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CHARACTERIZATION OF A *TRYPANOSOMA BRUCEI* ALKB HOMOLOG CAPABLE OF REPAIRING ALKYLATED DNA

Jana M. Simmons^{a,1}, Donna J. Koslowsky^b, and Robert P. Hausinger^{a,b,*}

^aDepartment of Biochemistry & Molecular Biology, Michigan State University, East Lansing, MI, United States

^bDepartment of Microbiology & Molecular Genetics, Michigan State University, East Lansing, MI, United States

Abstract

Trypanosoma brucei encodes a protein (denoted TbABH) that is homologous to AlkB of *Escherichia coli* and AlkB homolog (ABH) proteins in other organisms, raising the possibility that trypanosomes catalyze oxidative repair of alkylation-damaged DNA. *TbABH* was cloned and expressed in *E. coli*, and the recombinant protein was purified and characterized. Incubation of anaerobic TbABH with Fe^{II} and α -ketoglutarate (α KG) produces a characteristic metal-to-ligand charge-transfer chromophore, confirming its membership in the Fe^{II}/ α KG dioxygenase superfamily. The protein binds to DNA, with a clear preference for alkylated oligonucleotides according to results derived by electrophoretic mobility shift assays. Finally, the protozoan gene was shown to partially complement *E. coli alkB* cells when stressed with methylmethanesulfonate; thus confirming assignment of TbABH as a functional AlkB protein in *T. brucei*.

Keywords

Trypanosoma brucei; DNA repair; oxidative demethylation; α -ketoglutarate; ferrous ion

1. Introduction

Trypanosomes are eukaryotic parasites that cause diseases in various mammalian hosts (Barrett et al., 2003). They are evolutionarily divergent organisms that branched early from the phylogenetic tree, before fungi, plants, or animals (Pace, 2009). *Trypanosoma brucei*, the causative agent of African Sleeping Sickness in humans, is an extracellular parasite with a dense exterior protein coat that limits access to the parasite's cellular membrane (Field and Carrington, 2009, Roditi et al., 1998). In bloodstream form cells, the coat protein is expressed from a single allele and changes periodically in a population to evade detection by the host immune system; a process called antigenic variation (Cross et al., 1998). The genes encoding the repertoire of coat proteins are located primarily in the subtelomeric regions among large patches of repetitive sequence and are relocated by homologous recombination

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*Corresponding author: Tel.: (517)-884-5404; fax: (517)-353-8957; hausinge@msu.edu.

¹Current Address: Department of Biochemistry and Molecular Biology, Michigan State University, Grand Rapids, MI, 49503, United States.

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into a promoter-containing expression site as needed (Horn and Barry, 2005, Horn and McCulloch, 2010).

The many repetitive regions in the *T. brucei* genome and the high frequency of recombination events lead to the need for efficient DNA repair and maintenance mechanisms. Components for most of the common DNA repair pathways have been identified in trypanosomes and some have been characterized (Passos-Silva et al.). Proteins homologous to certain elements of the mammalian mismatch repair pathway (Bell et al., 2004), base excision repair (Castillo-Acosta et al., 2008), nucleotide excision repair (Lee et al., 2007) and homologous recombination (Glover et al., 2008) are present. One aspect of DNA repair not yet described in trypanosomes involves removal of aberrant methyl groups; such reactions are catalyzed by methylated-DNA glycosylases, alkyl transferases, and oxidative demethylases in other systems (Sedgwick et al., 2007). Of particular interest to the studies described here are the AlkB-type hydroxylases, which have been characterized in *Escherichia coli* and eukaryotes.

E. coli alkB has been studied for its role in the adaptive response to alkylation damage since the 1980s (Kataoka et al., 1983, Kondo et al., 1986). This gene was long known to confer resistance to certain methylating agents, and in 2002 the encoded protein was discovered to be a member of the Fe^{II}/α-ketoglutarate (αKG) dioxygenases (Falnes et al., 2002, Trewick et al., 2002). The enzyme catalyzes the N-dealkylation of 1-methyladenine and 3-methylcytosine in DNA by using the oxidative demethylase mechanism shown in Figure 1 in which the unstable hydroxylated intermediate spontaneously releases an aldehyde to regenerate the native base. AlkB repairs the analogous lesions in RNA (Aas et al., 2003), including mRNA and tRNA (Ougland et al., 2004). Furthermore, the enzyme dealkylates 1-methylguanine, 3-methylthymine, 3-methylguanine, and several etheno adducts of DNA (Delaney and Essigmann, 2004, Delaney et al., 2005, Koivisto et al., 2004, Mishina et al., 2005).

Eukaryotes often possess several AlkB homologs (termed ABH) that may be expressed in different tissues or localize differentially in the cell (Tsujikawa et al., 2007) and that function in a variety of different roles. Focusing just on the human proteins, ABH1 demethylates 3-methylcytosine, but not 1-methyladenine, in DNA and RNA (Westbye et al., 2008), and additionally it possesses a DNA lyase activity that is specific towards abasic sites (Müller et al., 2010). ABH2 and ABH3 catalyze the same type of oxidative dealkylation reactions as AlkB (Duncan et al., 2002, Koivisto et al., 2004), with ABH3 exhibiting preference for RNA substrates (Aas et al., 2003, Falnes et al., 2004, Ougland et al., 2004). No functional studies have yet been reported for ABH4, ABH5, ABH6, or ABH7. ABH8 is a multi-domain protein with tRNA methyltransferase (Fu et al., 2010) and 5-methoxycarbonylmethyluridine hydroxylase (Fu et al., 2010, van den Born et al., 2011) activities. Finally, the more distantly related *FTO* (fat mass and obesity associated) gene encodes an oxygenase that acts weakly on 3-methylthymine and 3-methyluracil in DNA and RNA (Gerken et al., 2007, Jia et al., 2008, Sanchez-Pulido and Andrade-Navarro, 2007) and more efficiently on 6-methyladenosine in RNA (Jia et al., 2011).

Here, we characterize a trypanosomal AlkB homolog (TbABH), confirm its membership in the family of non-heme iron and α-ketoglutarate dependent hydroxylases, and demonstrate its ability to functionally replace AlkB in *E. coli*.

