JBP1 and JBP2 Proteins Are Fe\(^{2+}\)/2-Oxoglutarate-dependent Dioxygenases Regulating Hydroxylation of Thymidine Residues in Trypanosome DNA*\(^2\)

Received for publication, January 14, 2012, and in revised form, April 17, 2012. Published, JBC Papers in Press, April 18, 2012, DOI 10.1074/jbc.M112.341974

Laura J. Cliffe\(^1\), Gwen Hirsch\(^1\), Jin Wang\(^5\), Dilrukshi Ekanayake\(^1\), Whitney Bullard\(^1\), Muhan Hu\(^1\), Yinsheng Wang\(^5\), and Robert Sabatini\(^1\)^\(^1\)

From the \(^1\)Department of Biochemistry and Molecular Biology, University of Georgia, Athens, Georgia 30602-7229 and the \(^5\)Department of Chemistry, University of California, Riverside, California 92521-0403

**Background:** Base J regulates Pol II transcription.

**Results:** JBP1 and -2 stimulate the first step of base J synthesis: hydroxylation of thymidine.

**Conclusion:** JBP are Fe\(^{2+}\)/2-OG-dependent dioxygenases sensitive to physiologically relevant O\(_2\) tensions.

**Significance:** These results predict that JBPs can act as oxygen sensors regulating trypanosome gene expression and adaption to different host niches.

We have recently demonstrated that O-linked glucosylation of thymine in trypanosome DNA (base J) regulates polymerase II transcription initiation. In vivo analysis has indicated that base J synthesis is initiated by the hydroxylation of thymidine by proteins (JBP1 and JBP2) homologous to the Fe\(^{2+}\)/2-oxoglutarate (2-OG)-dependent dioxygenase superfamily where hydroxylation is driven by the oxidative decarboxylation of 2-OG, forming succinate and CO\(_2\). However, no direct evidence for hydroxylase activity has been reported for the JBP proteins. We now demonstrate recombinant JBP1 hydroxylates thymine specifically in the context of dsDNA in a Fe\(^{2+}\), 2-OG-, and O\(_2\)-dependent manner. Under anaerobic conditions, the addition of Fe\(^{2+}\) to JBP1/2-OG results in the formation of a broad absorption spectrum centered at 530 nm attributed to metal chelation of 2-OG by the N-terminal thymidine hydroxylase domain of JBP1. This domain is sufficient for full activity and mutation of residues involved in coordinating Fe\(^{2+}\) inhibit iron binding and thymidine hydroxylation. Hydroxylation in vitro and J synthesis in vivo is inhibited by known inhibitors of Fe\(^{2+}\)/2-OG-dependent dioxygenases. The data clearly demonstrate the JBP enzymes are dioxygenases acting directly on dsDNA, confirming the two-step J synthesis model. Growth of trypanosomes in hypoxic conditions decreases JBP1 and -2 activity, resulting in reduced levels of J and changes in parasite virulence previously characterized in the JBP KO. The influence of environment upon J biosynthesis via oxygen-sensitive regulation of JBP1/2 has exciting implications for the regulation of gene expression and parasite adaptation to different host niches.

\(\beta\)-d-Glucopyranosylxylosymethyluracil (base J)\(^2\) is a hypermodified DNA base found in eukaryotes. This DNA modification is evolutionarily conserved within members of the kinetoplastid family, namely *Trypanosoma brucei*, *Trypanosoma cruzi*, and *Leishmania*, where J replaces about 1% of the total T in the genome and is predominantly present in repetitive DNA sequences, such as telomeric repeats (for review, see Ref. 1). However, more recently, we localized a minor fraction of J to chromosome-internal regions coinciding with RNA polymerase II (Pol II) transcription initiation and termination sites (2). Loss of base J synthesis at these chromosome-internal regions in *T. cruzi* led to increased Pol II transcription initiation and corresponding changes in gene expression and parasite virulence (3, 4). Thus, base J represents a novel epigenetic modification of kinetoplastid DNA involved in regulating gene expression.

Indirect evidence (for review, see Ref. 5) indicates J is synthesized in a two-step pathway (Fig. 1). Step one involves the hydroxylation of thymine in DNA by a thymidine hydroxylase (TH) enzyme, forming 5-hydroxymethyluracil (hmU). This intermediate is then glucosylated by a glucosyltransferase forming base J. Although the glucosyltransferase has not been identified, two proteins involved in the first step (JBP1 and JBP2) (6, 7) have been characterized. Both JBP1 and JBP2 (8, 9) contain a putative TH domain at the N terminus that has led to the designation of these enzymes belonging to the new TET/JBP subfamily of dioxygenases that require Fe\(^{2+}\) and 2-oxoglutarate (2-OG) for activity (10, 11). Family members are typically identified on a structural level by the presence of a jelly roll \(\beta\)-helix sheet that contains four key conserved residues involved in the binding of Fe\(^{2+}\) and 2-OG and are essential for catalytic activity (see for review, see Ref. 12). Mutation of these conserved residues within the TH domain of JBP1 and JBP2

---

* This work was supported, in whole or in part, by National Institutes of Health Grants 2R56AI063523-07A1 (to R. S.) and R01 CA101864 (to Y. W.).

† To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biology, University of Georgia, Davidson Life Science Bldg., 120 Green St., Athens GA 30602-7229. Tel.: 706-542-9806; Fax: 706-542-1738; E-mail: rsabatini@bmb.uga.edu.

\(^2\) The abbreviations used are: base J, \(\beta\)-d-glucopyranosylxylosymethyluracil; 2-OG, 2-oxoglutarate; DMOG, dimethyl oxoglycine; TH, thymidine hydroxylase; JBP base J-binding protein; rJBP1, recombinant JBP1; hmU, 5-hydroxymethyl-2-deoxyuridine; Pol, polymerase; TET, ten-eleven translocation-1 protein.
in histones and of N-methylated nucleic acids as well as hydroxylation of 5-methyl cytosine in DNA and 5-methoxycarbonylmethyluridine at the wobble position of tRNA. As such, dioxygenase enzymes have a broad range of biological roles, including DNA repair and O2 sensing and regulating gene expression (16, 17).

A distant homolog of the JBP1/2 TH domain was recently identified in the mammalian protein TET1, which is found fused to the histone methyltransferase MLL gene during acute myeloid leukemia (11, 18). TET1 (and the related TET2 and TET3 proteins) has been shown to convert 5-methylcytosine in DNA to 5-hydroxymethylcytosine. This conversion may play an important role in the epigenetic control of gene expression in mammals (11, 18). Based on the sequence similarity, JBP and TET proteins have been grouped together in the TET/JBP subfamily of dioxygenases (10).

