Identification of a Novel Prostaglandin F\(_{2\alpha}\) Synthase in 
*Trypanosoma brucei*

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Abstract

Members of the genus *Trypanosoma* cause African trypanosomiasis in humans and animals in Africa. Infection of mammals by African trypanosomes is characterized by an upregulation of prostaglandin (PG) production in the plasma and cerebrospinal fluid. These metabolites of arachidonic acid (AA) may, in part, be responsible for symptoms such as fever, headache, immunosuppression, deep muscle hyperaesthesia, miscarriage, ovarian dysfunction, sleepiness, and other symptoms observed in patients with chronic African trypanosomiasis. Here, we show that the protozoan parasite *T. brucei* is involved in PG production and that it produces PGs enzymatically from AA and its metabolite, PGH\(_2\). Among all PGs synthesized, PGF\(_{2\alpha}\) was the major prostanoid produced by trypanosome lysates. We have purified a novel *T. brucei* PGF\(_{2\alpha}\) synthase (TbPGFS) and cloned its cDNA. Phylogenetic analysis and molecular properties revealed that TbPGFS is completely distinct from mammalian PG synthases. We also found that TbPGFS mRNA expression and TbPGFS activity were high in the early logarithmic growth phase and low during the stationary phase. The characterization of TbPGFS and its gene in *T. brucei* provides a basis for the molecular analysis of the role of parasite-derived PGF\(_{2\alpha}\) in the physiology of the parasite and the pathogenesis of African trypanosomiasis.

Key words: *Trypanosoma brucei* • African trypanosomiasis • PG production • PGF\(_{2\alpha}\) synthase • aldo-keto reductase

Introduction

Prostaglandins (PGs)\(^1\) of the 2 series are synthesized by the oxygenation of AA. In this pathway, AA is converted to PGH\(_2\) by cyclooxygenases (COX-1 and -2). Subsequently, the resulting PGH\(_2\) is converted in vivo and in vitro to various arachidonate metabolites, such as PGD\(_2\), PGE\(_2\), and PGF\(_{2\alpha}\) (Fig. 1) by the action of their respective synthases (1).

PGs are actively produced and widely distributed in various tissues of mammals, where they are potent mediators of a large variety of physiological and pathological responses including regulation of vascular tone, miscarriage, ovarian dysfunction, infertility, sleepiness, inflammation, bronchoconstriction, pain, fever, immunosuppression, and other symptoms (2–7). However, PG production is not restricted to mammals. Studies have shown the production of PGs by parasites such as cestodes, nematodes, protozoa (8–13) in response to the addition of AA or calcium ionophore. In *Schistosoma mansoni*, PG production has been reported to be associated with the transformation of cercaria into schistosomules (14), whereas in the protozoan parasite *Amoeba proteus*, PGs may play a signal-cou-
plunging role during phagocytosis, because they elicit vacuole formation (15). PGF\textsubscript{2\alpha} and PGD\textsubscript{2} are also found to be consistently elevated in the plasma of animals experimentally infected with *Trypanosoma congolense* (16) and in the cerebrospinal fluid of humans with chronic infection of *T. brucei gambiense* (17), respectively. These results suggest an upregulation of PG production during African trypanosomiasis. However, the molecular basis for this upregulation has not yet been elucidated. In addition, despite the obvious importance of PGs in the pathogenesis of parasitic infections and of PG production in parasitic protozoa, little is known about the molecular mechanisms of PG production in these organisms. The results presented here identify for the first time a *T. brucei* protein that exhibits a PG synthase activity capable of specifically converting PGH\textsubscript{2} to PGF\textsubscript{2\alpha}.

**Materials and Methods**

**Nucleotide Sequence Data.** The nucleotide sequence data reported in this paper is available from EMBL/GenBank/DDBJ under accession number AB034727.

*T. brucei* Cells. Bloodstream forms of *T. brucei* clone MITat 1.4 were isolated from infected rats as described previously (18). Trypanosome cells were cultivated in the presence or absence of 66 \( \mu \)M AA in a modified minimum essential medium supplemented with 10% FCS. The culture was incubated in a 5% CO\textsubscript{2} atmosphere at 37\textdegree C as described previously (18–20). Cells were harvested from the logarithmic growth phase or from the late stationary phase by centrifugation (1,500 \( g \)) at 4\textdegree C for 5 min. Cells were lysed and used as enzyme sources. After centrifugation, the supernatants were used for the determination of PGs secreted into the culture medium by live trypanosomes.