2. Materials and methods

2.1. Gene identification and multiple sequence alignment

The Basic Local Alignment Search Tool (Altschul et al., 1997) was utilized to search the protein-encoding sequences of the *Trypanosoma brucei brucei* genome with the protein sequence of *E. coli* AlkB as the query, resulting in the identification of a sequence with the NCBI accession number XP_844196. The sequence of the identified trypanosomal AlkB homolog (TbABH) was aligned by using Clustal W (Thompson et al., 1993) to presumed orthologs from *Trypanosoma cruzi* and *Leishmania major* (EAN89336.1 and CAJ03488.1, respectively), representative group 1A AlkB sequences (van den Born et al., 2009) of *E. coli* (NP_416716), *Brucella abortus* (ZP_05894130.1), *Pseudomonas putida* (AAN69003.1), *Pseudomonas syringae* (NP_792910.1), five human AlkB paralogs (ABH1, AAH25787.1; ABH2, Q6NS38.1; ABH3, Q96Q83.1, ABH8, Q96BT7.2; and FTO, NP_001073901.1), and related proteins from a variety of eukaryotes (*Ixodes scapularis*, XP_0002405982.1; *Drosophila melanogaster*, LD02396p; *Schizosaccharomyces pombe*, CAA18657.3; and *Arabidopsis thaliana*, AEE28784.1). The TbABH sequence also was analyzed by using several online servers to predict the protein's subcellular location: LOCTree (Nair and Rost, 2005), PSORTII (<http://psort.hgc.jp/form2.html>), SubLoc (Hua and Sun, 2010), and ESLPred (Bhasin and Raghava, 2004).

2.2. Cloning

A 991-bp DNA fragment containing *TbABH* was amplified by PCR using genomic *T. b. brucei* strain 427 DNA as a template, a forward primer (5'-AGGATATACCCATGGAAGACCC-CGTGC-3' which introduces an NcoI restriction site, underlined), a reverse primer (5'-GAGCA-TCCTCGAGTTCGTTAAGGAACCTCAC-3' with a XhoI site), and a *Taq* polymerase master mix kit (Promega) which leaves a single 3' adenine nucleotide overhang. The PCR product was treated with a PCR clean up kit (Qiagen, Inc.) and ligated into pGEM-T Easy (Promega) to create pGEM-TbABH. The pGEM-TbABH plasmid was transformed into *E. coli* DH5 α (Invitrogen), isolated from several transformants, and sequenced (Davis Sequencing). *TbABH* was excised from the pGEM-T backbone by NcoI and XhoI restriction and ligated into pET28b (Novagen) which had been cut previously with the same enzymes, creating pET-TbABH and putting the coding sequence in frame with a sequence encoding a C-terminal 6-histidine tag. This plasmid was transformed into the expression strain *E. coli* BL21 (DE3).

2.3. Protein production and purification

E. coli BL21 (DE3) cells containing pET-TbABH encoding TbABH-His₆ (hereafter referred to simply as TbABH) were grown at 30 °C in lysogeny broth (LB) supplemented with 100 μ g/mL kanamycin while shaking at ~160 rpm to an optical density of 0.4 to 0.6 at 600 nm. Cultures were induced to overexpress the desired gene by addition of isopropyl- β -D-thiogalacto-pyranoside (IPTG) to 0.1 mM, and grown for an additional 4 h, then harvested at 4 °C by centrifugation at ~8,000 *g* for 8 min. The cell paste was either used immediately for protein purification or stored at -80 °C.

In a typical purification, 3 mL of binding buffer (30 mM imidazole, 10 mM Tris, 150 mM NaCl, pH 7.9) was added per g of cell paste for resuspension. The protease inhibitor phenylmethylsulfonyl fluoride was added to 0.5 mM, cells were lysed by sonication (Branson Sonifier, 3 pulses of 1 min each, 3 W output power, duty cycle 50%, with cooling on ice), and the cell lysates were ultracentrifuged at 100,000 *g* for 1 h. Soluble cell-free extracts were loaded onto a Ni-bound nitrilotriacetic acid column (Qiagen) pre-equilibrated with binding buffer. The column was washed with binding buffer until the baseline was reestablished, and proteins were released with elution buffer (150 mM imidazole, 10 mM

Tris, 150 mM NaCl, pH 7.9). Fractions containing the purified proteins, as determined by denaturing sodium dodecyl sulfate-poly-acrylamide gel electrophoresis (SDS-PAGE, 12% acrylamide) (Laemmli, 1970) and Coomassie staining, were pooled and dialyzed into binding buffer by using 12–14 kDa molecular weight cutoff dialysis tubing (Fisher) at 4 °C overnight with stirring. The concentration of protein was determined by using its calculated molar absorptivity at 280 nm ($45,350 \text{ M}^{-1} \text{ cm}^{-1}$ according to the ExPasy protein parameters prediction server (<http://ca.expasy.org/tools/protparam.html>) (Hulo et al., 2006). The protein was either used immediately for assays or was stored at 4 °C, discarding after two weeks or if precipitation developed. A typical purification yielded approximately 3 mg protein per g of cell paste.

2.4. Gel filtration chromatography and native gel electrophoresis

To determine the native size and oligomeric state of the protein, purified TbABH was concentrated to 200 μM (7.4 mg/mL) of protomer in an Amicon centrifugal filter unit with a 10 kDa molecular weight cutoff and chromatographed on a Superdex®75 size exclusion column pre-equilibrated with binding buffer. Retention times were compared to those of gel filtration standards (BioRad). The collected fractions also were examined by native PAGE using a 3–12% Bis-Tris Blue native gradient gel (Invitrogen) and (50 mM bis-Tris, 50 mM tricine, pH 6.8) to verify the major oligomeric forms of TbABH.