Although the characterization of the JBP enzymes in vivo has proven to be useful in confirming their importance in J biosynthesis and elucidating the function of base J, TH activity has not yet been demonstrated for JBP in vitro. Here we develop an in vitro assay to demonstrate that JBP hydroxylation of thymine residues in dsDNA is dependent on Fe$^{2+}, 2$-OG, and O$_2$. We show rJBP1 binds Fe$^{2+}$, and mutation of the two conserved metal binding ligands of dioxygenases results in the loss of Fe$^{2+}$ binding and inability to hydroxylate thymidine. Competitive inhibitors of 2-OG significantly reduce JBP activity both in vivo and in vitro. Taken together, this data confirm the identity of the JBPs as Fe$^{2+}/2$-OG-dependent dioxygenases and provide direct evidence for the two-step J-biosynthesis pathway of modifying T-residues in kinetoplastid DNA. Furthermore, the O$_2$ requirement for JBP1-stimulated hydroxylation in vitro and J synthesis in vivo and the corresponding changes in T. cruzi virulence suggest the oxygen regulation of JBP enzyme activity to be important in allowing the parasite to adapt to changing host conditions during its lifecycle.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Blood stream form T. brucei cell line 221a of strain 427 were cultured as described previously (7). Promastigote L. major cells were grown at 26 °C in M199 media supplemented with 10% FBS as described (19). Y strain T. cruzi wild-type (WT) and JBP1 double knock-out epimastigotes were grown in liver infusion tryptose medium containing 10% fetal bovine serum as previously described (4). Epimastigotes were differentiated to infective metacyclics as described (20). For in vivo inhibition of TH activity, cells were grown in the presence of dimethylxoglycine (DMOG) (Frontier Scientific), 2,4-pyridinedicarboxylic acid hydrate (Acros Organics), or DMSO as indicated in Fig. 4 (inhibitor stocks were made up in 25% DMSO). As a control, parasites were grown in medium with comparable DMSO concentrations as cultures with the highest level of diluted inhibitor.

**Preparation of Recombinant JBP1**—Recombinant His-tagged L. tarentolae JBP1 (Lt-JBP1) was produced essentially as previously described (8) with the following modifications. The Lt-JBP1 vector was transformed to BL21-DE3 T7$^{	ext{th}}$ (21). Protein production was induced when cells reached an optical density of 0.6–0.9 with 0.3 mM isopropyl-1-thio-β-D-galactopyranosidase (IPTG), a L. tarentolae T. brucei T. cruzi L. major JBP1 KO cell line has elucidated an important function of base J in trypanosomes. Anti-J ChiP analysis indicated that whereas the remaining J in the T. cruzi JBP1 KO is located in telomeric DNA, base J is lost from the divergent and convergent strand-switch regions involved in Pol II initiation and termination, respectively. The loss of base J at transcription start sites coincides with a decrease in nucleosome abundance, increased histone acetylation, and increased Pol II occupancy at promoter regions (3, 4). This increase in Pol II recruitment correlates with an increased rate of transcription initiation and changes in gene expression (3). These studies indicate the importance of epigenetic regulation of Pol II transcription via DNA modification and chromatin structure in kinetoplastids as well as provide a mechanism for J regulation of trypanosome gene expression. Thus, characterization of the enzyme(s) regulating the hydroxylation of specific T-residues along the chromosome is critical for understanding the control of trypanosome gene expression.

The Fe$^{2+}/2$-OG-dependent dioxygenase enzyme family encompasses a large group of enzymes that catalyze the hydroxylation of a diverse variety of substrates, including but not limited to DNA, protein, RNA, and lipid (for review, see Ref. 12). Most dioxygenases utilize Fe$^{2+}$ as cofactor and 2-OG and oxygen (O$_2$) as co-substrates with the reaction coupling oxidative decarboxylation of 2-OG to the hydroxylation of substrate. Succinate and carbon dioxide are released as byproducts. The requirement of enzyme activity for molecular O$_2$ has suggested many of these dioxygenase function as direct O$_2$ sensors (14, 15). They may also be activated by the co-substrate 2-OG, a Krebs cycle intermediate, ascorbate, and Fe$^{2+}$ and inhibited by the product succinate. Members of this dioxygenase enzyme family catalyze the demethylation of N$^6$-methyl lysine residues....
side at 16 °C for 16 h. rJBP1 was purified over a Talon resin column eluted with 600 mM NaCl, 50 mM HEPES, and 200 mM imidazole followed by dialysis in 50 mM HEPES (pH 8.0), 100 mM NaCl, and 5 mM EDTA for 3 h. Protein was then subject to further dialysis (ON) in 50 mM HEPES and 100 mM NaCl. Purified protein, >95% pure as judged by SDS-PAGE, was concentrated by Centricon.

Analysis of Fe$^{2+}$/Binding to JBP1 Using UV-Visible Spectroscopy—Fe$^{2+}$/ binding analysis was carried out using 250 μM rJBP1, 100 mM dithionite, 240 μM Fe$_2$SO$_4$, 1 mM 2-OG, and 1 mM ascorbic acid essentially as previously described (22). All solutions were made anaerobic by several rounds of vacuum degassing and flushing with argon using a vacuum manifold and sealed serum vials. The reaction mixture was then read from 200 to 800 nm in a Shimadzu UV-visible spectrophotometer using a quartz cuvette fitted with a stopper and purged with argon. After blanking against JBP1, we recorded spectra for samples to which anaerobic aliquots of Fe$^{2+}$/ and 2-OG were added. No significant absorption between 400 and 750 nm was detected for the reaction buffer containing Fe$^{2+}$/ and 2-OG without JBP1.

TH Assay—Reaction conditions were 50 mM HEPES (pH 8.0), 50 mM NaCl, 8 mM ascorbic acid, 4 mM 2-OG, 1 mM Fe$_2$SO$_4$, 1 mM ATP, 20 μg/ml BSA, 0.5 mM DTT, 3.5 μg of telomeric duplex DNA substrate, and 2.2 μM JBP1 in a total reaction volume of 50 μl. DNA duplexes were made by boiling complementary telomere oligos (top strand 5'-TGGGATTGGATTGGATTGGAT-3'; bottom strand 5'-ATCCCAAATCCAATCCCAATCCCA-3') for 5 min and allowed to cool overnight. Samples were incubated at 37 °C for 30 min, and DNA was isolated using the Qiagen nucleotide removal kit. Detection of dsDNA substrate with or without JBP1 enzyme in the digestion mixture were then added alkaline phosphatase (2 U) and 0.2 units of bovine spleen phosphodiesterase (SPD) and 50 units of micrococcal nuclease (MNase) (Worthington) overnight at 37 °C, and nucleotides were phosphorylated with T4 polynucleotide kinase (New England Biolabs). Nucleotide substrate (3.5 μg) was incubated with antibody for 2 h at room temperature with agitation. One hundred microliters of magnetic beads (Genscript) were then added for an additional 2 h. After stringent washing, beads were added to scintillation fluid, and cpm was measured. Assays were performed in triplicate.

