A Key Role for Old Yellow Enzyme in the Metabolism of Drugs by *Trypanosoma cruzi*

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Abstract

Trypanosoma cruzi is the etiological agent of Chagas' disease. So far, first choice anti-chagasic drugs in use have been shown to have undesirable side effects in addition to the emergence of parasite resistance and the lack of prospect for vaccine against *T. cruzi* infection. Thus, the isolation and characterization of molecules essential in parasite metabolism of the anti-chagasic drugs are fundamental for the development of new strategies for rational drug design and/or the improvement of the current chemotherapy. While searching for a prostaglandin (PG) $F_{2\alpha}$ synthase homologue, we have identified a novel "old yellow enzyme" from *T. cruzi* (TcOYE), cloned its cDNA, and overexpressed the recombinant enzyme. Here, we show that TcOYE reduced 9,11-endoperoxide PGH_2 to $PGF_{2\alpha}$ as well as a variety of trypanocidal drugs. By electron spin resonance experiments, we found that TcOYE specifically catalyzed one-electron reduction of menadione and β-lapachone to semiquinone-free radicals with concomitant generation of superoxide radical anions, while catalyzing solely the two-electron reduction of nifurtimox and 4-nitroquinoline-*N*-oxide drugs without free radical production. Interestingly, immunoprecipitation experiments revealed that anti-TcOYE polyclonal antibody abolished major reductase activities of the lysates toward these drugs, identifying TcOYE as a key drug-metabolizing enzyme by which quinone drugs have their mechanism of action.

Key words: Chagas' disease • enzyme • chagasic drug reduction • redox cycling • PG production

Introduction

Old yellow enzyme (OYE)* was discovered in the 1930s and first used to demonstrate the requirement of a cofactor for catalysis by enzymes (1, 2). This enzyme has since been identified in yeasts (1, 3), plants (4), and bacteria (5, 6) but not in animals. Despite extensive biochemical and spectroscopic characterization, the physiological function of OYE has remained obscure and the possible involvement of it in the pathological processes of human disease is unknown.

Chagas' disease affects more than 20 million people in South America (7) where the disease is a major public health and economic problem. The limited success and liability of the current treatments for Chagas' disease has led to the search for new anti-trypanosomal drugs. Naphthoquinones such as menadione (2-methyl-1,4-naphthoquinone) and β-lapachone (3,4-dihydro-2,2-dimethyl-2*H*-naphtho[1,2-b]pyran-5,6-dione), and nitroheterocycles, i.e., 4-nitroquinoline-*N*-oxide, nifurtimox (3-methyl-*N*-

[(5-nitro-2-furanyl)-methylene]-4-thiomorpholinamine 1,1-dioxide), and benznidazole (2-nitro-*N*-[phenylmethyl]-1H-imidazole-1-acetamide) have been used for several decades as trypanocidal drugs (nifurtimox and benznidazole provided by S.R. Wilkinson, London School of Hygiene and Tropical Medicine, London, United Kingdom; references 8–11). These compounds are thought to exert their trypanocidal action by the generation of free radicals through a redox cycling process in which the drugs are enzymatically reduced to form drug anion radicals. Under aerobic conditions these radicals reduce oxygen nonenzymatically, leading to drug regeneration and formation of superoxide radical anions that cause oxidative stress and cell death in susceptible parasites. Several studies have reported free radical production upon the addition of these drugs to either intact cells or lysates of *Trypanosoma cruzi* (12–15). Although naphthoquinones and nitroheterocyclic drugs have been shown to undergo the redox cycling process within the parasite, the precise mechanism by which the drugs act and the involvement of parasite molecules in the redox cycling process have not yet been fully elucidated.

We have been investigating the metabolism of arachidonic acid (AA) in parasitic protozoa and have previously shown PG production in *Plasmodium falciparum* (16) and *Trypanosoma brucei* (17). To gain additional insight into PG synthesis and function in trypanosomatids, we extended our investigation to *T. cruzi* and identified a *T. cruzi* OYE (TcOYE) whose gene shares limited sequence identity (16–28%) with OYEs described earlier. In this study, we show that TcOYE catalyzes $PGF_{2\alpha}$ synthesis in addition to the reduction of a variety of trypanocidal drugs. Moreover, anti–TcOYE polyclonal antibody abolishes the reductase activity of *T. cruzi* epimastigote lysates toward naphthoquinone and nitroheterocyclic drugs, implicating TcOYE for the first time as a subversive target by which quinone drugs have their mechanism of action.

Materials and Methods

Sequence Data. The nucleotide sequence data reported in this paper is available from GenBank/EMBL/DDBJ under accession no. AB075599.