2.5. Spectroscopy

UV/visible spectra were obtained by using an HP 8453 spectrophotometer (Hewlett Packard) equipped with a circulating water bath and magnetic stirrer. TbABH in binding buffer was concentrated to 265 μM of protomer in an Amicon centrifugal filter unit with a 10 kDa cutoff. Stock solutions of 10 mM Fe^{II} and 50 mM αKG were prepared by adding the dry reagents to vials, subjecting them to repeated cycles of vacuum and argon, and adding anaerobic water by syringe. Protein was added to a 1-cm path length, 1.5 mL, quartz cuvette, the sample was made anaerobic by gentle vacuum/argon cycling, and it was scanned while stirring at 9 °C. Four equivalents of αKG were added (1 mM final concentration) and the sample was re-scanned, followed by titration with iron in 8 increments up to 500 μM with scanning after each addition. Data were corrected for dilution in Excel and plotted as difference curves with the absorbance from the protein alone set as the baseline. The difference absorbance of the feature observed at 530 nm (ΔA) was plotted versus the concentration of total added iron ($[\text{L}_t]$) to calculate a K_d for the binding of iron to the αKG -bound active site by using equation 1 (Ryle et al., 1999). In this equation, ΔA_{max} is the predicted maximal absorbance change, $[\text{E}_t]$ is the total enzyme concentration, and n is the number of iron binding sites per protein molecule.

$$\Delta A = \Delta A_{\text{max}} ((K_d + [\text{L}_t] + n[\text{E}_t]) - ((K_d + [\text{L}_t] + n[\text{E}_t])^2 - (4[\text{L}_t]n[\text{E}_t]))^{0.5}) / 2n[\text{E}_t] \quad (\text{Eq. 1})$$

2.6. Electrophoretic mobility shift assays (EMSA)

Gel band shift assays were conducted by incubating 50 ng of a plasmid ds-DNA substrate (7.5 nM in assay) with increasing amounts of TbABH (up to 20 μM) for 1–2 h at 30 °C. This maximum amount of TbABH equates to a final ratio of protein to DNA base pairs of 1:2. These assays were analyzed by electrophoresis in TBE buffer (89 mM each of Tris and borate, 1 mM EDTA, pH 8) using a 0.8% agarose gel and visualized via staining with ethidium bromide. DNA binding was evidenced by a shift in the electrophoretic mobility of the DNA band in the gel upon increasing protein concentration.

Other gel band shift assays were conducted by incubating ^{32}P -labeled oligonucleotides with increasing amounts of TbABH and monitoring the effects on electrophoretic mobility when using a 4% polyacrylamide gel examined via autoradiography. Briefly, oligonucleotides (forward 5'-CGA TCC AGA CTC GAC TAC GCC ATC CGA TCC-3' and reverse 5'-GGA TCG GAT GGC CTA CTC CAC TCT GGA TCG-3') were treated with methyl methane-sulfonate (MMS, to 1.5%) in 20 mM cacodylate buffer, pH 7.5, for 5 h at 31 °C. Ethanol precipitation was used to recover the DNA. 50 pmol of the forward oligo (either untreated or methylated) was treated with 50 μCi of γ - ^{32}P -ATP and 10 units of T4 polynucleotide kinase (Gibco) in the provided buffer for 50 min at 37 °C. DNA was denatured and purified on a 12% agarose gel containing 7 M urea. Gel slices containing the labeled DNA oligonucleotides were excised and the DNA extracted, ethanol precipitated to purify, and quantified by scintillation counting. 50 nM stocks of the DNA substrates used in the binding assays were prepared using 10 kcpm/ μL . For ds-substrate, the labeled forward probe and the reverse oligonucleotide were combined in binding buffer (30 mM imidazole, 150 mM NaCl, 10 mM Tris, pH 7.9) and incubated at 70 °C for 1 min, and then slow cooled to 27 °C to allow hybridization.

For binding assays, 10 nM oligonucleotide was combined with increasing concentrations of TbABH (0–30 μM in 10 μL , up to a 100-fold excess of protein subunit per base pair) containing 20% glycerol as a molecular crowding agent for 1 h at room temperature. Samples were electrophoresed on a 4% polyacrylamide gel containing a 37.5:1 ratio of acrylamide/bisacrylamide in Tris-glycine in a BRL V16-2 system at 20 mA per gel until the dye front was about half way through the gel. Gels were fixed in a solution containing 10% each of methanol and acetic acid and dried by vacuum. Dried gels were exposed to a phosphorescence screen for at least 2 h on a Typhoon 9200 variable mode imager. The images were analyzed using Image Quant, with the count values of the shifted bands normalized to the total counts in each lane, converted to nM shifted DNA, and plotted against the total TbABH concentration in the sample. The resulting data were fit in Sigma Plot to equation 2 for hyperbolic single-site saturation, where B_{max} is the theoretical maximum DNA bound, for estimation of the K_d of DNA binding. The results shown are the average of duplicate experiments.

$$y = B_{\text{max}}[E]/(K_d + [E]) \quad (\text{Eq. 2})$$

To investigate binding of TbABH to another form of damaged DNA, oligonucleotides containing abasic sites were created and used in analogous EMSA studies. Briefly, oligonucleotides (forward 5'-AGTAGACAGCTACCATGCCTGCACGAAGUTAGCAATTTCGTAATCATGGTCATAGCTAGTA-3' and reverse 5'-TACTAGCTATGACCATGATTACGAA-TTGCTAGUTTTCGTGCAGGCATGGTAGCTGTCTACT-3', each containing a single deoxyuridine, in bold) were hybridized by heating to 95 °C for 1 min then allowed to slow cool to room temperature. The ds-DNA was treated with uracil-DNA glycosylase (New England Biolabs) following the manufacturer's instructions to induce formation of abasic sites. For the assays, 1 μM of treated or untreated oligonucleotides was incubated with increasing amounts of TbABH (0–30 μM) in 25 mM Tris, pH 8, containing 50 mM KCl for 20 min at 37 °C. Samples were loaded onto a 6% polyacrylamide gel (19:1 acrylamide:bisacrylamide ratio) and electrophoresed at 90 V in TBE buffer and visualized by staining with ethidium bromide.