Hypoxia—To grow cells in a hypoxic environment, 50-ml culture flasks were placed in anaerobic chambers (BD Biosciences) with an Aneropak pouch (Mitsubishi Gas Chemical Co.). Generation of hypoxic environment was confirmed using indicator strip. For growing cells at 1 or 3% O$_2$, culture flasks were placed in anaerobic chambers and gas-exchanged by an evacuation-replacement procedure using premixed 1 or 3% O$_2$ with 5% CO$_2$ and balanced nitrogen. A continuous flow of gas was then applied for at least 5 min followed by daily gassing. To maintain hypoxic environment, hypoxia-treated cells were harvested and lysed inside the anaerobic chamber (Coy) to avoid reoxygenation. Samples were then processed for RNA/DNA under atmospheric O$_2$ conditions.

T. cruzi Invasion Assay—T. cruzi epimastigotes grown under hypoxic conditions as described above for 10 days were transferred to hypoxic Grace’s insect media (Invitrogen); made anaerobic by vacuum degassing and flushing with argon) for 14 days to differentiate to infective metacyclics as previously described (4). Epimastigotes that were grown at 21% O$_2$ were differentiated in a similar procedure but under atmospheric O$_2$ conditions. Differentiated metacyclic parasites (from 21% O$_2$ and hypoxic conditions) were then purified, equal numbers of infective metacyclic parasites were incubated with vero cells for 3 h, and parasite invasion was quantitated as previously described at 21% O$_2$ conditions (4). Briefly, vero cells were plated on 13-mm round coverslips at a density of 3 × 10$^4$ cells in Eagle’s Minimum Essential Medium with 10% fetal calf serum and cultivated in 24-well plates for 24 h at 37 °C in a 5% CO$_2$, atmosphere and 21% oxygen. Coverslips with attached cells were then washed three times with phosphate-buffered saline (PBS) to remove the unattached cells and debris. Purified metacyclic forms were used for invasion assays. Purified metacyclics from each growth condition were centrifuged to remove the cell debris and seeded onto the Vero cells in equal numbers (5 × 10$^6$ parasites/well). After 3 h at 37 °C, the interaction was stopped by removing the parasites, and washing the cells three times with PBS. Monolayers were fixed and stained with Giemsa stain. Invasion was quantified using two methods. The percentage of infected cells was calculated by counting the number of intracellular parasites per coverslip and expressed as the number of parasites per 100 vero cells. We also determined the average number of parasites per infected cell by counting the number of parasites in ∼90 infected vero cells for each experiment.

Detection of hmdU by Mass Spectrometry—After the incubation of dsDNA substrate with or without JBP1 enzyme in the TH assay as described above, the purified DNA was digested to nucleosides and subjected to liquid chromatography-mass spectrometry (LC-MS) analysis. The enzymatic digestion of the duplex DNA substrate was conducted following previously described procedures with some modifications (23). Nuclease P1 (4 units), phosphodiesterase 2 (0.005 units), erythro-9-(2-hydroxy-3-nonyl) adenine (20 nmol), and a 20-μl solution containing 300 mM sodium acetate (pH 5.6) and 10 mM zinc chloride were added to the DNA and incubated at 37 °C for 48 h. To the digestion mixture were then added alkali phosphatase (2 units), phosphodiesterase 1 (0.005 units), and 40 μl of 0.5 M Tris-HCl (pH 8.9). The digestion was continued at 37 °C for 2 h and subsequently neutralized with formic acid. To the mixture were then added 1.5 pmol of isotopically labeled hmdU (24). The enzymes in the digestion mixture were subsequently removed by chloroform extraction twice, and the resulting aqueous layer was subjected to off-line HPLC enrichment of hmdU. A 4.6 × 250-mm Alltima HP C18 column (5 μm in particle size, Grace Davison, Deerfield, IL) was used for the enrichment of hmdU from the enzymatic digestion products of DNA. The mobile phases were 10 mM ammonium formate in water (solution A) and methanol (solution B), and the flow rate was 1 ml/min. A gradient of 42 min at 0% B, 1 min at 0–5% B, 22 min at 5% B, 5 min at 5–20% B, and 10 min at 20% B was employed. A typical HPLC trace is shown in supplemental Fig. S3A. The HPLC fractions eluting at 18.0–24.0 min were pooled.
for hmdU. The collected fractions were dried in the SpeedVac, redissolved in H₂O, and subjected for LC-MS/MS analysis.

For LC-MS/MS analysis, a 3.0 × 100-mm Hypersil GOLD column (particle size, 5 μm, Thermo Scientific) was used for the separation of the fractions containing hmdU, and the flow rate was 50 μl/min. A solution of 0.1% (v/v) formic acid in water (solution A) and a solution of 0.1% (v/v) formic acid in methanol (solution B) were employed as mobile phases, and a gradient of 35 min 0–70% at B, 1 min 70–0% at B, and 14 min at 0% B was used for the separation. The effluent from the LC column was directed to a TSQ Vantage triple quadrupole mass spectrometer (Thermo Fisher Scientific), which was set up for monitoring the fragmentation of the [M-H]⁻ ions of the unlabeled and labeled hmdU in the multiple-reaction monitoring mode. The S-lens radio frequency amplitude was maintained at 97 V, the capillary temperature was maintained at 270 °C, and the vaporizer temperature was 153 °C. The sheath gas flow rate was 20 arbitrary units, the auxiliary gas flow rate was 0.3 arbitrary units, the spray voltage was 2.7 kV, and the scan time was 100 ms. The multiple-reaction monitoring transitions were m/z 257 → 124 and m/z 260 → 126 for unlabeled and labeled hmdU, respectively (supplemental Fig. S3, C and D). The collision energy was set at 17 V.

Determination of Genomic Level of J—To quantify the genomic J levels, DNA was isolated and utilized in the anti-J DNA immunoblot assay as described (25). Briefly, 2-fold serially diluted genomic DNA was blotted to nitrocellulose followed by incubation with anti-J antisera. Bound antibodies were detected by a secondary goat anti-rabbit antibody conjugated to HRP (horseradish peroxidase) and visualized by ECL (enhanced chemiluminescence). The membrane was stripped and hybridized with a probe for the β-tubulin gene to control for DNA loading.

Quantitative Reverse Transcription-PCR—Total RNA was obtained using Qiagen RNeasy kits according to the manufacturer’s instructions. First-strand cDNA was synthesized from 1 μg of total RNA using an iScript cDNA synthesis kit (Bio-Rad) per the manufacturer’s instructions. Heat-inactivated cDNA reaction mixtures were finally treated with RNase H at 37 °C for 45 min. Quantification of selected genes was performed on an iCycler with an iQ5 multicolor real-time PCR detection system (Bio-Rad). Primer sequences used in the analysis are available upon request. The reaction mixture contained 5 pmol of forward and reverse primer, 2× iQ SYBR Green super mix (Bio-Rad), and 2 μl of template cDNA. Standard curves were prepared for each gene using 10-fold dilutions of known quantity (15 ng/μl) of WT DNA. The quantities were calculated using iQ5 optical detection system software. Each sample was normalized to 245 rRNA (T. cruzi) or tubulin (L. major). Statistical analysis was performed using Student’s t test. A value of p < 0.05 was considered significant.