**Incubation of T. brucei Lysates and Enzyme Assay.** Lysates from logarithmic growth phase *T. brucei* (2.5 \( \times \) 10\textsuperscript{8} cells) isolated from infected rats and from the logarithmic growth and late stationary phase (8.3 and 5.4 \( \times \) 10\textsuperscript{7} cells, respectively) organisms isolated from bloodstream-form cultures were prepared by hypotonic lysis using double-distilled water containing a cocktail of reversible and irreversible inhibitors (one tablet in 25 ml) of pancreas extract, pronase, thermolysin, chemotrypsin, trypsin, and papain (Complete\textsuperscript{TM}, Roche Diagnostics). For PG production from AA, we used the reaction mixture described by Ujihara et al. (21) with the following modifications: 100 mM sodium phosphate, pH 7.0, 2 \( \mu \)M hematin, 5 mM tryptophan, 1 mM AA, and 300 \( \mu \)l of the respective *T. brucei* lysates in a final volume of 500 \( \mu \)l. The mixture was incubated at 37\textdegree C for 30 min, and then the reaction was stopped by addition of 100 \( \mu \)l of 1 M HCl and 6 vol of cold ethyl acetate.

For PGF\textsubscript{2\alpha} synthesis from PGH\textsubscript{2}, a standard reaction mixture that contained 100 mM sodium phosphate, pH 7.0, 20 \( \mu \)M NADP\textsuperscript{+}, 100 \( \mu \)M glucose-6-phosphate, 1 U of glucose-6-phosphate dehydrogenase, and a diluted amount of enzyme in a final volume of 100 \( \mu \)l was used. The reaction was started by the addition of 1 \( \mu \)l of 500 \( \mu \)M 1-[\textsuperscript{14}C]PGH\textsubscript{2} (2.04 Gbq/mmol) and was carried out at 37\textdegree C for 2 min and terminated by the addition of 250 \( \mu \)l of a stop solution (30:4:1 vol/vol/vol diethyl ether/methanol/2 M citric acid). To test for the nonenzymatic formation of PGF\textsubscript{2\alpha}, we incubated the reaction mixture containing all of the components in the absence of the enzyme. The organic phase (50 \( \mu \)l) was applied to 20 \( \times \) 20-cm silica gel plates (Merck) at 4\textdegree C, and the plates were developed with a solvent system of 90:2:1 vol/vol/vol diethyl ether/methanol/acetic acid at –20\textdegree C. The radioactivity on the plates was monitored and analyzed by a Fluorescent Imaging Analyzer FLA 2000 and Mac Bas V2.5 software (Fuji Photo Film Co.). For assessment of substrate specificity, the reaction mixtures consisted of 100 mM sodium phosphate, pH 7.0, the purified recombinant enzyme, 100 \( \mu \)M NADPH, and substrates at various concentrations, as indicated in Table II, in a total volume of 500 \( \mu \)l. Reactions were initiated by the addition of substrate, and the decrease in absorbance at 340 nm was monitored at 37\textdegree C. Blanks without enzyme or without substrate were included. The 9,10-phenanthrenequinone reductase activity was chosen to represent 100% activity. For 1-[\textsuperscript{14}C]-PGD\textsubscript{2} and PGE\textsubscript{2} production, 40 \( \mu \)M 1-[\textsuperscript{14}C]PGH\textsubscript{2} was incubated with either 250 \( \mu \)g of recombinant hematopoietic PGDS.
(22) or 500 μg of recombinant PGES (23). After incubation at 25°C for 5 min, the resulting PGs were extracted three times with cold ethyl acetate, dried at a low temperature under vacuum, and used as substrates for the T. brucei PGF<sub>2α</sub> synthase (Tb-PGFS) specificity study.

**Extraction and Quantification of PGs.** After addition of [H]PGD<sub>2</sub>, [H]PGE<sub>2</sub>, and [H]PGF<sub>2α</sub> (60 Bq each per assay; NEN Life Science Products), used as tracers to determine the recovery during extractions, PGs recovered from the trypomastigote culture medium (10 ml) and those from incubation of parasite lysates (300 μl) were extracted and separated by HPLC as described previously (9, 21). The resulting PGD<sub>2</sub>, PGE<sub>2</sub>, and PGF<sub>2α</sub> were quantified by enzyme immunoassay (EIA) with their respective EIA kits (Cayman Chemical).

**Gas Chromatography–Mass Spectrometry Analysis.** Gas chromatography–selected ion monitoring (GC-SIM) analyses were run on a Hitachi M-80B double-focused mass spectrometer equipped with a Van den Berg’s solventless injector and a fused silica capillary column (Ultra no. 1; 25 m length, 0.32 mm internal diameter, 280°C column temperature). PGs recovered from culture media and those formed by the incubation of parasite lysates with AA were fractionated by HPLC and converted into their corresponding methyl ester (ME)-dimethylisopropylsilyl (DMiPS) ether or ME-methoxime ether derivatives according to the method described previously (24).