2.7. In vitro enzyme assays

Production of formaldehyde from methylated DNA was monitored by using a formaldehyde dehydrogenase-coupled assay which converts the product into formate under conditions that are essentially irreversible (Lizcano et al., 2000). The reaction was monitored spectrophotometrically at 340 nm to follow the coupled reduction of NAD^+ to NADH. Assays containing 1 μM TbABH, 100 μM αKG , 5 μM Fe^{II} , 13 μM methylated 25-mer poly-dA oligonucleotide (~30 μM methylated bases assuming a typical ~10% methylation efficiency), 1 mM NAD^+ , and 0.05 units of formaldehyde dehydrogenase in 50 mM Hepes, pH 8, were mixed in a 300 μL quartz, black walled cuvette and continuously monitored at 340 nm in a Shimadzu spectrophotometer. A series of control experiments were carried out, omitting individual components of the TbABH assay. A standard curve demonstrated that formaldehyde was detectable in a linear fashion from 150 μM down to 5 μM in the assay volume.

Consumption of αKG was monitored by using *ortho*-phenylenediamine (OPDA) which is known to react with α -keto acids (Hayashi et al., 1982). Assays containing 1 μM TbABH, 13 μM methylated poly-dA oligonucleotide, 5 μM Fe^{II} , and 100 μM αKG in 50 mM Hepes, pH 8, were incubated at 37 °C. Samples (225 μL) were taken at selected time points and quenched by addition of 75 μL OPDA solution (10 mg/mL OPDA in 1 M phosphoric acid adjusted to pH 2 with 0.25% β -mercaptoethanol). Quenched samples were heated at 100 °C for 3 min, cooled to room temperature, and monitored at 340 nm in a quartz, black-walled, microcuvette by using a Shimadzu spectrophotometer. The standard curve for the OPDA reaction with αKG was linear from 5 μM to 100 μM .

2.8. Complementation assays

TbABH was PCR amplified from pET-TbABH by using forward (5' - CGTGGAAATTCATGGAAGACCCCGTGC-3') and reverse 5' - GCTCCAAGCTTTCATTCGTTAAGGAACCTCAC-3') primers that introduced EcoRI and HindIII restriction sites (underlined). The DNA containing the gene was excised with these enzymes and ligated into pUC18 to create pUC-TbABH. Because *TbABH* was out of frame in pUC-TbABH, this plasmid was mutagenized by using PCR amplification primers (forward 5' -GATTACGAATTCG ATGGAAGACCCCG-3' and reverse 5' -CGGGGTCTTC CATCGAATTCGTAATC-3') that inserted a base downstream of the EcoRI site (underlined) and before the *TbABH* start site (boxed), placing the gene in-frame with the *lacZa* start site which added 7 extra residues to the N-terminal end of the produced protein. The new construct, denoted pUC-TbABH2, was verified by sequencing (Davis Sequencing). For complementation studies, wild-type BW25113 and BW25113 $\Delta alkB$ cells (Keio collection; <http://ecoli.naist.jp>) (Datsenko and Wanner, 2000) were used as obtained or after being transformed with either pUC18 or pUC-TbABH2. Cultures were grown to exponential phase before plating 100 μL on LB agar containing 200 $\mu\text{g/mL}$ of ampicillin. After allowing the plates to dry, sterile paper discs soaked in 1% or 5% MMS were placed on top of the agar, the plates were incubated overnight at 37 °C, and the resulting zones of inhibition were analyzed to monitor cell stress according to the Kirby-Bauer disc diffusion method (Bauer et al., 1966).

3. Results and discussion

3.1. Analysis of the TbABH Sequence

The genome of *T. brucei brucei* encodes a full length ortholog of bacterial AlkB or eukaryotic ABH proteins (Fig. 2) that we have termed *T. brucei* AlkB homolog (TbABH). This protein was predicted to be localized to the nucleus by the ESLPred, SubLoc, and PSORTII online prediction servers, and further identified as a DNA-binding protein by the

LocTree program. The most conserved regions are located in the predicted double-stranded β -helix core, a defining feature of this enzyme family, which includes the putative metal-coordinating facial triad residues (His 213, Asp 215, and His 269) as well as the probable α KG-stabilizing Arg 305 and Arg 311 (Fig. 2). The sequences shown include orthologs of TbABH present in two other kinetoplastids (*Trypanosoma cruzi* and *Leishmania major*), a range of other eukaryotes [i.e., proteins of yet undefined function in tick (*I. scapularis*), fruit fly (*D. melanogaster*), fungus (*S. pombe*), and plant (*A. thaliana*)], selected human homologs (ABH1, ABH2, ABH3, ABH8, and FTO are shown, but ABH4-ABH7 also are related), and representative bacterial AlkB. TbABH is most closely related to the other protozoan proteins, followed by the set of eukaryotic proteins along with ABH1 [a closer relationship to ABH1 than ABH3 or ABH8 was previously noted (van den Born et al., 2008)], then the bacterial sequences (e.g., *E. coli* AlkB is 23% identical), and finally the other human ABH proteins.