RESULTS

JBP Is Fe²⁺/2-OG-dependent Dioxygenase Hydroxylating Thymine in dsDNA to hmlU—We have previously demonstrated the ability to detect hmlU-modified DNA using an anti-hmlU pulldown assay that is highly specific for hmlU over base J or unmodified thymidine (Ref. 9 and supplemental Fig. S1A).

We now utilize this hmlU detection system to set up an in vitro TH assay, incubating recombinant JBP1 with a 24-mer duplex oligodeoxynucleotide in the presence of Fe²⁺, 2-OG, and ascorbic acid. As predicted, incubation of JBP1 with duplex DNA resulted in the formation of hmlU (Fig. 2C). The addition of JBP1 caused a significant increase in cpm, reflective of the production of hmlU by >9-fold over background (no JBP1). A titration of increasing amounts of JBP1 protein corresponded with an increase in hmlU formation, the peak of which was used for subsequent assays (supplemental Fig. S1B). To confirm the species of product generated in our in vitro TH reaction, we measured hmlU in the DNA substrate upon incubation with JBP1 using LC-MS/MS with the stable isotope-dilution method. In this LC-MS/MS method, we monitored the m/z transitions for unlabeled and labeled hmdU (26), respectively, in the multiple-reaction monitoring mode. We found that the level of hmdU in the DNA substrate after incubation with JBP1 was ~7 lesions/10⁴ dT, which was ~7-fold higher than that in the DNA from the control reaction (Fig. 2D). Interestingly, the degree of hydroxylation in the presence of JBP1 over the negative control measured by LC-MS/MS is very similar to the increase detected by the hmlU pulldown assay. These results demonstrate that JBP1 can induce the oxidation of thymine in duplex DNA to give hmlU in vitro. The data also confirm the use of our anti-hmlU pulldown assay for following in vitro hmlU production.

Utilizing the hmlU pulldown assay, we demonstrate that the TH domain is within the N terminus of JBP1. The N-terminal half of JBP1 (amino acids 1–450) is capable of hydroxylating thymidine (Fig. 2, A and E) and is as active as the full-length enzyme (Fig. 2, F and G). Thymine in the context of single-stranded DNA substrate, rather than duplex DNA, is not hydroxylated (Fig. 3A), demonstrating that the TH activity of JBP1 is specific for double-stranded DNA substrate. Consistent with other members of the Fe²⁺/2-OG-dependent dioxygenase family, optimal JBP1 activity requires O₂, Fe²⁺, and 2-OG, with a significant reduction in thymidine hydroxylation seen when the assay is performed in the absence of each (Fig. 3, B–D). As observed for other dioxygenases (27), the TH activity of JBP1 was stimulated by ascorbate (data not shown), potentially reflecting the regeneration of Fe³⁺ from Fe²⁺ (28).

The requirement for both Fe²⁺ and 2-OG for optimal thymidine hydroxylation along with the stimulation by ascorbate strongly supports the proposal that JBP1 is a member of the dioxygenase family. Direct evidence that JBP1 binds Fe²⁺ and 2-OG was provided by examining the absorption spectrum of the anaerobic protein in the presence and absence of Fe²⁺ and 2-OG. The protein with bound Fe²⁺ and cofactor had an absorption peak at 530 nm (Fig. 2B), similar to the transition observed in other family members. This chromophore is due to a weak charge transfer from Fe²⁺ to 2-OG and is a spectroscopic signature of Fe²⁺/2-OG-dependent dioxygenases (12). The two highly conserved histidine metal binding ligands in the core jelly roll domain of Fe²⁺/2-OG enzymes have been shown to be essential for Fe²⁺ binding and hydroxylase function (12, 17). Consistent with this finding, mutation of residues H189A and H239A in JBP1 ablated J synthesis in vivo (8). We see here that in vitro Fe²⁺ binding and thymidine hydroxylation is ablated in the H189A/H239A mutant, demonstrating that Fe³⁺

JUNE 8, 2012•VOLUME 287•NUMBER 24
JOURNAL OF BIOLOGICAL CHEMISTRY 19889
binding is critical for TH activity of JBP1 (Fig. 2, B and C). Taken together, we have unequivocally shown that JBP1 is a Fe$^{2+}$/2-OG-dependent dioxygenase, requiring Fe$^{2+}$, O$_2$, and 2-OG for activity. The data also support the previous sequence and in vivo analyses, indicating the TH domain at the N terminus of JBP1 and JBP2.

**Dioxygenase Inhibitors Reduce JBP Activity Both in Vitro and in Vivo**—Many inhibitors of Fe$^{2+}$/2-OG dioxygenases have been well characterized (17). Two of these characterized inhibitors, DMOG and 2,4-pyridinedicarboxylic acid hydrate, are competitive inhibitors of 2-OG. These compounds have been shown to be effective inhibitors of several members of the Fe$^{2+}$/2-OG dioxygenases (17) including (but by no means limited to) the DNA modifying enzymes such as fat mass and obesity-associated protein (FTO) (29). When the JBP1 TH assay was performed in the presence of either DMOG or 2,4-pyridinedicarboxylic acid hydrate, we saw a highly significant inhibition of JBP1 activity (Fig. 4A and data not shown). Also consistent with other dioxygenases, we saw inhibition with succinate by product inhibition (Fig. 4B).
As base J is the downstream product of thymidine hydroxylation in vivo, we predicted that the treatment of J containing kinetoplastids with these cell-permeable inhibitors would result in a reduction in J biosynthesis. Indeed the treatment of T. brucei, L. major, and T. cruzi with DMOG caused a significant inhibition in J synthesis (Fig. 4, C–E and supplemental Fig. 2). The ability to generate a cell line that lacks base J in T. brucei after the inhibition of JBP TH activity with DMOG is consistent with our ability to delete both enzymes and the apparent non-essential nature of base J in this parasite (9). The generation of J null cells through DMOG feeding of T. brucei also demonstrates that both JBP1 and JBP2 are 2-OG dioxygenase enzymes. Similarly, DMOG reduces J synthesis in the JBP1KO and JBP2 KO T. cruzi cell lines (data not shown).