**Purification and Determination of the Partial Amino Acid Sequences of TbPGFS.** Bloodstream-form T. brucei cells (5 × 10<sup>11</sup>) were isolated from infected rats and lysed by hypotonic lysis. Soluble proteins, obtained by differential centrifugation at 3,000 g for 15 min and then at 100,000 g for 1 h at 4°C, were fractionated with ammonium sulfate. The active fractions (40–100% saturation) were resuspended in 0.1 M sodium phosphate, pH 7.0, and loaded onto a HiloLoad 16/60 Superdex 75 pg gel filtration column (Amersham Pharmacia Biotech) and eluted with 20 mM NaCl in 20 mM Tris/Cl, pH 8.0. Active fractions were pooled, dialyzed against 20 mM sodium phosphate, pH 7.0, and concentrated by use of Centricon centrifugal filters with a molecular mass cut-off of 3,000 daltons (Millipore). The concentrated active fraction was further loaded onto a Resource PHE Hydrophobic interaction column (Amersham Pharmacia Biotech), which had been equilibrated with 2 M ammonium sulfate in 20 mM sodium phosphate, pH 7.0, and eluted with a decreasing linear gradient of 2–0 M ammonium sulfate in the same buffer containing 1% (vol/vol) Tween 20. The active peak was dialyzed against 20 mM Tris/Cl, pH 8.0, and applied to a Mono Q HR 10/10 ion exchange column (Amersham Pharmacia Biotech) equilibrated with the same buffer. The elution was carried out with an increasing linear gradient of 0–400 mM NaCl in the same buffer. The active fraction was further purified by gel filtration on a HiloLoad 16/60 Superdex 200 pg column (Amersham Pharmacia Biotech).

Protein concentration was determined by use of bicinechonic acid reagent (Pierce Chemical Co.) with BSA as a standard following the manufacturer’s protocol. The purity of the protein was assessed by SDS-PAGE on 14% (wt/vol) gels, and the gels were stained with silver (Daichi Pure Chemicals), sypro orange (Bio–Rad Laboratories), or Coomassie Brilliant Blue (Daichi Pure Chemicals). Pure protein (220 μg) was subjected to in-gel digestion with lysyl-endopeptidase for the determination of the internal amino acid sequences according to Rosenfeld et al. (25).

**PCR Amplification, cDNA Cloning, and Sequencing.** Total RNA was extracted from bloodstream-form T. brucei cells (4 × 10<sup>8</sup>) by sour phenol, pH 4.5, saturated with TE buffer. First-strand cDNA was synthesized by avian myeloblastosis virus reverse transcriptase after annealing 1 μg of T. brucei total RNA with Oligo DT- Adaptor Primer (Takara Shuzo). For 3′ rapid amplification of cDNA ends (RACE), the first PCR amplification was carried out with gene-specific primers, i.e., sense primer 5′-AGGGTTATCCGACACATGGAGGCG-3′ and antisense primer M13 M4 (5′-GTATTCTCCAGTACGAC-3′) and the first-strand cDNAs as the template by the following program: after initial denaturation at 95°C for 5 min, the PCR reaction proceeded at 94°C for 20 s, 55°C for 20 s, and 74°C for 30 s for 30 cycles. PCR products from the first PCR amplification were used as the templates for nested PCR amplification with gene-specific primers, i.e., sense primer 5′-CTGTACGGCGATAAGAGGTCGGCGCC-3′ and antisense primer M13 M4. For 5′ RACE, the resulting PCR product was amplified with a 23-mer sense primer from T. brucei spliced leader sequence, 5′-CGCTATTTTATAGAAGCTTTTG-3′ (26) and a gene-specific antisense primer, 5′-GTGTTACTACTCGAAGACCCCGA-3′. The amplified fragments were cloned into pGEM-T Easy vector (Promega). DNA sequences were determined from both strands with a SequiTherm cycle sequencing kit (Epiconcept Technologies) and a LI-COR automated DNA sequencer (model 4000; LI-COR Inc.).

**Alignment of Primary Structures and Phylogenetic Analysis.** The sequences of 17 members of the aldo–keto reductase (AKR) superfamily were retrieved from public databases. The amino acid sequences were aligned with the TbPGFS sequence by using the CLUSTAL W program (27). A neighbor-joining method (28) of protein phylogeny was used to infer phylogenetic relationships among the sequences.

**Expression and Purification of the Recombinant Enzyme.** The coding region of TbPGFS cDNA was amplified by PCR using sense primer 5′-CGGAAATTCATGGCTTCTACATCCC-3′ and antisense primer 5′-CCGTCGCCACGTTACATCTCGAAGACCCCGA-3′, which carried EcoRI and Sall restriction sites at their respective 5′ ends. The amplified fragments containing the entire open reading frame were digested with EcoRI and Sall and then cloned into the corresponding sites of pMAL-c2 vector (New England Biolabs, Inc.). The resultant expression vector was used for transformation of Escherichia coli DH5α. Transformed cells were cultured for 6–7 h in the presence of 0.5–1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and then harvested and sonicated. The recombinant protein in the soluble fraction was purified according to the manufacturer’s protocol (New England Biolabs, Inc.).

