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The characterization of a unique *Trypanosoma brucei* β-hydroxybutyrate dehydrogenase

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ABSTRACT

A putative β-hydroxybutyrate dehydrogenase (βHBDH) ortholog was identified in *Trypanosoma brucei*, the unicellular eukaryotic parasite responsible for causing African Sleeping Sickness. The trypanosome enzyme has greater sequence similarity to bacterial sources of soluble βHBDH than to membrane-bound Type I βHBDH found in higher eukaryotes. The βHBDH gene was cloned from *T. brucei* genomic DNA and active, recombinant His-tagged enzyme (His_{10}-*Tb*βHBDH) was purified to approximate homogeneity from *E. coli*. βHBDH catalyzes the reversible NADH-dependent conversion of acetoacetate to D-3-hydroxybutyrate. In the direction of D-3-hydroxybutyrate formation, His_{10}-*Tb*βHBDH has a k_{cat} value of 0.19 s^{-1} and a K_M value of 0.69 mM for acetoacetate. In the direction of acetoacetate formation, His_{10}-*Tb*βHBDH has a k_{cat} value of 11.2 s^{-1} and a K_M value of 0.65 mM for D-3-hydroxybutyrate. Cofactor preference was examined and His_{10}-*Tb*βHBDH utilizes both NAD(H) and NADP(H) almost equivalently, distinguishing the parasite enzyme from other characterized βHBDHs. Furthermore, His_{10}-*Tb*βHBDH binds $NAD(P)^+$ in a cooperative fashion, another unique characteristic of trypanosome βHBDH. The apparent native molecular weight of recombinant His_{10}-*Tb*βHBDH is 112 kDa, corresponding to tetramer, as determined through size exclusion chromatography. RNA interference studies in procyclic trypanosomes were carried out to evaluate the importance of *Tb*βHBDH *in vivo*. Upon knockdown of *Tb*βHBDH, a small reduction in parasite growth was observed suggesting βHBDH has an important physiological role in *T. brucei*.

1. Introduction

Beta hydroxybutyrate dehydrogenase (βHBDH, E.C. 1.1.1.30) catalyzes the NAD(H)-dependent interconversion of acetoacetate to D-3-hydroxybutyrate. βHBDH is involved in the synthesis of ketone bodies, namely acetoacetate, D-3-hydroxybutyrate, and acetone, from acetyl-CoA. Ketone body production occurs when carbohydrates are scarce and acetyl-CoA is present in excess, such as during periods of starvation. When acetyl-CoA is in excess it does not enter the TCA cycle; rather, it is metabolized to yield ketone bodies. D-3-Hydroxybutyrate synthesized by βHBDH in the liver is exported to extrahepatic tissues, where βHBDH participates in the pathway that converts D-3-hydroxybutyrate back to acetyl-CoA, by catalyzing the conversion of D-3-hydroxybutyrate to acetoacetate. Acetyl-CoA is subsequently utilized in the TCA cycle in extrahepatic tissues, ultimately providing fuel for the organism.

There are fundamental differences between eukaryotic and bacterial βHBDHs. Eukaryotic βHBDH (Type 1) is bound to the inner mitochondrial membrane and requires lipid for activity [1,2]. More recently, a cytosolic eukaryotic Type 2 βHBDH (human DHRS6) has been discovered, although its physiological function is yet to be determined [3]. Bacterial βHBDHs, by contrast, are not membrane bound and do not require lipid for activation [4]. In bacteria, βHBDH is part of the poly-hydroxybutyrate (PHB) cycle. Many bacteria possess a complement of enzymes to polymerize D-3-hydroxybutyrate to form PHB and subsequently breakdown the PHB polymer according to the nutritional state of the bacteria [5]. Poly-hydroxybutyrate serves as an energy store, protects bacteria from stresses [6], and functions as an electron and carbon sink [7].