**JBP Activity Is Regulated by Oxygen Levels in Vivo—**Given that O2 is critical for JBP1 to hydroxylate thymidine in vitro (Fig. 3B), we hypothesized that O2 levels would impact J synthesis in vivo. To address this, we grew T. cruzi and L. major at atmospheric O2 concentrations (21%) and under low O2 (1%) or hypoxic (<0.1%) conditions. Consistent with inhibition of JBP TH activity and loss of J after DNA replication, we saw significant reductions in J synthesis in T. cruzi when grown under 1% O2 and <1% hypoxic conditions (Fig. 5A). We did not detect any change in mRNA levels of the JBP enzymes in hypoxia, and J synthesis was fully restored within 30 min of re-exposure of the cells to 21% O2 (Fig. 5, A and B). This supports the reduction in J biosynthesis being at the level of inhibition of TH activity rather than a down-regulation of the synthesis machinery and that JBP-stimulated thymidine hydroxylation and thus J synthesis occurs independently of DNA replication. Reduced O2 levels affected the enzymatic activity of both JBP1 and JBP2 in T. cruzi, as significant reductions in J levels were seen in both JBP1KO and JBP2 KO cell lines (Fig. 5C). Low O2 had similar effects on JBP function in L. major (supplemental Fig. 5, A and B). Our inability to grow T. brucei in 3% or less O2 conditions (data not shown) precluded similar analyses of JBP function in this parasite.

We have previously demonstrated that reduced J levels in T. cruzi results in increased Pol II recruitment to promoter regions and an increase in the rate of transcription initiation, which in turn causes global changes in gene expression (3, 4). Notably, surface proteins involved in parasite pathogenesis (including members of the trans-sialidase and mucin gene family) are significantly affected during reduced J levels. Accordingly, we measured a 5-fold increase in the ability of T. cruzi to invade the mammalian cell after the 20-fold reduction in base J (4). Therefore, we hypothesized that the 5-fold reduction in J levels associated with cells grown in hypoxic conditions would also result in an enhanced invasion phenotype. When T. cruzi epimastigotes were grown under hypoxia for 10 days and differentiated to infective metacyclics, and the mammalian cell invasion assay was performed in 21% O2, we saw a significant increase in both the percent of host cells infected as well as the number of parasites within each cell compared with parasites grown and differentiated under 21% O2 (Fig. 5, D and E). Taken together, the data suggest the JBP TH enzymes may act as O2 sensors, allowing the parasite to respond to changes in O2 levels, and regulate J synthesis, gene expression, and (among other phenotypes) virulence.

**DISCUSSION**

Sequence analysis indicated that JBP1/2 contain a Fe2+/2-OG-dependent dioxygenase-like domain at the N termini, including the presence of four conserved catalytic residues, critical in binding nonheme iron, 2-OG, and enzyme activity (12). The recent identification and characterization of the TET enzymes catalyzing the hydroxylation of 5-MeC in DNA strengthened the identification of JBPs as dioxygenases (11), characterizing them as members of the new TET/JBP subfamily of Fe2+/2-OG dioxygenase (10, 11). However, no direct analysis of JBP thymidine hydroxylation has been demonstrated despite a clear in vivo gene knock out and mutagenesis studies demonstrating the importance of JBP1 and JBP2 in J biosynthesis (8, 9, 30). The oxidation of thymidine residues by a thymidine hydroxylase is unusual. Thymine hydroxylase enzymes that oxidize the free base are known (31, 32) but thymidine hydroxylases that oxidize the base in DNA have not previously been characterized. Here we utilize an in vitro thymidine hydroxylase assay and in vivo inhibition studies to provide direct evidence for hydroxylation of T-residues in DNA during J synthesis by JBP1 and JBP2 and identify them as members of the Fe2+/2-OG family of dioxygenases. We also now provide conclusive evidence for the two-step J biosynthesis mechanism (Fig. 1) by demonstrating the ability of JBP to modify T-residues specifically in dsDNA substrate in vitro and regulating J synthesis in a DNA replication-independent manner in vivo.

The in vitro TH assay clearly demonstrates JBP1 is a Fe2+/2-OG dioxygenase that requires Fe2+, 2-OG, and O2 for hydroxylating thymine in the context of dsDNA. Extension of these analyses in vivo indicates both JBP1 and JBP2 utilize similar co-factors for T-hydroxylation and J synthesis. The sensitivity of J synthesis in various WT and JBP KO kinetoplastid cell
lines to 2-OG inhibitors and hypoxia confirms that both JBP
are Fe$^{2+}$/2-OG dioxygenase enzymes. This is further supported
by the ability of both JBP1 and JBP2 to stimulate hmU modifi-
cation of genomic DNA when expressed in
Escherichia coli.3
The rapid generation of a cell line that completely lacks base J
upon DMOG treatment of
T. brucei
supports the nonessential
nature of the modified base in this species as demonstrated by
our previous generation of a J-null trypanosome by deleting
both JBP1 and JBP2 (9). The inability to delete both JBP1 and
JBP2 in
T. cruzi
(3, 4) and inability to delete JBP1 from
L. taren-
tolae
(33) have suggested the essential nature of J in these spe-
cies. Future studies will utilize DMOG to elucidate the essential
nature and function of J in these and other J-containing
organisms.

The hydroxylation activity of JBP1 is specific for dsDNA,
correlating with the specificity of J-DNA binding domain at the
C terminus of JBP1 (34, 35). Inactivation of the N-terminal TH
domain has no effect on J-DNA binding (8). Thus, JBP1 binding
to J-DNA is independent from TH activity. We demonstrate
here that the TH domain at the N terminus is functional
in vitro
without the C-terminal J-DNA binding domain. However, it is
possible that
in vivo
TH activity of the JBP1s is stimulated by
their C-terminal domain. In fact, the low level of
in vitro
conversion of thymidine residues suggests that the ability of JBP1 to
bind J residues in dsDNA may enhance TH activity. This “prop-
gagation” activity may represent an essential function for JBP1
in vivo
(7, 35). We have previously demonstrated that JBP1 and
JBP2 operate optimally in different chromatin environments;
JBP1 at internal regions involved in Pol II transcription and
JBP2 within telomeric-localized repetitive DNA (2). Presum-
ably this functional difference is due to distinct C-terminal
domains on each enzyme, including the SWI/SNF2 chromatin
remodeling domain in JBP2 (2, 5, 7). The apparent low activity
of JBP1-stimulated T-hydroxylation on synthetic DNA may
reflect the optimal substrate for these enzymes
in vivo
is chro-
matin or, as mentioned above specifically for JBP1, the stimu-
latory effects of initial levels of J. Furthermore, we cannot rule
out the possibility that additional factors stimulate JBP recruit-
ment and/or TH activity
in vivo.