In an extension of the sequence analysis, we examined TbABH for matches to key residues of related proteins of known three-dimensional structures. *E. coli* AlkB crystal structures (Holland and Hollis, 2010, Yang et al., 2008, Yu et al., 2006) identified a Thr 51-Pro 52-Gly 53 motif important to sugar ring inversion and methylbase flipping, showed Asp 135 and Glu 136 are involved in methyladenine binding, indicated that Trp 69 and the His 131 metal ligand acted to sandwich the modified base, and found that a ~90-nucleotide recognition lid assists in binding ss-DNA, ss-RNA, or one strand of ds-DNA. Overall, 15 AlkB residues interact directly or indirectly with the lesion strand (Thr 51, Gly 53, Thr 56, Ser 58, Val 59, Arg 73, Gln 74, Tyr 76, Lys 127, Ser 129, Leu 130, Gln 132, Lys 134, Asp 136, and Arg 210) and another six (Phe 156, Arg 161, Asn 162, Asp 163, Arg 167, and Gln 190) with the complementary strand (Sundheim et al., 2008). These key AlkB amino acids are underlined Fig. 2. TbABH lacks the triplet motif, has a similar Ser 217-Glu 218 pair, contains a corresponding Trp 151 and His 213 sandwich, and lacks almost all comparable residues specific to the lesion strand while retaining two matches for residues that bind the complementary strand (Arg 143 and Asp 145). ABH2 uses 24 residues to bind ds-DNA substrate: Arg 110, Gln 112, Tyr 122, Ser 125, His 167, Gly 169, Glu 170, Arg 172, Asp 174, and Glu 175 on the lesion strand and Gln 100, Val 101, Phe 102, Gly 103, Trp 105, Ser 107, Arg 198, Gly 204, Arg 215, Tyr 235, Val 240, Arg 241, Lys 242, and Lys 2443 on the complementary strand (see underlined residues in Fig. 2)(Yang et al., 2008). Phe 102 is of special interest because it intercalates into the DNA to induce base flipping. Only three potentially corresponding residues are found in TbABH. ABH3 was crystallized in the absence of substrate; however, a potential ss-DNA or RNA binding groove was noted with Arg 122, Glu 123, and Asp 124 forming an extended hairpin that serves as an outer wall of the groove and three residues (Glu 123, Asp 189, and Asp 194) positioned at the groove entrance (Sundheim et al., 2006). Again, these residues are not obviously found in TbABH. ABH8, which acts on tRNA, has an RNA recognition motif in a C-terminal domain and a disordered nucleotide recognition lid (Pastore et al., 2012). These features are not apparent in TbABH. Finally, the RNA-binding protein FTO was reported to possess an extra 12-residue loop (residues Pro 213 – Gly 224) that protects against binding to ds-DNA (Han et al., 2010). As seen in Fig. 2, TbABH includes three residues of this loop sequence. The limited sequence similarities noted above make it difficult to speculate on the specific contributions of particular TbABH residues to substrate binding. Thus, identification of residues conferring substrate specificity to TbABH awaits analysis of its crystal structure.

3.2. General biochemical properties of TbABH

To characterize this protein, *TbABH* was cloned from *T. b. brucei* genomic DNA, the gene was expressed recombinantly in *E. coli*, and the protein was purified by affinity chromatography to ~90% purity as visualized via SDS-PAGE and Coomassie staining (data

not shown). Significantly, endogenous AlkB (subunit M_r of approximately 24,000) was absent in the purified TbABH sample (subunit M_r of ~37,000). PAGE analysis indicates that multiple forms of the protein were present as purified from *E. coli* (Fig. 3). The predominant peak (panel A) chromatographed as a monomer while the less prominent peak eluted as a dimer; however, blue native gel electrophoresis of the fractions was most consistent with each protein sample equilibrating to form equivalent mixtures of 37 kDa monomers, 74 kDa dimers, and 148 kDa tetramers. Of interest, human ABH8 resembles this behavior: it equilibrates between a monomer and dimer in solution, with a tetrameric species observed by crystallography (Pastore et al., 2012). In contrast, other characterized AlkB and ABH proteins are monomers. The significance of TbABH multimerization, if any, remains unknown.

To confirm proper folding of the recombinant TbABH, purified protein was examined for its ability to form a characteristic chromophore associated with binding of the iron cofactor and the α KG cosubstrate in other members of the $\text{Fe}^{\text{II}}/\alpha\text{KG}$ -dependent dioxygenases (Ryle et al., 1999). The protein was monitored by UV-visible spectroscopy under anaerobic conditions, using low temperature to maintain stability, while titrating in αKG and Fe^{II} . As shown in Figure 4, anaerobic TbABH generated the diagnostic metal-to-ligand charge-transfer transition at 530 nm when both metal and cofactor were present. The extinction coefficient for this feature was approximately $190 \text{ M}^{-1} \text{ cm}^{-1}$, consistent with values previously described for members of this enzyme family (Hegg et al., 1999, Ryle et al., 1999). By plotting the relative absorbance change at 530 nm versus the total concentration of iron titrated into the protein sample and fitting to equation 1, an Fe^{II} K_d of $\sim 4 \mu\text{M}$ was calculated along with an estimation of 1.3 ± 0.07 iron atoms binding per active site.

3.3. DNA binding by TbABH

A DNA repair protein is expected to bind DNA; thus, the ability of TbABH to bind DNA substrates was tested. TbABH bound to supercoiled ds-plasmid (pGEX5) DNA as evidenced by a shift in the electrophoretic mobility of the DNA band in an agarose gel (Fig. 5A). The development of multiple TbABH-DNA complexes confounded efforts to quantify the thermodynamics of this interaction; therefore, EMSA assays were performed using ^{32}P -labeled oligonucleotides to obtain more quantitative binding data. Of great significance, such studies included untreated ss-DNA and ds-DNA as well as DNA samples that had been subjected to methylation by MMS. TbABH addition weakly shifted ss-polynucleotides with no significant effect of methylation (data not shown), but showed little binding to non-methylated ds-DNA while exhibiting a marked preference for binding to methylated ds-DNA (Fig 5B). The band intensities were quantified, normalized to the total ^{32}P counts in each lane, and converted to nM DNA bound. These values were plotted against the concentration of added TbABH and the resulting curve was fit to a hyperbolic single-site binding equation (equation 2). This analysis allowed estimation of a K_d of $7.1 \pm 1.9 \mu\text{M}$ (Fig 5C). Although these assays contained 10 nM DNA, the process used for methylating the substrate typically exhibits poor efficiency ($\sim 10\%$); thus, the finding that saturation (B_{max}) corresponded to $0.79 \pm 0.08 \text{ nM}$ DNA indicates near stoichiometric binding ($\sim 80\%$) of the methylated substrate. In additional EMSA studies, the presence of an abasic site in DNA was shown to not enhance binding to TbABH, whereas this type of interaction has been noted for human ABH1 (Müller et al., 2010).

3.4. Examination of DNA repair by TbABH

Two *in vitro* assays were used in attempts to obtain direct evidence for the demethylation reaction expected of a functional AlkB. A formaldehyde dehydrogenase-coupled reaction detects released formaldehyde by monitoring increases in absorbance at 340 nm due to the reduction of NAD^+ to NADH as part of the coupled reaction. In addition, αKG consumption

was monitored by a colorimetric OPDA assay. Unfortunately, neither assay provided conclusive evidence for oxidative demethylation of methylated DNA. These negative results indicate that TbABH either cannot catalyze an AlkB-like reaction or that the purified protein is compromised in some manner and that another cellular factor may be required.