Trypanosoma brucei is the eukaryotic, unicellular protozoan that causes African Sleeping Sickness. The parasite's lifecycle is split between its tsetse fly vector and its vertebrate host. There are unique features to the parasite's energy metabolism in these two different environments. In the vertebrate host, the bloodstream form of the parasite relies solely upon glycolysis for energy generation; pyruvate is the excreted end product [8,9]. The tsetse fly midgut procyclic form of the parasite employs a more complex energy metabolism, wherein both glucose and amino acids are degraded. In procyclic parasites, acetate and succinate are the major

Abbreviations: βHBDH, beta-hydroxybutyrate dehydrogenase; *Tb*, *Trypanosoma brucei*; RNAi, RNA interference.

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end products excreted [10,11]. Acetyl-CoA is not degraded to CO_2 through the TCA cycle; rather, acetyl-CoA is metabolized through a two-enzyme cycle comprised of succinate:acetate CoA-transferase (ASCT) and succinyl CoA synthetase, giving rise to the production of ATP and succinate [12,13]. Another unique aspect of trypanosome metabolism is the ability of procyclic parasites to utilize acetate for the production of cytosolic acetyl-CoA by the enzyme acetyl-CoA synthetase to meet the demands of de novo fatty acid synthesis [14].

We have identified a putative βHBDH ortholog in *Trypanosoma* species that resembles the bacterial sources of the enzyme, but there are no apparent orthologs of the enzymes involved in the bacterial PHB cycle present in trypanosomes. *T. brucei* has been reported to excrete low levels of D-3-hydroxybutyrate [12,15], indicating a functional βHBDH. Curiously, the related trypanosomatid *Leishmania* species do not possess an identifiable βHBDH ortholog, based on database mining. Moreover, the vast majority of single-celled eukaryotes lack a βHBDH ortholog. Among protists, only *T. brucei*, *Trypanosoma cruzi*, *Tetrahymena thermophila*, and *Dictyostelium discoideum* have an apparent βHBDH ortholog, while among fungi, only *Aspergillus fumigates* contains an identifiable βHBDH ortholog [16]. Thus, the function of βHBDH in trypanosomatid metabolism is unclear.

To characterize the trypanosome βHBDH, a recombinant form of the *T. brucei* βHBDH protein was expressed, isolated, and characterized kinetically to assess the activity of the putative trypanosome βHBDH. An unusual cofactor preference was observed and cooperative binding of the oxidized form of the cofactor was experimentally determined, both traits unique to the parasite enzyme. The importance of *Tb*βHBDH *in vivo* was assessed through RNA interference studies in procyclic parasites. Parasites in which *Tb*βHBDH is depleted exhibit slowed growth, indicating an important role for this enzyme *in vivo*.

2. Materials and methods

2.1. Cloning, expression and purification of T. brucei βHBDH

The full length *Tb*βHBDH gene (GeneDB ID: Tb927.10.11930) was amplified from *T. brucei* 427 wild-type genomic DNA and cloned into pET16b (Novagen). The construct, pMH1, encodes for an amino-terminal 10 histidine-tagged *Tb*βHBDH. The sequences of all constructs were verified by DNA sequencing (Genewiz, Inc.).

Soluble His_{10}-*Tb*βHBDH was overexpressed in Rosetta 2 cells (Novagen) grown at 27 °C, induced with 50 μM IPTG for 12 h, and purified to approximate homogeneity using Ni affinity chromatography. Purified His_{10}-*Tb*βHBDH was stored stably without loss of activity at −20 °C in 50 mM sodium phosphate, 120 mM sodium chloride, and 250 mM imidazole, pH 8.5 containing 50% glycerol (storage buffer). Recombinant His_{10}-*Tb*βHBDH was used to generate custom polyclonal antibodies in rabbits (Lampire Biologicals, Pipersville, PA).