As documented for other Fe$^{2+}$/2-OG oxygenases, our anal-
yses indicated a role of intracellular levels of 2-OG and succi-
nate in regulating thymidine hydroxylation by JBP. The
requirement of 2-OG and inhibition by succinate in JBP1-stim-
ulated TH activity indicate the 2-OG/succinyl ratio within the
parasite as a critical factor for J synthesis and may help explain
the lack of base J in the procyclic
T. brucei
life stage (36). It is

3 T. Southern, M. Marshall, and R. Sabatini, unpublished results.

FIGURE 4. Thymidine hydroxylase activity of JBP1 and JBP2 is reduced by dioxygenase inhibitors. The
in vitro TH assay was performed using JBP1 in the
presence/absence of 0.5 mM DMOG (A) and 1 mM succinate (B). Error bars represent the S.D. of triplicate experiments. Data are expressed as % activity relative
to the control reaction. C. T. brucei cells were grown in the presence or absence of 0.5 mM DMOG or 0.5 mM 2,4-pyridinedicarboxylic acid hydrate for 6 days, and
DNA was isolated for anti-J dot blot analysis. Samples were 2-fold serially diluted. The same blots were hybridized with a tubulin probe to control for DNA
loading. DMSO is provided as a control where parasites were grown in medium with comparable DMSO concentrations as cultures with highest level of diluted
inhibitor. Similar DMOG treatment was also performed in L. major (D) and T. cruzi (E).
well characterized that procyclic *T. brucei* cells, in contrast to the long-slender bloodstream life stage that contains base J, produce high concentrations of succinate representing incompletely oxidized product of aerobic fermentation (37, 38). The pathway(s) leading to lifecycle-specific production of succinate are not well understood, but the unique absence of a tricarboxylic acid cycle in bloodstream form *T. brucei* is presumably a key factor (39, 40). Our data suggest that the developmental increase in succinate within the parasite residing in the tsetse fly (and associated shift in the 2-OG/succinate ratio) would prevent optimal hydroxylation of thymidine by the Fe$^{2+}$/2-OG dioxygenases, reducing J synthesis and presumably leading to the eventual down-regulation of JBP expression.4 Interestingly, upon re-expression of identical levels of JBP1 enzyme in the procyclic and bloodstream form *T. brucei* is presumably a key factor (39, 40). Our data suggest that the developmental increase in succinate within the parasite residing in the tsetse fly (and associated shift in the 2-OG/succinate ratio) would prevent optimal hydroxylation of thymidine by the Fe$^{2+}$/2-OG dioxygenases, reducing J synthesis and presumably leading to the eventual down-regulation of JBP expression. Interestingly, upon re-expression of identical levels of JBP1 enzyme in the procyclic and bloodstream form trypanosome, an 800-fold decrease in the succinate/2-OG ratio in bloodstream versus procyclic parasites corresponds to an ~15-fold increase in J synthesis in bloodstream forms.4 The response of JBP TH activity and J synthesis to levels of O$_2$, 2-OG, and succinate implicate a role for metabolic flux as well as the environment to which the parasite is exposed in epigenetic regulation of gene expression in these human pathogens.

The functional dependence on O$_2$ has implicated a number of Fe$^{2+}$/2-OG dependent dioxygenase enzymes to operate as O$_2$ sensors, playing crucial roles in cellular function (14). Perhaps the best studied is prolyl hydroxylase, which is an Fe$^{2+}$/2-OG dioxygenase responsible for hydroxylation of the transcription factor, hypoxia-inducible factor on its proline residues at positions 402 and 564 (41). Hydroxylated hypoxia-inducible factor is then recognized by the VHL subunit of the E3(UBC)-ubiquitin ligase followed by degradation within the 26 S proteasome. However, hypoxia renders the prolyl hydroxylase inactive, and therefore, hypoxia-inducible factor-$\alpha$ is stabilized, allowing translocation to the nucleus, where it dimerizes with hypoxia-inducible factor-$\beta$, regulating gene transcription (for review, see Ref. 41 and 42). These changes in gene transcription include genes that allow cell adaptation and survival in a hypoxic environment, such as genes involved in glycolysis or angiogenesis (43).

Oxygen occurs in varying concentrations within the human host, ranging from 0 to 21% (anoxic and aerobic environments) (44). Not only do O$_2$ levels vary between human tissues but also an O$_2$ gradient within the tissue depending upon the proximity to blood vessels and to the O$_2$ consumptive activity of the cell. As a consequence, in contrast to human cells, pathogens have evolved to adapt and prevail in a diverse array of oxygen con-

---

4 L. J. Cliffe and R. Sabatini, unpublished data.

**FIGURE 5. JBP as oxygen sensors in vivo regulating J synthesis and virulence.** A, *T. cruzi* epimastigotes were grown in atmospheric (21% O$_2$), 1% O$_2$, and hypoxic (<0.1% O$_2$) conditions for 10 days. A sample of cells grown at <0.1% O$_2$ was re-exposed to 21% O$_2$ for 30 min (R). DNA was isolated, and J levels were assessed by anti-J dot blot. Blots were stripped and probed for tubulin as a loading control. B, RNA was isolated from the same *T. cruzi* cells, and the relative amounts of mRNA for JBP1 and JBP2 were calculated by quantitative RT-PCR as described under “Experimental Materials.” Black bars, 21%; white bars, <0.1% O$_2$. Values were normalized to 24 S control. C, *T. cruzi* cells that have either JBP1 (JBP1KO) or JBP2 (JBP2KO) deleted from the genome were grown in the indicated oxygen conditions, and levels of J were detected as described in A. D and E, epimastigotes grown in 21% or <0.1% O$_2$ were then differentiated into metacyclics and purified, and equal numbers of differentiated metacyclic parasites were allowed to invade mammalian cells in the presence of 21% O$_2$ as described under “Experimental Procedures.” Slides were then Giemsa-stained and assessed for the presence of intracellular parasites. Data are expressed as the number of parasites per 100 cells (D) and the number of parasites per infected cell (E). Error bars represent the S.D. of triplicate experiments. *p < 0.0001.
centrations. This adaptation is especially important for pathogens, such as *T. cruzi*, that experience varying oxygen concentrations from high levels on the skin to low levels within various tissues (i.e. gut and muscle) of the human host and insect vector.

We have shown that in *T. cruzi*, decreased J levels after the loss of JBP function result in increased Pol II recruitment and transcription initiation and corresponding genome wide changes in gene expression, including genes involved in pathogenesis (4). This in turn has direct effects on the host-parasite relationship, with increased mammalian cell invasion and delayed egress (4). These data combined with the analysis of JBP TH activity presented here suggest JBP1/2 can act as O₂ sensors in vivo to modulate J biosynthesis and gene expression, allowing the parasite to adapt to changing conditions within the mammalian host. To examine this hypothesis, we exposed *T. cruzi* parasites to hypoxia. To rule out the potential effects of the mammalian cell hypoxia-inducible factor response to hypoxia, the invasion assay itself was performed at 21% O₂. Therefore, rapid recovery of TH activity and J synthesis upon re-exposure of the cells to 21% O₂ (Fig. 5) during the 3-h *T. cruzi* invasion assay may have reduced the effects of hypoxia. Regardless, the data clearly demonstrate a parasite response to low O₂ in both levels of J synthesis and virulence phenotypes we have previously directly linked to epigenetic regulation by J (4). Therefore, we propose that the thymidine hydroxylase enzymes, JBP1 and JBP2, can act as O₂ sensors allowing the parasite to respond to changes in O₂ levels and regulate J synthesis, thus providing at least one mechanism of hypoxic signaling regulating gene expression and, among other potential phenotypes, virulence. Although we have not fully characterized the sensing capability of JBP by measuring the *Kₘ* for O₂, we do demonstrate significantly reduced enzyme activity (i.e. J synthesis) upon shifting the parasite to physiologically relevant low oxygen tensions. Although previous analysis of JBP loss of function mutants supports the proposed model of hypoxia regulation of *T. cruzi* virulence via JBP, we acknowledge that the current data do not rule out other potential O₂ sensing pathways influencing gene expression and corresponding cellular response. This is especially relevant when we consider trypanosomes encode putative JmjC domain-containing dioxygenase enzymes that may serve as histone lysine demethylases. To better describe the mechanistic link between hypoxic signaling and JBP function, future experiments will explore the relationship between length of time parasites are grown under different levels of O₂ and corresponding changes in J synthesis, chromatin structure, Pol II occupancy, transcription initiation, and gene expression profiles.