As an alternative approach to investigate possible AlkB-like activity by TbABH, the *T. brucei* gene was tested for its ability to complement an *alkB* knockout in an *E. coli* cell line. The *alkB* gene of BW25113Δ*alkB* cells is replaced with a kanamycin resistance cassette, resulting in 3.5-fold greater susceptibility to methylation damage by MMS as evidenced by the decreased viable cell counts over time when exposed to this methylating agent (data not shown). The *alkB* knockout cell line was transformed with pUC-TbABH2 (encoding the trypanosomal protein preceded by 7 extra residues at its N-terminus) or pUC18, and the two transformed cultures and wild-type strain were compared for MMS sensitivity by the Kirby-Bauer disk diffusion method. At 1% MMS, wild-type cells showed no inhibition of growth while the *alkB* knockout exhibited an average zone of inhibition (the distance between the edge of the paper disc and the edge of non-growth) of 4.0 ± 0.7 mm in this particular experiment. The complemented strain containing pUC-TbABH2 partially complemented the knockout strain in a reproducible manner, as evidenced by the reduced zone of inhibition in this particular experiment of 1.5 ± 0.3 mm (Fig. 6).

3.5. Conclusions

Trypanosomes encode a full-length ortholog of *E. coli* AlkB. The protozoan protein exhibits characteristics representative of the Fe^{II} and αKG dependent dioxygenase superfamily of enzymes, including the formation of a diagnostic metal-to-ligand charge-transfer chromophore when incubated with the metal and cofactor under anaerobic conditions. Further, while *E. coli* AlkB binds DNA only very weakly (Dinglay et al., 2000), TbABH forms a tight complex and exhibits a clear preference for alkylated ds-DNA. Although direct *in vitro* evidence for DNA repair was not obtained, an *E. coli* cell line deficient in *alkB* was partially complemented by the expression of *TbABH* under alkylation stress conditions confirming its assignment as a functional AlkB protein.

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Abbreviations

αKG	α-ketoglutarate
MMS	methyl methanesulfonate
ODPA	<i>ortho</i> -phenylenediamine
TbABH	AlkB homolog from <i>Trypanosoma brucei</i>

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Highlights

- *T. brucei* encodes a homolog of *E. coli* AlkB, which repairs alkylation damage of DNA.
- TbABH belongs to the Fe(II) and alpha-ketoglutarate dioxygenase superfamily
- TbABH binds DNA, with a preference for alkylated DNA
- *TbABH* complements an *E. coli alkB* knockout

Fig. 1.
Reactions of AlkB with 1meA and 3meC in DNA or RNA.

EcAlkB	-----MDDLFADEP----	10
BaAlkB	-----MKDLFDNLN----	9
PsAlkB	-----MQRKGDTPATLDFADQTPQPR	22
PpAlkB	-----MIQSDLDLFGPQP----	13
IsABH	-----MEAQSDPFKEEFKFKYKR-RLVRPDLTGVVDALRDDC	35
ABH1	-----MGKMAAAVGSVATLATEPGEDAFRKLFRFYRQSRPGTADLEGVIDFSAAHA	51
DmABH	-----MFKLVFKQYKC-KSTSPDLTEVVKIDDCVV	29
TbABH	MEDPVRCECQKHAQDDGYSQGELLPLTAFRQVEKRYKLYRYDRRTSRLQ-VPETDFSDV	59
TcABH	MEE-----RDSTSPQYVGGENLALTPFRLAEKRYKLYRFDRRAARRQQ-LPETDFSDV	52
LmABH	-----MAEKRYKLYEFSRRETRRRPPPPETDLTDV	30
SpABH	-----MLAGNMEQANVFRLEEKRYKCRADTI PDMSEVLDPNDFQS	40
AtABH	-----MYESANVSDDADRTAFRRAEKKYKLYEYQDSKFSRKKKLPKPIDLSE	47
ABH8	-----MDSNHQSNYKLSKTEKKFLRKQIKAKHTLLRHGDIETVSYATQSLV	46
ABH3	-----MEEKRRRARVQGAWAAPVKSQAIAQATTAKSHLHQKPGQTWKNK	45
ABH2	-----MDRFLVKGAGGGLLRKQEEQEPTGEEPVLGGDKESTRKRP	41
FTO	-----MKRTPTAEEEREREAKKLRLLEELEDTWLPLYLTPKDDIFYQQW	42
EcAlkB	-----WQEPLAAGAVILRRFAFN	28
BaAlkB	-----TREIMAPGAVLLRGFALA	27
PsAlkB	-----RDEQIGPGSWLFSGFALP	40
PpAlkB	-----QRLASHTVLLPGFALA	29
IsABH	SSALRP---RALSEHL-----SQADCEGLGLCAERLPAAYELVDAPGLLLL	78
ABH1	ARGKGPGAQKVIKSQLNVSSV-----SEQNAYRAGLQPVSKWQAYGLKGYPGFIFI	102
DmABH	KGNDQEKSDNNIVQRLSISN-----VESLQTMGPVKSPKDWRTYTLTNHPGIIVI	79
TbABH	VDFRNLEANTAANL-----ACISTRECHEDDLVKCCTFAGVPGMEVF	102
TcABH	VDFRCPDANTDQNR-----SLIRG-VSTLYDSQVRCFEFVAVPGLLFF	94
LmABH	VQLHDLDRNTDENKRLIHRVVLPRKPQHNDGASLSAADAQPGKEVLCTYFAGVPGLLL	90
SpABH	FG-----FEALVEIKPRVFSFQKAPGLLIL	65
AtABH	LLDFNLISQNFNND-----GVLPGDGRVSKVDSSVFCIDNRPGFYFI	90
ABH8	VANGGLGNGVSRNQLLPVLEKGLVDALLMPPNPKPYSFARYRTTESKRAYVTLNKEVV	106
ABH3	EHHLSDREFVFKEPQQ-----VVRRAPEPRVIDREGVYEISLSPTGVSRV	90
ABH2	RR-----EAPGNGGHSAGPSWRHRAEGLDCS	68
FTO	QLKYPKILIREASSVS-----EELHKEVQEAFLTLHKHGCLFRDLVRIQKDLL	91
EcAlkB	AAE-----QLIRDINDVASQSPFRQMVTPGGYTMSVAMTN-----	63
BaAlkB	CEE-----SILSAVARVCAAAPFRHMTTPGGYRMSVAMTN-----	62
PsAlkB	AMP-----TLLLALEQTLGHSPFRHMLTPGGLSMSAALSS-----	75
PpAlkB	ETE-----ALLDALRPVLRAPFRHMYTPGGLRMAVALTN-----	64
IsABH	PNP-----FTAEGQRLWVRRCLEEYTRPPHVTNVKAPAT-----	112
ABH1	PNP-----FLPGYQWHWVKQCLKLYSQPNVCNLDKHSKEETQDLWEQSKEFLRY----	153
DmABH	RNP-----FSEGRRYWSARCLRDFPRTPNIVNLNERLFDESVRSDWWKQLN-----	126
TbABH	PNF-----LTDAEQQEFCRAALLEYGDSSRRHPNHLSTHAS-----	137
TcABH	PDA-----ISEEEQQAFCRDAILRYGDSSQHPNHLSTHAS-----	129
LmABH	PGV-----LSEAEQQRWCRAVLDYADSAKHPNLSLTHAK-----	125
SpABH	KNY-----VSSELOMQLLKSIMFTQIQDPENKTNLSPFYQLPLGNDSIWRRYYNGDGESI	120
AtABH	PDA-----LSLKEQCKWIKESLTSFPQPPNRTNHNAIYGPIDDLFDSAKENKVLVQDDL	145
ABH8	DDLQKITLYLNFVEKVQWKELRPQALPPGLMVVEEIISSSEEEKMLLESVDWT-----	159
ABH3	CLYPG---FVDVKEADWILEQLCQDVPWKQRTGIREDITYQ-----	128
ABH2	YTV-----LFGKAEADEIFQELEKEVEYFTGALARVQVFG-----	103
FTO	TPVSRILIGNPGCTYKYLNTRLFTVPWPVKGSNIKHTAEIAAACETFLKLN-----	143