**Northern Blot Analysis.** Total RNA from 10<sup>8</sup> cells of each growth stage was isolated as described above, separated on 1.2% (wt/vol) agarose–2% formaldehyde gels, and blotted onto a Hybond N<sup>+</sup> membrane (Amersham Pharmacia Biotech). RNA was crosslinked by UV radiation for 6 min, and the membrane was then dried for 2 h at 80°C. After prehybridization, hybridization was performed for 3 h in 5× SSPE (sodium saline phosphate EDTA), 5× Denhardt’s solution, 0.5% (wt/vol) SDS, and 0.5 mg sonicated salmon sperm DNA using the DIG system (Roche Diagnostics) according to the manufacturer’s protocol. After high-stringency washing, the blot was pretreated with 0.1 M maleic acid, 0.15 M NaCl, pH 7.5, containing 1% blocking reagent before the antidigoxigenin–AP Fab fragment was added and was then developed with CDP-Star (all reagents from Roche Diagnostics). Chemiluminescence was detected by use of BioMax Light-1 film (Sigma–Aldrich).
Results and Discussion

Production and Secretion of PGD₂, PGE₂, and PGF₂α from AA by T. brucei. To assess possible PG production and secretion by live trypanosomes, we cultured T. brucei in vitro in a chemically defined medium supplemented with 10% FCS. PGD₂, PGE₂, and PGF₂α secreted into the culture medium were quantified by EIA. As shown in Fig. 2A, live trypanosomes secreted small amounts of PGs: 75 ± 10 pg/ml for PGD₂, 100 ± 9.4 pg/ml for PGE₂, and 156 ± 20 pg/ml for PGF₂α (n = 3). As with other parasitic protozoa, T. brucei does not synthesize AA de novo from acetate (29), but it does take up this fatty acid from its environment and does incorporate it into its total and separate classes of lipids (30, 31). The presence of phospholipase A₂ (32, 33), an enzyme that catalyzes the release of AA from phospholipids, in T. brucei indicates the existence of a free intracellular AA source in this parasitic protozoan. Thus, the small amounts of PGs, secreted by T. brucei in the absence of exogenously added AA, were most likely produced from the intracellular AA and from the trace amount of AA present in the serum used for the medium preparation. We then grew trypanosomes in the presence of 66 μM AA exogenously added and which had no effect on cell growth (our unpublished observations). PG accumulation in the media increased to 875 ± 16 pg/ml for PGD₂, 447 ± 11 pg/ml for PGE₂, and

![Figure 2](image-url)

**Figure 2.** PG production by T. brucei and mass spectra of trypanosomal PGs. (A) The PGs secreted into the culture media by live trypanosomes when the organisms were cultured with or without AA were quantified. − AA and + AA indicate growth in the absence and presence of 66 μM AA, respectively. PG detection limits were <25, 11, and 10 pg/assay for PGD₂, PGE₂, and PGF₂α, respectively. The values shown are the means from three independent experiments along with SE. (B) PG production by T. brucei lysates. PGs were produced during the incubation of 1 mM AA with lysates of bloodstream-form trypanosomes. The values shown are the means from three independent experiments along with SE. (C–E) To identify and quantify the prostanoids by GC-MS, PGs from the incubation of trypanosome lysates with AA were extracted and resolved into PGD₂, PGE₂, and PGF₂α as described in Materials and Methods. After treatment (see Materials and Methods), the prostanoids were subjected to GC-MS. (C) Selected ion recordings monitoring characteristic ions for PGD₂ at m/z 595. (D) Those for PGE₂ at m/z 595. (E) Those for PGF₂α at m/z 668. The number indicates the ion intensity for each peak. Upper mass spectra indicate traces of the selected ion recordings of the ME-MO-DMiPS ether derivatives of authentic PGD₂ (C), PGE₂ (D), and PGF₂α (E); and lower ones, traces of the selected ion recordings of the ME-MO-DMiPS ether derivatives of PGD₂ (C), PGE₂ (D), and PGF₂α (E) produced by trypanosome lysates. (F) Effects of heat treatment and of NSAIDs on PG production by trypanosome lysates. Nonboiled (control) and boiled T. brucei lysates were incubated with 1 mM AA at 37°C for 30 min in the absence of NSAIDs, and PGs produced were measured by EIA. To investigate the effects of NSAIDs, we incubated nonboiled T. brucei lysates with 1 mM AA in the absence (control) or presence of the indicated concentrations of aspirin or indomethacin. The values shown are the means from three independent experiments along with SE.
211 ± 6 pg/ml for PGF$_{2\alpha}$ ($n = 3$), suggesting that, in vivo, *T. brucei* would rely on AA uptake from its environment (host tissues) to release significant amounts of PGs.