The ability of O₂ to modulate epigenetic regulation of gene expression is an important factor to consider when evaluating transcriptome profiles of various parasite life stages. All studies thus far grow kinetoplastids under atmospheric 21% O₂ conditions that are far removed from physiological normoxia. Because changes in levels of base J significantly affect the transcription profile of *T. cruzi* and J levels are responsive to O₂ levels, we believe care and consideration should be given when evaluating transcriptional profiling of kinetoplast parasites grown at 21% O₂, as this may well not reflect the “true” transcriptome profile of the parasite life stage. The epigenetic control of gene expression and its regulation through O₂ levels and other metabolites present exciting new possibilities for Pol II regulatory mechanisms in early-divergent organisms where transcriptional regulation is poorly understood.

Acknowledgments—We especially thank Bill Lanzollotta, John Demmick and other members of the Lanzollotta laboratory for assistance and use of their anaerobic chamber and spectrophotometer. Robert Arnold provided assistance in the initial hypoxia experimental design. We also thank Marion Marshall and Timothy Southern for preliminary work on development of ihmUIP assay and rJBP1 protein purification. We also thank Steve Hajduk, Rudo Kieß, Piet Borst, and John Harrington for useful comments on the manuscript.

REFERENCES

1. Borst, P., and Sabatini, R. (2008) Base J. Discovery, biosynthesis, and possible functions. *Annu. Rev. Microbiol.* 62, 235–251

2. Cliffe, L. J., Siegel, T. N., Marshall, M., Cross, G. A., and Sabatini, R. (2010) Two thymidine hydroxylases differentially regulate the formation of glucosylated DNA at regions spanning polymerase II polycistronic transcription units throughout the genome of *Trypanosoma brucei*. *Nucleic Acids Res.* 38, 3923–3935

3. Ekanyake, D., and Sabatini, R. (2011) Epigenetic regulation of polymease II transcription initiation in *Trypanosoma cruzi*. Modulation of nuclide some abundance, histone modification, and polymerase occupancy by O-linked thymine DNA glucosylation. *Eukaryot. Cell* 10, 1465–1472

4. Ekanyake, D. K., Minning, T., Weatherly, B., Gunasekera, K., Nilsson, D., Tarleton, R., Ochsenreiter, T., and Sabatini, R. (2011) Epigenetic regulation of transcription and virulence in *Trypanosoma cruzi* by O-linked thymine glucosylation of DNA. *Mol. Cell. Biol.* 31, 1690–1700

5. Sabatini, R. C., L. J. Vainio, S., and Borst, P. (2008) in *DNA and RNA Modification Enzymes; Comparative Structure, Mechanism, Function, Cellular Interactions. and Evolution* (Grosjean, H., ed) pp. 120–131, Landes Biosciences, Austin, TX

6. Cross, M., Kieß, R., Sabatini, R., Wilm, M., de Kort, M., van der Marel, G. A., van Boom, J. H., van Leeuwen, F., and Borst, P. (1999) The modified base J is the target for a novel DNA-binding protein in kinetoplastid protozoans. *EMBO J.* 18, 6573–6581

7. DiPaolo, C., Kieß, R., Cross, M., and Sabatini, R. (2005) Regulation of trypanosome DNA glycosylation by a SWI2/SNF2-like protein. *Mol. Cell* 17, 441–451

8. Yu, Z., Genest, P. A., ter Riet, B., Sweeney, K., DiPaolo, C., Kieß, R., Christodoulou, E., Perrasik, A., Simmons, J. M., Hausinger, R. P., van Luenen, H. G., Rigden, D. J., Sabatini, R., and Borst, P. (2007) The protein that binds to DNA base J in trypanosomatids has features of a thymidine hydroxylase. *Nucleic Acids Res.* 35, 2107–2115

9. Cliffe, L. J., Kieß, R., Southern, T., Birdon, L., Sweeney, K., and Sabatini, R. (2009) JBP1 and JBP2 are two distinct thymidine hydroxylases involved in J biosynthesis in genomic DNA of African trypanosomes. *Nucleic Acids Res.* 37, 1452–1462

10. Iyer, L. M., Tahiliani, M., Rao, A., and Aravind, L. (2009) Prediction of novel families of enzymes involved in oxidative and other complex modifications of bases in nucleic acids. *Cell Cycle* 8, 1698–1710

11. Tahiliani, M., Koh, K. P., Shen, Y., Pastor, W. A., Bandukwala, H., Budno, Y., Agarwal, S., Iyer, L. M., Liu, D. R., Aravind, L., and Rao, A. (2009) Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. *Science* 324, 930–935

12. Hausinger, R. P. (2004) Fe(II)/2-OG-dependent Dioxygenases. *Crit. Rev. Biochem. Mol. Biol.* 39, 21–68

13. Vainio, S., Genest, P. A., ter Riet, B., van Luenen, H., and Borst, P. (2009) Evidence that J-binding protein 2 is a thymidine hydroxylase catalyzing the first step in the biosynthesis of DNA base J. *Mol. Biochem. Parasitol.* 164, 157–161