EcAlkB	-----CGHLGWTTHROGYLYSPIDPQTNKFWPA-MEQ	94
BaAlkB	-----CGPVGWTDRTGYRSPADPETGKFWPS-MPD	93
PsAlkB	-----CGPLGWITDRHGYSVDVDPQTGGQFWPA-MPD	106
PpAlkB	-----CGTLGWSDQHGYRSPSPDFVSGQFWPA-LPP	95
IsABH	-----VLRAGDPFYGALRWATVGLHHDWDT-KVYDKTRRSP-FPD	150
ABH1	-----KEATKRRPRSLEKLRLWTVGYHYNWDS-KKYSADHYTP-FPS	194
DmABH	-----LCSDGVFEQRIKSAMRWTTFGYHHNWDT-KIYDEEMQSP-FPE	167
TbABH	-----EPRETRYEPPMRWATVGFYSQWSS-KSYCKEKYSH-FPR	175
TcABH	-----KPKCTKRYEAPMRWATLGFSYDWT-S-KTYTRENYS-A-FPP	167
LmABH	-----APQSTSCYQPPMRWATLGYSYEWTO-KVYCRDRYST-FPS	163
SpABH	-----IDGLGETKPLTVDRLVHKKLRWVTLGEQYDWTTKKEYPDPSPKSPG-FPK	167
AtABH	NNKWKFYEEVDIEKATRSSCKSVSASVLLRKLRLSTLGLQFDWSKRNVDVSLPHNN-IPD	204
ABH8	-----EDTDNQNSQKSLKHRRVKHFGYEFHYENNNVDKDKPLSGGLEP	202
ABH3	-----QPRLTAWYGELPYTYSRITMEPNPHWHP----	156
ABH2	-----KWHSVPRKQATYGDAGLTYYTFSGLTSPKWPWIP----	136
FTO	-----DYLQIETIQALEELAKEKANEDAVPLCMSADFPVRVGMG	182
EcAlkB	SFHNLCQRAATAAGYPD-----FQPDACLINRYAPC-AKLSLHODKDEPDLRAP	142
BaAlkB	TFLALAQDAARQAGYPD-----FTPDACLINRYEPC-ARLSLHODKDERNFSNP	141
PsAlkB	AFMQLAQSAALAAGYRG-----FVPDACLINRYIEPC-AKMSLHODKDEHHRWP	154
PpAlkB	VLLALASRAAMAGFDG-----FVPDACLVNHYLPC-TRLSLHODRDEQDFGQP	143
IsABH	CLRELATGLARLAGFSA-----FOPEAAIVNYYAMD-SALGGHVDNSELALDAP	198
ABH1	DLGFLSEQVAAACGFED-----FRAEAGILNYYRLD-STLGTHVDRSELDHSPK	242
DmABH	DLSSSLCGLFAQALGYAD-----FKPEAAIVNYYPPVC-STLSGHTDHSEPNKSAP	215
TbABH	RLRKCVIERIARLCNVS-----QP--YEPQTSIVNYYFPVC-AMMAHODVSEEVLOQP	224
TcABH	ALKRRIEEILHLCSSSTD-----LKDVPNSIYEPQTAIVNYYFVC-SMMAHODVSEESMOPH	224
LmABH	ALRTRMCDLVSLVAGVRQDGFCCAYPDVYEPQTAIVNYYFVC-SMMCHODISEETLEOP	222
SpABH	DLGDFVEKVVKESTDFLH-----WKAEAAIVNYYFVC-DTLTSAHIDESEEDLTLP	216
AtABH	ALCQLAKTHAAIAMPDGE-----EFRPEGAIVNYYFGIC-DTLGGHLDMEADWSKP	254
ABH8	ICESFLEKWLKGYIKHK-----PDQMTINYYEPC-QGIPAHIDTHS-AFEDE	248
ABH3	VLRTLKNRIEENTGHTFN-----SLLCNLYRNEKDSVDWHSDEPSLGRCP	202
ABH2	VLERIRDHVSQVGTGQTFN-----FVLINRYKDCDHI GEHRD DERELAPGS	182
FTO	SSYNGQDEVDIKSRAAYNVTLLNFMDPQKMEYLKEEYPFGMCKMAVSWHHDENLVDRSAV	242
EcAlkB	IVS-VSLGLPAIEQFGGLKRNPLK-----RLLEHFGDVVWVGCESR	183
BaAlkB	IVS-VSLGLPATFQFGGMKRTDPIT-----KYILHHGDDVVWVGCEPSR	182
PsAlkB	VVS-VSLGIPAIQFQGMQSRDKTR-----RISLHFGDVVWVGCEDR	195
PpAlkB	IVS-VSLGLPAVLEFGGLQRADKAQ-----RIALSHGDDVLWVGCVDR	184
IsABH	VVS-ASFGQTAVELVVGATRERRPR-----ALLRSGDVLVMSGPAP	239
ABH1	LIS-FSFGQSAIEELLEGLQDEAPT-----AMFMHSGDIMIMSCFSR	283
DmABH	LIS-FSFGQTAIEELIGRSLEEKPT-----AIYLOSQDVMIMSCFSR	256
TbABH	LIS-ISLGCSCVELMGTESRDDTPC-----AFWIRSGDVIVVSCASR	265
TcABH	LIS-ISLGCSCVELMGTSSRDDAPY-----AFWIRSGDVAVFSCPSR	265
LmABH	LMS-LSLGCSCVELMGTQSREDAPY-----AFLIRSGDVAAFTGCPSR	263
SpABH	LIS-LSMGLDCIYLIETESRSEKPS-----ALRTHSGDVVIMTCTSR	257
AtABH	IVS-MSLGCCKATIELLGKSKDDPPH-----AMYIRSGDVVLMAGEAR	295
ABH8	IVS-LSLGCSEIVMDFKHP--DGIAP-----PVMIPRRSLVMTGESR	287
ABH3	IIASLSFGATRTEMRKKPPPEENGDTYV-----ERVKIPDHGTLTIMECATQ	252
ABH2	PIASVSGACRDEVFRHKDSRGKSPS-RRV-----AVVRLPFAHGSLLMNHPTN	231
FTO	AVYSYSCGPEEESDDSHLEGRDPDIWVGFKISWDIETPGLAIPHQDCYFMLDDLN	302