When the cell lysates were prepared from *T. brucei* grown in vitro in the presence or absence of AA and incubated with 1 mM AA, PGs were actively produced in a time- and dose-dependent manner (data not shown). PGF$_{2\alpha}$ was the major prostanoid synthesized in both cases, followed by PGE$_2$ and PGD$_2$ (Fig. 2 B). The addition of AA during the cultivation of trypanosomes increased PG formation by the lysates ~12-fold for PGD$_2$, 11-fold for PGE$_2$, and 5-fold for PGF$_{2\alpha}$.

The molecular masses and the characteristic fragment ions of PGs produced by trypanosomes were determined by GC-SIM analysis of the DMiPS ether derivatives. All of the materials eluted from HPLC had exactly the expected molecular masses: $m/z$ 595 for PGD$_2$ (Fig. 2 C) and PGE$_2$ (Fig. 2 D) and $m/z$ 668 for PGF$_{2\alpha}$ (Fig. 2 E). Relative ion intensity ratios of individual characteristic fragments of PG derivatives in the eluates were identical to those of authentic standards. Quantification by gas chromatography–mass spectrometry analysis (GC-MS) of PGs extracted from culture medium or trypanosome lysates incubated with AA gave values of the same order of magnitude as those determined by EIA, and finally PGs derived from trypanosomes coeluted with authentic standards on HPLC and showed the same titration curves as authentic PGs by EIA (data not shown).

To determine the nature of the protozoan catalyst and to evaluate whether or not the catalyst shares a kinship with COX-1 and COX-2 from mammalian tissues, we investigated the effects of heat and nonsteroidal antiinflammatory drugs (NSAIDs) on PG production by *T. brucei* lysates. Heating the lysates for 5 min at 100°C before incubation with 1 mM AA reduced the levels of PGD$_2$, PGE$_2$, and PGF$_{2\alpha}$ synthesis to <10% as compared with control values, whereas the addition of 3 mM aspirin or 42 µM indomethacin to the reaction mixture had no inhibitory effects on PG formation (Fig. 2 F). The results reported here show that PG-producing activities in *T. brucei* were heat sensitive and unaffected by aspirin or indomethacin. As NSAIDs are well established inhibitors of mammalian COX-1 and COX-2 (34), the trypanosomal enzyme system involved in PG synthesis from AA is markedly different from its mammalian counterpart. Future investigations in our laboratory will probably unravel the identity of the enzymes involved in PG generation in this organism.

**Enzymatic Formation of PGD$_2$ and PGF$_{2\alpha}$ from PGH$_2$ by *T. brucei* Lysates.** Another approach to demonstrate de novo synthesis of PGs in *T. brucei* was to use 1-[¹⁴C]PGH$_2$ as a substrate. As an initial attempt to identify PG-producing enzymes in *T. brucei*, we prepared membrane and cytosolic fractions from bloodstream-form trypanosomes grown in rats and incubated them with 1-[¹⁴C]PGH$_2$ in the presence or absence of various cofactors. Two major PG synthase activities were identified (Fig. 3, A and B). Membrane proteins isomerized PGH$_2$ to PGD$_2$ (Fig. 3 A, lane 2). No PGD$_2$ was formed during incubation of PGH$_2$ with heat-inactivated membrane fractions (data not shown) or in the absence of membrane proteins, though a small nonenzymatic production of PGE$_2$ was observed under the same conditions (Fig. 3 A, lane 1).

On the other hand, in the presence of a NADPH-generating system, cytosolic proteins (Fig. 3 B, lane 2), but not heat-inactivated ones (Fig. 3 B, lane 3), synthesized PGF$_{2\alpha}$ from PGH$_2$. PGH$_2$ was converted nonenzymatically to PGD$_2$ but not to PGF$_{2\alpha}$ (Fig. 3 B, lane 1). Also, no PGF$_{2\alpha}$ formation was observed in the absence of the NADPH-generating system or in the presence of 5 µM of other cofactors such as dithiothreitol and glutathione (data not shown). The profile of PG production from PGH$_2$ is consistent with that of PG synthesis from AA. Our data show that PGH$_1$ may be used as a substrate for PG synthesis by a protozoan parasite. Earlier studies on cell–cell interactions in the eicosanoid pathway have demonstrated the enzymatic cooperation between cell types (35). It has been shown that platelets can transfer PGH$_2$ to cultured endothelial cells (36) and vascular tissue (37) for efficient conversion to PGI$_2$ and thromboxane, respectively, and that platelets can utilize AA released by endothelial cells for lipoygenase metabolism (36). Additional evidence has also shown that such an exchange may occur in vivo (38, 39). However, it is not yet clear whether *T. brucei* utilizes PGH$_2$ as a predominant pathway for PGF$_{2\alpha}$ generation in host tissues, because the nature of the trypanosomal catalyst that converts AA to PGs is not known. The results presented here show also that PGD$_2$ and PGF$_{2\alpha}$ synthases from PGH$_2$ are catalyzed by trypanosomal enzymes. PGH D-isomerase (PGD synthase) activity is localized in the membrane proteins, a fact that made its purification difficult, and PGH F$_{2\alpha}$ reductase (PGF$_{2\alpha}$ synthase) activity is in soluble pro-
teins. As PGF$_{2\alpha}$ was the major prostanoid synthesized by trypanosome lysates, we started with the cytosolic activity to purify TbPGFS.