14. Ozer, A., and Bruick, R. K. (2007) Non-heme dioxygenases. Cellular sen-
sors and regulators jelly rolled into one? Nat. Chem. Biol. 3, 144–153
15. Kaelin, W. G., Jr., and Ratcliffe, P. J. (2008) Oxygen sensing by metazoans. The central role of the HIF hydroxylase pathway. J. Biol. Chem. 303, 393–402
16. Loenarz, C., and Schofield, C. J. (2008) Expanding chemical biology of 2-oxoglutarate oxygenases. Nat. Chem. Biol. 4, 152–156
17. Rose, N. R., McDonough, M. A., King, O. N., Kawamura, A., and Schofield, C. J. (2006) Structural studies on 2-oxoglutarate-dependent dioxygenases. J. Biol. Chem. 281, 130,098–130,104
18. Wilson, D. M., 3rd, Takeshita, M., and Demple, B. (1997) Basic site binding by the human apurinic endonuclease, Ape, and determination of the DNA contact sites. Nucleic Acids Res. 25, 933–939
19. Sigman, D. S., Kuwabara, M. D., Chen, C. H., and Bruice, T. W. (1991) Nuclease activity of 1,10-phenanthroline-copper in study of protein-DNA interactions. Methods Enzymol. 208, 414–433
20. Ryle, M. J., Padmakumar, R., and Hausinger, R. P. (1999) Stopped-flow quantification of a guanine-thymine intrastrand cross-link lesion induced by Cu(II)/H2O2/ascorbate. Biochemistry 38, 15278–15286
21. Wang, J., Yuan, B., Guerrero, C., Bahde, R., Gupta, S., and Wang, Y. (2011) High performance liquid chromatography-tandem mass spectrometry coupled with stable isotope-dilution method. Anal. Chem. 83, 2201–2209
22. Kaelin, W. G., Jr., and Ratcliffe, P. J. (2008) Oxygen sensing by metazoans. The central role of the HIF hydroxylase pathway. J. Biol. Chem. 303, 393–402
23. Wang, J., Yuan, B., Guerrero, C., Bahde, R., Gupta, S., and Wang, Y. (2011) High performance liquid chromatography-tandem mass spectrometry coupled with stable isotope-dilution method. Anal. Chem. 83, 2201–2209
24. Hong, H., Cao, H., Wang, Y., and Wang, Y. (2006) Identification and quantification of a guanine-thymine intranstand cross-link lesion induced by Cu(II)/H2O2/ascorbate. Chem. Res. Toxicol. 19, 614–621
25. van Leeuwen, F., Wijsman, E. R., Kieft, R., van der Mareel, G. A., van Boom, J. H., and Borst, P. (1997) Localization of the modified base J in telomeric VSG gene expression sites of Trypanosoma brucei. Genes Dev. 11, 3232–3241
26. Frelon, S., Douki, T., Ravant, J. L., Pouget, J. P., Tornabene, C., and Cadet, J. (2000) High performance liquid chromatography-tandem mass spectrometry measurement of radiation-induced base damage to isolated and cellular DNA. Chem. Res. Toxicol. 13, 1002–1010
27. Clifton, I. J., McDonough, M. A., Ehrismann, D., Kershaw, N. J., Granatino, N., and Schofield, C. J. (2006) Structural studies on 2-oxoglutarate oxygenases and related double-stranded β-helix fold proteins. J. Inorg. Biochem. 100, 644–669
28. Trewick, S. C., Henshaw, T. F., Hausinger, R. P., Lindahl, T., and Sedgwick, B. (2002) Oxidative demethylation by Escherichia coli AlkB directly reverts DNA base damage. Nature 419, 174–178
29. Gerken, T., Girard, C. A., Tung, Y. C., Webby, C. J., Saudak, V., Hewitson, K. S., Yeo, G. S., McDonough, M. A., Cunliffe, S., McNeil, L. A., Galvanovskis, J., Rorsman, P., Robins, P., Prieur, X., Coll, A. P., Ma, M., Jovanovic, Z., Farooqi, I. S., Sedgwick, B., Barroso, L., Lindahl, T., Ponting, C. P., Ashcroft, F. M., O’Rahilly, S., and Schofield, C. J. (2007) The obesity-associated FTO gene encodes a 2-oxoglutarate-dependent nucleic acid demethylase. Science 318, 1469–1472
30. Kieft, R., Brand, V., Ekanayake, D. K., Sweeney, K., DiPaolo, C., Reznikoff, W. S., and Sabatini, R. (2007) JBP2, a SWI2/SNF2-like protein, regulates de novo telomeric DNA glycosylation in bloodstream form Trypanosoma brucei. Mol. Biochem. Parasitol. 156, 24–31
31. Holme, E., Lindstedt, G., Lindstedt, S., and Toftt, M. (1970) 7-Hydroxylatio
32. Simmons, J. M., Müller, T. A., and Hausinger, R. P. (2008) Fe(II)/α-ketoglutarate hydroxylases involved in nucleobase, nucleoside, nucleotide, and chromatin metabolism. Dalton Trans. 38, 5132–5142
33. Genest, P. A., ter Riet, B., Dumas, C., Papadopoulou, B., van Luenen, H. G., and Borst, P. (2005) Formation of linear inverted repeat amphicons after targeting of an essential gene in Leishmania. Nucleic Acids Res. 33, 1699–1709
34. Sabatini, R., Meeuwenhoor, N., van Boom, J. H., and Borst, P. (2002) Recognition of base J in duplex DNA by J-binding protein. J. Biol. Chem. 277, 958–966
35. Heidebrecht, T., Christodoulou, E., Chalmers, M. J., Jan, S., Ter Riet, B., Grover, R. K., Joostten, R. P., Littler, D., van Luenen, H., Griffin, P. R., Wentworth, P., Jr., Borst, P., and Perrakis, A. (2011) The structural basis for recognition of base J containing DNA by a novel DNA binding domain in JBP1. Nucleic Acids Res. 39, 5715–5728
36. van Leeuwen, F., Dirks-Mulder, A., Dirks, R. W., Borst, P., and Gibson, W. (1998) The modified DNA base β-glucosyl-hydroxymethyluracil is not found in the tsetse fly stages of Trypanosoma brucei. Mol. Biochem. Parasitol. 94, 127–130
37. Tielens, A. G., and van Hellemmond, J. J. (2009) Surprising variety in energy metabolism within Trypanosomatidae. Trends Parasitol. 25, 482–490
38. Cazzulo, J. J. (1992) Energy metabolism in Trypanosoma cruzi. Subcell. Biochem. 18, 235–257
39. Fairlamb, A. H., Henderson, G. B., and Cerami, A. (1986) The biosynthesis of trypanothione and N1-glutathionylspermidine in Crithidia fasciculata. Mol. Biochem. Parasitol. 21, 247–257
40. Michels, P. A., Hannaert, A., Dirks, R. W., Borst, P., and Gibson, W. (2000) Metabolic aspects of glycosomes in trypanosomatidae. New data and views. Parasites. Today 16, 482–489
41. Masson, N., and Ratcliffe, P. J. (2003) HIF prolyl and asparaginyl hydroxylase levels. J. Cell Science 116, 3041–3049
42. Majmundar, A. J., Wong, W. J., and Simon, M. C. (2010) Hypoxia-inducible factors and the response to hypoxic stress. Mol. Cell 40, 294–309
43. Wengen, R. H. (2000) Mammalian oxygen sensing, signaling, and gene regulation. J. Exp. Biol. 203, 1253–1263
44. Ernst, J. F., and Tielker, D. (2009) Responses to hypoxia in fungal pathogens. Cell. Microbiol. 11, 183–190