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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], Robert P. Hausinger [a,b,*]

[a] Department of Biochemistry & Molecular Biology, Michigan State University, East Lansing, MI, United States
[b] Department of Microbiology & Molecular Genetics, Michigan State University, East Lansing, MI, United States

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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 (αKG) produces a characteristic metal-to-ligand charge-transfer chromophore, confirming its membership in the Fe^{II}/αKG 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*.

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 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., 2010). 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 Fig. 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

Abbreviations: αKG, α-ketoglutarate; MMS, methyl methanesulfonate; ODPA, *ortho*-phenylenediamine; TbABH, AlkB homolog from *Trypanosoma brucei*.

* Corresponding author at: Department of Microbiology & Molecular Genetics, 567 Wilson Road, Michigan State University, East Lansing, MI, United States. Fax: +1 517 353 8957.
E-mail address: hausinge@msu.edu (R.P. Hausinger).
[1] Department of Biochemistry & Molecular Biology, Michigan State University, Grand Rapids, MI 49503, United States.

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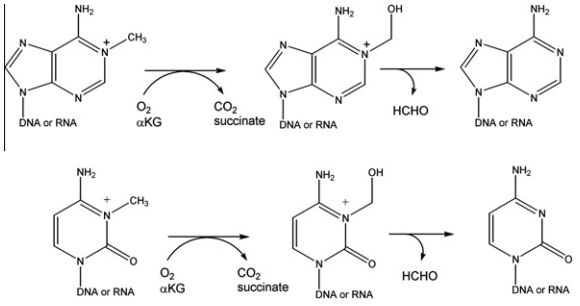

Fig. 1. Reactions of AlkB with 1meA and 3meC in DNA or RNA.

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., 2010a) and 5-methoxycarbonylmethyluridine hydroxylase (Fu et al., 2010b; 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 *T. 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; *Schizosaccharomcyes pombe*, CAA18657.3; and *Arabibdopsis 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′-AGGATATACCATGGAAGACCCCGTGC-3′ which introduces an NcoI restriction site, underlined), a reverse primer (5′-GAGCA-TCCTCGAGTTCGTTAAGGAACTCAC-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* DH5α (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-His_6 (hereafter referred to simply as TbABH) were grown at 30 °C in lysogeny broth (LB) supplemented with 100 μg/mL kanamycin while shaking at ~160 rpm to an optical density of 0.4–0.6 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 ~8000*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 M^{-1} 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