**Purification and Partial Amino Acid Sequencing of TbPGFS.** We purified TbPGFS from the soluble fraction of early logarithmic growth phase bloodstream trypanosomes by ammonium sulfate (40–100% saturation) fractionation and sequential column chromatographies. TbPGFS activity was monitored by the reduction of PGH$_2$ in the presence of a NADPH-generating system. Table I summarizes the results of a typical purification procedure. Active fractions from the final gel filtration (Hiload 16/60 Superdex 200 pg)

| Fraction       | Protein mg | Total activity nmol/min | Specific activity nmol/min/mg | Purification fold | Recovery % |
|----------------|------------|-------------------------|-----------------------------|-------------------|-----------|
| Cytosol        | 512        | 410                     | 0.80                        | 1                 | 100       |
| 40–100% (NH$_4$)$_2$SO$_4$ | 42          | 392                     | 9.30                        | 12                | 96        |
| Superox 75     | 15         | 328                     | 22                          | 28                | 80        |
| Resource PHE   | 4.9        | 255                     | 52                          | 65                | 62        |
| Mono Q         | 0.2        | 93                      | 465                         | 581               | 23        |
| Superdex 200   | 0.06       | 52                      | 860                         | 1,075             | 13        |

**Figure 4.** Sequence homology of TbPGFS with members of the AKR superfamily. (A) Multiple sequence alignment of deduced TbPGFS amino acid sequence with representative members of the AKR superfamily. The amino acid sequences were taken from the sequence databases SwissProt (SP), Protein Identification Resource (PIR), and GenBank (GB). TbPGF synthase is aligned with: L major-putRed, *L. major* putative reductase (SP P22045) (57); BovPGFS, bovine lung PGFS (GB J03570) (58); Bsub-put-Mordh, *B. subtilis* putative morphine dehydrogenase (GB AF008220) (59); Pstipitis-XR, *Pichia stipitis* xylose reductase (GB AE008220) (59); Ps put-Mordh, *Pseudomonas putida* morphine dehydrogenase (GB M94775); and Therm-OxidoRed, *Thermotoga maritima* oxidoreductase (GB AE001762).
were pooled and analyzed by SDS-PAGE. Upon silver staining, a single band with a molecular mass of 33 kD was detected (Fig. 3 C, lane 2). Finally, TbPGFS was purified 1,075-fold with a recovery of ~13% and a specific activity of 860 nmol/min/mg of protein.

We subjected the purified protein to partial amino acid sequencing. Although the NH₂ terminus was blocked, we identified two amino acid sequences, LWNSDQGYES and NIAVTAWSPL (Fig. 4 A), of two internal peptide fragments from in-gel digestion of the purified TbPGFS with lysyl-endopeptidase.

Cloning of TbPGFS cDNA. From a database search using the two amino acid sequences mentioned above, we identified the corresponding 538-bp gene fragment in public databases (available from EMBL/GenBank/DBJ under accession number AQ945329) that encoded the amino acid sequence NIAVTAWSPL. As this genomic clone was truncated at the NH₂ terminus, we isolated a 1,164-bp full-sequence NIAVTAWSPL. As this genomic clone was identified two amino acid sequences, LWNSDQGYES and NIAVTAWSPL (Fig. 4 A), of two internal peptide fragments from in-gel digestion of the purified TbPGFS with lysyl-endopeptidase.