Fig. 2.

Multiple sequence alignment of selected bacterial and eukaryotic AlkB-like proteins. Residues that are conserved among at least 12 of the sequences are shaded black. The metal-coordinating triad and the α KG-stabilizing arginines are indicated by asterisks and pound signs. Residues conserved among all kinetoplastid and/or bacterial sequences are shaded gray. Residues proposed to be significant to substrate specificity in structurally characterized proteins (see main text) are underlined. The arrows above the sequences represent the 8 β -strands that make up the core fold of *E. coli* AlkB (Yu et al., 2006). The sources of the AlkB and ABH sequences are EcAlkB, *E. coli*; BaAlkB, *Brucella abortus*; PsAlkB, *Pseudomonas syringae*; PpAlkB, *Pseudomonas putida*; IsABH, *Ixodes scapularis*; ABH1, human; DmABH, *Drosophila melanogaster*; TbABH, *T. brucei*; TcABH, *Trypanosoma cruzi*; LmABH, *Leishmania major*; SpABH, *Schizosaccharomyces pombe*; AtABH, *Arabidopsis thaliana*; and ABH8, ABH3, ABH2, and FTO from human.

Fig. 3.

Native size and oligomeric state determination of TbABH. **A.** Chromatogram of TbABH analyzed by using a Superdex[®]75 column and compared with BioRad gel filtration standards (retention times of γ -globulin, 158 kDa; ovalbumin, 44 kDa; and myoglobin, 17 kDa, are shown as diamonds above the chromatogram). The bar on the chromatogram indicates the fractions analyzed by gel electrophoresis. **B.** Blue native polyacrylamide gradient gel showing the oligomeric states and native sizes of TbABH. Lanes: M, molecular mass markers; numbers, 5 fractions corresponding to the bar in panel A.

Fig. 4. Spectroscopic evidence for binding of Fe^{II} and αKG by TbABH. **A.** The UV/visible spectrum of anaerobic TbABH (266 μM protomer in binding buffer) was obtained for the sample as isolated (baseline), after adding 1 mM αKG , and while titrating in Fe^{II} with stirring at 9 °C. The (Fe^{II} - αKG -protein minus protein) difference spectra shown correspond to the addition of 0, 63, 125, 188, 250, 313, 375, 438, and 500 μM metal ions. **B.** The intensity of the absorbance difference at 530 nm was examined as a function of added Fe^{II} . The data were fit to equation 1.

Fig. 5. EMSA studies of TbABH and various DNA substrates. **A.** Agarose gel depicting the shift in mobility of supercoiled pGEX5 plasmid (7.5 nM) with increasing TbABH concentration (0 to 20 μM). **B.** Native polyacrylamide gel showing the shift in mobility of ³²P-labeled ds-oligonucleotide (7.5 nM; untreated on left and methylated on right) with increasing TbABH concentration (0 to 30 μM). **C.** Fit to equation 2 of data from methylated oligonucleotide in B.

Fig. 6.

Complementation of an *E. coli alkB* mutant with *TbABH* under alkylation stress. BW25113 wild-type cells containing either pUC18 or pUC-TbABH2 were stressed with 1% MMS according to the Kirby-Bauer disk diffusion method (Bauer et al., 1966). Plates were incubated overnight at 37 °C and the resulting zones of inhibition were measured. A representative experiment is shown for the wild-type cells containing pUC18, $\Delta alkB$ cells containing pUC18, and $\Delta alkB$ cells containing pUC-TbABH2. The zones of inhibition are shown by black lines and white dashed circles.