2.2. Substrate determination, kinetic analysis and inhibition studies

All substrates were of reagent grade. pH rate profiles were carried out to determine the optimal pH for maximal activity in both directions. For the enzyme activity assay in the direction of acetoacetate formation, the 1 mL reaction contained 70 μg of His_{10}-*Tb*βHBDH, and varying amounts of either D-3-hydroxybutyrate or $NAD(P)^+$ in 50 mM sodium phosphate, pH 8.5. Enzyme activity was monitored spectrophotometrically at 340 nm (Beckman D 640 Spectrophotometer), using a molar extinction coefficient of 6220 M^{-1} cm^{-1} for NAD(P)H. In the direction of hydroxybutyrate formation, the reaction contained 70 μg of His_{10}-*Tb*βHBDH and varying amounts of either acetoacetate or NAD(P)H in 50 mM citric acid, pH 6.0. D-3-Hydroxybutyrate formation was monitored spectrophotometrically at 370 nm due to high absorbance limitations at 340 nm. We determined and used a molar extinction coefficient of 2567 M^{-1} cm^{-1} for NAD(P)H at 370 nm. Kinetic constants were determined by using either a Lineweaver–Burk plot or by fitting the Hill equation to the data (SigmaPlot v. 11, Jandel Corporation). All kinetic constants were determined in triplicate.

All other potential substrate molecules were examined for NAD^+-dependent dehydrogenase activity. L-3-Hydroxybutyrate, lactate, malonate, and cacodylate were tested as inhibitors. Enzyme activity was monitored in the presence of 70 μg of His_{10}-*Tb*βHBDH, 4 mM NAD^+, 0.01–3.0 mM D-3-hydroxybutyrate and one of the following inihibtors: 10 mM L-hydroxybutyrate, 15 mM lactate, 1 mM cacodylate, or 15 mM malonate. Results were analyzed using Lineweaver–Burk plots.

2.3. Molecular mass determination

The oligomeric state of His_{10}-*Tb*βHBDH was determined through size exclusion chromatography using Ultrogel AcA-34 resin equilibrated with storage buffer. Blue dextran, bovine intestinal alkaline phosphatase, gamma-globin, bovine albumin, and egg grade III albumin (all from Sigma), were applied to the column at 3 mg/mL and their elution monitored by absorbances at either 600 nm (blue dextran) or 280 nm. His_{10}-*Tb*βHBDH was applied to the column at 1.0 mg/mL in storage buffer. His_{10}-*Tb*βHBDH elution was monitored by β-hydroxybutyrate dehdyrogenase activity.

2.4. RNA interference studies

To target *Tb*βHBDH by RNAi, a 400 bp region from nucleotide 173 to 572 of the open reading frame of Tb927.10.11930 was amplified from *T. brucei* genomic DNA and inserted into p2T7-177 [17] to yield pMH2. The RNAi construct for *Tb*ASCT (GeneDB ID: Tb11.02.0290) targeted the same region described in Ref. [12], which was cloned into p2T7-177 and named pMH3. Both *Tb*βHBDH and *Tb*ASCT were targeted simultaneously by inserting the *Tb*βHBDH target region into pMH3 at the HindIII site to yield pMH4. All primers can be found in supplemental Table 1. These constructs were then transfected into *T. brucei* procyclic cell line 29-13 [18] according the protocol of McCulloch et al. [19]. Clonal cell lines were generated through limiting dilution.

RNA interference was induced through the addition of 2 μg/mL tetracycline to the media daily. RNAi-induced and non-induced cells were grown in parallel and in replicate. Cell density was determined every 24 h and 8×10^6 cells were removed from the RNAi-induced and RNAi-non induced cell cultures daily for Western blot analysis. *Tb*βHBDH was detected using custom *Tb*βHBDH rabbit polyclonal antibodies (serum used at a dilution of 1:500) and goat anti-rabbit-IgG conjugated with alkaline phosphatase used at a 1:20,000 dilution.

3. Results and discussion

3.1. Identification of a Trypanosome βHBDH

BLAST searches of the *T. brucei* database (www.genedb.org) revealed one potential βHBDH ortholog, Tb927.10.11930, annotated as an NAD or NADP dependent oxidoreductase, putative, short chain dehydrogenase. In an attempt to identify as many βHBDH orthologs as possible, BLAST searches of the *T. brucei* genome with several bacterial, Type I and Type II βHBDHs still yielded only Tb927.10.11930 as a significant match. This is in contrast to multicellular eukaryotes, which contain both Type I