Relationship of the TbPGFS to Other Members of the AKR Superfamily. Sequence analysis, database search, and alignment of the TbPGFS amino acid sequence (Fig. 4 A) revealed that the TbPGFS is a member of subfamily 5A of the AKR superfamily (40). The TbPGFS amino acid sequence showed 61% identity to that of a Leishmania major putative reductase and between 48 and 55% identity to bacterial protein sequences. In contrast, TbPGFS showed rather low identities to AKRs from mammals and plants (39–40%). Furthermore, phylogenetic analysis (Fig. 4 B) confirmed that TbPGFS formed a clade together with L. major putative reductase and Bacillus subtilis putative morphine dehydrogenase. However, bootstrap proportions of the clades forming Trypanosomatid-Bacillus and Leishmania-Trypanosoma were relatively low (500–600), and thus the statistical support for any particular topology linking TbPGFS to a mammal, plant, yeast, or bacteria AKR cluster was rather weak. These results demonstrate that TbPGFS is different not only from other AKRs of mammals, including mammalian PGFS, but from AKRs of plants, yeast, and prokaryotes, indicating that it is a novel enzyme. The fact that this enzyme forms a distinct and distant clade with the statistical support for any particular topology linking TbPGFS to a mammal, plant, yeast, or bacteria AKR cluster was rather weak. These results demonstrate that TbPGFS is different not only from other AKRs of mammals, including mammalian PGFS, but from AKRs of plants, yeast, and prokaryotes, indicating that it is a novel enzyme.

Expression and Characterization of Recombinant TbPGFS Protein. TbPGFS protein was heterologously expressed in a pMAL-c2 expression system. The recombinant enzyme was produced as a fusion protein with maltose-binding protein (MBP) in the cytosolic fraction of E. coli after IPTG induction (Fig. 5 A, lane 3). Upon SDS-PAGE, the fusion protein, MBP–TbPGFS, exhibited a molecular mass of ~76 kD (Fig. 5 A, lane 3). Crude proteins from E. coli expressing MBP–TbPGFS (Fig. 5 B, lane 4), but not lysates from E. coli host or from E. coli transformed with empty pMAL-c2 vector (Fig. 5 B, lanes 2 and 3), converted PGH₂ to PGF₂α only in the presence of a NADPH-generating system, indicating that the detected synthase activity depended on T. brucei protein and not on any contaminating activity from the host or from the vector. This is in good agreement with early results demonstrating de novo synthesis of PGF₂α from PGH₂ by the isolated enzyme (Figs. 3 A and 4 A).
B and 5 B), and these findings show that the cloned *T. brucei* cDNA encoded a bona fide PGF synthase and provide conclusive evidence for the existence of specific PG biosynthetic pathways in a pathogenic parasite. The fusion protein was purified by amylose affinity chromatography (Fig. 5 A, lane 4), gel filtration after factor Xa cleavage to separate TbPGFS from MBP (Fig. 5 A, lane 5), and ion exchange chromatography (Fig. 5 A, lane 6). The purified recombinant TbPGFS had an apparent molecular mass of 33 kD (Fig. 5 A, lane 6), identical to that of the wild-type protein (Fig. 3 C, lane 2).

Characterizing the purified recombinant enzyme and comparing its molecular properties with those of a number of mammalian PGF synthases purified and characterized from our laboratory (41–45), we found that TbPGFS exhibited a high and specific PGFS activity toward PGH2 (2 mmol/min/mg), a broad range of temperatures (25–40°C), and pH (6–9) optima, the lowest *K*ₘ (1.3 μM) for PGH₂, and the highest *k*₅₀ value of 63 min⁻¹ (Table II). As a member of the AKR superfamily, the purified TbPGFS showed AKR activity toward other substrates as well. It exhibited significantly high activity toward 9,10-phenanthrenequinone.

| Substrate                  | Concentration | Relative activity | *K*ₘ  | *V*₅₀      |
|---------------------------|---------------|------------------|-------|------------|
| 9,10-Phenanthrenequinone  | 40 μM         | 100%             | 0.42  | 48.0       |
| p-Nitrobenzaldehyde       | 40 μM         | 64%              | 0.80  | 22.0       |
| PGH₂                      | 5 μM          | 44%              | 1.30  | 2.0        |
| Progesterone              | 40 μM         | 2%               | ND    | ND         |
| Sodium glucuronate        | 40 μM         | 1%               | ND    | ND         |
| Testosterone              | 40 μM         | 0.4%             | ND    | ND         |
| NADPH                     | –             | –                | –     | 5.7        |
| NADH                      | –             | –                | –     | nd         |

ND, not determined; nd, not detected.

