviations and could be fit to Michaelis–Menten equation or its alternate forms (Figures 4 and S3). It is seen that (Figures 4 and S3, insets) the $[S]/V$ versus [*S*] plots are linear suggesting that the enzyme follows Michaelis–Menten kinetics under these conditions. The k_{cat} and K_m values for AlkB with the four substrates were calculated based on a fit of the *V* versus [*S*] plot to the Michaelis–Menten equation and these values are presented in Table 1. The reproducibility of the data collected using the new assay is evidenced by the small standard deviations seen in the Figures 4 and S3, and in Table 1.

Table 1. Kinetics constants for AlkB

Substrate	Structure	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m (min^{-1} μM^{-1})
1mA	SS	3.7 ± 0.2	5.4 ± 0.9	0.68
	DS	3.1 ± 0.2	6.2 ± 1.3	0.48
3mC	SS	2.2 ± 0.1	3.4 ± 0.6	0.65
	DS	3.3 ± 0.2	9.3 ± 2.4	0.35

The values for k_{cat} and K_m were calculated from *V* versus [*S*] plots shown in Figure 4 and Supplementary Figure S3. Mean and standard deviation from three or more independent sets of data are shown. SS is single-stranded and DS is double-stranded.

Comparison of AlkB kinetic parameters

The k_{cat} and K_m values for the four substrates tested were fairly similar and the range of variation in catalytic specificity of the enzyme for the substrates was <2-fold (Table 1). While this shows that the enzyme does not have a strong preference for any one of the substrates, it did show a small preference for 1mA over 3mC and SS DNA over DS DNA. These results are qualitatively similar to some of the reported substrate preferences for AlkB, but they do show some important differences from the previous reports.

Falnes and colleagues (13) reported that AlkB prefers SS DNA over DS by a factor of 2 to 5. This conclusion was based on experiments where specific activity of AlkB was determined over a range of concentrations using DNA oligomers containing both A's and C's that were randomly labeled with ^{3}H-methyl groups. In contrast, we find that the ratios of catalytic specificities (k_{cat}/K_m) of the enzyme for the SS DNA to DS DNA substrates is only 1.4 for the 1mA and 1.9 for 3mC (Table 1). It is possible that these differences in the conclusions from the two studies may reflect the differences in the base sequences of DNA used in the two studies. Additionally, we also note that the experiments reported by Falnes *et al.* (13) were only semi-quantitative and hence the numbers presented in Table 1 are likely to be more representative of substrate preferences of AlkB.

Koivisto and colleagues (14) studied AlkB using a randomly methylated poly-dA substrate and reported k_{cat} and K_m values respectively to be $11.7 \pm 1.3\,min^{-1}$ and $1.4 \pm 0.2\,\mu M$. This k_{cat} value is ~3-fold higher, while the K_m value is ~4-fold lower than the values reported for SS 1mA-containing DNA we studied (Table 1). Thus the catalytic specificity of AlkB for methylated poly-dA substrate appears to be ~11-fold higher than the 1mA-containing substrate used here. These differences may reflect sequence differences between the two DNAs, but may also be due to secondary structure differences. While the substrate we used is likely to form secondary structure through intra- and inter-molecular hydrogen bonds, poly-dA cannot adopt such conformations. Thus the preference of AlkB for SS DNA may partly explain the differences between the two studies. Additionally, AlkB may act processively on substrates containing multiple methylated DNA bases. If so, the catalytic efficiency of the enzyme for demethylation of randomly labeled substrate would be

higher than that containing a single methylated base such as the substrates used in our studies.

The results reported above add two additional useful insights into AlkB action. First, they show that both 1mA- and 3mC-containing DNA substrates are better substrates in SS form than DS form. Previous work on substrate specificity of AlkB have generally used DNAs containing more than one methylated base. Second, no catalytic constants have been reported for AlkB using a substrate containing 3mC as the only methylated base. The results reported here show that AlkB acts with roughly the same efficiency on the two methylated bases tested, regardless of whether they are in SS or DS form (Table 1).

Summary and future prospects

We have presented here a new assay for DNA demethylating enzymes and illustrated its use by determining AlkB kinetic parameters. The assay uses subnanomole amounts of DNA substrate per reaction, is continuous, rapid and non-toxic. We have done preliminary experiments with human orthologs of AlkB, ABH2 and ABH3 and these experiments show that these enzymes should also be amenable to study using this technique (data not shown). The assay may be adapted to stopped-flow studies and hence should be particularly useful to study the reaction mechanism of this class of enzymes. It may also be used to study mutant enzymes or to study inhibitors of this class of DNA repair enzymes. We have also adapted this assay to 96-well microtiter plate reader (data not shown) and in this format it may be used for high-throughput analysis of demethylating enzymes or their mutants.

We note that this assay is not specific for enzymes that demethylate DNA. Oxidative demethylation of histones methylated at lysines can also generate formaldehyde (24), which can be detected using FDH and $APAD^+$ as described here. In fact Shi *et al.* (25) used FDH and the generation of NADH to monitor the production of formaldehyde by the histone demethylase LSD1. However, they used UV absorption at 340 nm to quantify NADH. Instead, using $APAD^+$ and measuring fluorescence would improve the sensitivity and stability of this assay. Finally, many oxidative detoxification reactions in the liver also create formaldehyde and these enzymes could also be studied using the same assay. Thus the fluorescence-based assay for DNA demethylation described here may be adapted to the study of a wide variety of cellular enzymes.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Conflict of interest statement. None declared.

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