Table II. *Substrate Specificity and Kinetic Parameters of TbPGFS*

Figure 6. PG production, differential expression of TbPGFS mRNA, and TbPGFS activity during the *T. brucei* life cycle. (A) PG secretion by bloodstream-form live trypanosomes from early logarithmic growth and late stationary phases. (B) PG production by lysates from bloodstream-form trypanosomes from early logarithmic and late stationary growth phases. (C) Northern blot analysis. *T. brucei* total RNA (3.9 μg) was isolated from the bloodstream-form population from infected rats (left panel) and from early logarithmic (right panel, 19 h) and late stationary growth phase (right panel, 44 h) cells of the cultured bloodstream-form population. Total RNA was hybridized with a probe derived from the bloodstream-form population from infected rats and from early logarithmic and late stationary growth phase (right panel, 44 h) cells of the cultured bloodstream-form population. Total RNA was hybridized with a probe derived from the bloodstream-form population from infected rats and from early logarithmic and late stationary growth phase trypanosomes. Lysates of respective cells were incubated with 5 μM PGH₂. The reaction products were analyzed by thin-layer chromatography as described in the legend to Fig. 4 (*n* = 3).
thenequinone, p-nitrobenzaldehyde (the common substrates for this superfamily), and PGH₂ but remarkably weak activity toward progesterone, sodium glucuronate, or testosterone (Table II). However, the AKR substrates did not compete with PGH₂ when added simultaneously in excess (Fig. 5 C), suggesting the existence of different catalytic sites for both types of substrate.

PGF₂α is synthesized either by the 9,11-endoperoxide reduction of PGH₂ or by the 9-keto reduction of PGE₂ (Fig. 1; reference 46). These reactions are catalyzed by PGH 9,11-endoperoxide reductase (41) and PGE₂ 9-keto reductase (46, 47) in mammals. A third pathway, the 11-keto reduction of PGD₂ catalyzed by PGD 11-keto reductase, does not produce PGF₂α as initially thought. Instead, it leads to the formation of 9α,11β-PGF₂α, a stereoisomer of PGF₂α (48). PGFSs described to date are enzymes that exhibit two activities on the same molecule, i.e., PGH 9,11-endoperoxide reductase activity and PGD 11-keto reductase activity (41, 48). To elucidate the catalytic properties of TbpGFS, we incubated the recombinant TbpGFS with either 1-[^14C]PGD₂ (Fig. 5 D, lanes 1 and 2) or 1-[^14C]PGE₂ (Fig. 5 D, lanes 3 and 4). No 9α,11β-PGF₂α or PGF₂α formation was observed with either substrate. This finding demonstrates that TbpGFS is a rather specific PGH 9,11-endoperoxide reductase devoid of any PGD 11-keto reductase or PGE 9-keto reductase activity on the same molecule.

PG Production and Expression of TbpGFS mRNA during T. brucei Cell Growth. In the protozoan parasites Trypanosoma and Leishmania, several enzymes involved in citric acid cycle (49), respiratory chain activities (50–54), lysosomal function, or in the metabolism of protein, carbohydrate, and purine are developmentally regulated. To investigate PG production and the expression of TbpGFS mRNA during the T. brucei life cycle, we cultured trypanosome cells in the presence of 66 μM exogenous AA and harvested them at 19 h for early logarithmic phase or at 44 h for late stationary phase. We then used EIA to quantify PGs secreted by live trypanosomes into the media or produced by trypanosome lysates. Trypanosome cells from both the early logarithmic and late stationary phases released PGD₂, PGE₂, and PGF₂α (Fig. 6 A). No significant difference was observed between the levels of PGD₂ and PGE₂ secreted by the two growth stages. However, early logarithmic phase trypanosomes released PGF₂α fourfold more than those in late stationary phase. On the other hand, lysates from early logarithmic and late stationary phase trypanosomes produced different levels of all three PGs (Fig. 6 B). Early logarithmic phase trypanosomes produced a significantly high level of PGF₂α as compared with lysates from late stationary phase organisms (Fig. 6 B).

Northern blot analysis revealed that TbpGFS mRNA was abundantly expressed during the early logarithmic phase of trypanosomes grown in rats (Fig. 6 C) or in vitro (Fig. 6 C, 19 h) and was downregulated during the late stationary growth stage of these organisms (Fig. 6 C, 44 h). The expression profile, shown in Fig. 6 C, was found to be in good agreement with the TbpGFS activity in the respective cells (Fig. 6 D), which was higher during the early logarithmic growth stage of cells from rats (1.00 ± 0.07 nmol/min/mg) and during the early logarithmic growth stage of cells from in vitro culture (0.42 ± 0.02 nmol/min/mg) but lower during the late stationary growth stage of these organisms (0.04 ± 0.01 nmol/min/mg). Taken together, our data show that PG production as well as TbpGFS mRNA expression are developmentally regulated.

Infection of mammals by African trypanosomes results in the release of high levels of PGs (16, 17) that may, in part, be involved in the pathogenesis of the disease. It is generally believed and accepted that the PGs are produced by host cells after their stimulation with trypanosome products (55, 56). We have shown that the parasite itself produces PGs and may contribute directly to the production of these mediators in mammals. However, the physiological relevance of PG production in T. brucei remains unknown. The findings reported here open up ways to investigate the role of TbpGFS in T. brucei replication and development as well as provide a new tool to study the role of parasite-derived PGs in the pathogenesis of African trypanosomiasis.

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