Parasites and Cultivation. Epimastigotes (the insect form) of *T. cruzi* clone Y_{NIH} (18) were grown at 26°C in liver infusion tryptose liquid medium, supplemented with 20 μg/ml hemin, 10% heat-inactivated fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin as previously described (19).

Enzyme Assays, PG Extraction, Analysis, and Quantification. For PG production from AA, *T. cruzi* epimastigotes (2–4×10^9 cells) were ruptured as previously described (17) and the lysates were used in a reaction mixture containing 100 mM sodium phosphate, pH 7.0, 2 μM hematin, 5 mM tryptophan, 1 mM AA, and 200 μl lysates in a final volume of 500 μl. The mixture was incubated at 37°C for 30 min, and then the reaction was stopped by the addition of 100 μl of 1 M HCl and 6 vol cold ethyl acetate. After the addition of [³H]PGD₂, [³H]PGE₂, and [³H]PGF₂α (60 Bq each per assay; NEN Life Science Products) used as tracers to determine the recovery during extractions, PGs recovered from the incubation of parasite lysates were extracted

and separated by HPLC as previously described (16, 17, 20). The resulting PGD_2, PGE_2, and $PGF_{2\alpha}$ were quantified by enzyme immunoassay with their respective EIA kits (Cayman Chemical).

Aerobic and/or anaerobic synthesis of $PGF_{2\alpha}$ from PGH_2 was performed using a standard reaction mixture that contained 100 mM sodium phosphate, pH 7.0, a diluted amount of enzyme, and the cofactor, i.e., NADPH–generating system (100 μM $NADP^+$, 100 μM glucose-6-phosphate, and 1 unit glucose-6-phosphate dehydrogenase) or 100 μM NADPH or NADH, in a final volume of 100 μl. For anaerobic reactions, mixtures were bubbled with argon gas for 5 min. The reaction was started by the addition of 1 μl of 500 μM 1-[¹⁴C]PGH₂ (2.04 Gbq/mmol) performed at 37°C for 2 min and was terminated by the addition of 250 μl of a stop solution (diethyl ether/methanol/2 M citric acid [30:4:1 vol/vol/vol]). To test the nonenzymatic formation of $PGF_{2\alpha}$, we incubated the reaction mixture containing all the components in the absence of the enzyme. The organic phase (50 μl) was applied to 20 × 20-cm silica gel plates (Merck) at 4°C and the plates were developed with a solvent system of diethyl ether/methanol/acetic acid (90:2:1 vol/vol/vol) at −20°C. The radioactivity on the plates was monitored and analyzed by Fluorescent Imaging Analyzer FLA 2000 and Mac Bas V2.5 software (Fuji Photo Film).

For nifurtimox inhibition of TcOYE reductase activity, various concentrations of the drug were preincubated with an appropriate amount of enzyme and the reaction was started by the addition of NADPH and PGH₂. Spectrophotometric assays were performed in a standard reaction mixture (1 ml) containing 100 mM sodium phosphate, pH 7.0, an appropriate amount of enzyme, and 100 μM NADPH or NADH. We incubated the mixture at 37°C for 2 min under anaerobic conditions before adding substrates to initiate the reaction. NADPH and/or NADH oxidation was monitored by the decrease in absorbance at 340 nm. Blanks without enzyme or substrate were included. To investigate the conversion of PG carbonyl or hydroxyl groups into alcohol or keto-oxo groups, 1-[¹⁴C]PGD₂, PGE₂, and PGF₂α (40 μM each) produced as previously described (17) were incubated with TcOYE and the resulting products were analyzed by thin layer chromatography.

Liquid Chromatography-Mass Spectrometry (LC-MS) Analysis. LC-MS analyses were run on a Waters Alliance LC-MS system equipped with 2690 separation module, 996 photodiode array, ZQ4000 mass detectors, and fused with an Inertsil-ODS3 column (GL Sciences Inc.) that had a 2.1 mm ID, 250-mm length, and 37°C column temperature. We produced $PGF_{2\alpha}$ by incubating 80 μg cold 9,11-endoperoxide PGH₂ (Cayman Chemical) with 100 μg TcOYE as described above. After stopping the reaction, $PGF_{2\alpha}$ was extracted, dried, redissolved in 100 μl ethanol, and then analyzed by LC-MS.

Protein Fractionation. *T. cruzi* epimastigotes (10^{10} cells) were ruptured by hypotonic lysis. Soluble proteins resulting from 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 fraction (20–80% saturation) resuspended in PBS was loaded onto a Hiload 16/60 Superdex 200 pg (Amersham Biosciences) gel filtration column and eluted with the same buffer. Active fractions were pooled, concentrated by the use of Centricon centrifugal filters with a molecular weight cut-off of 3,000 (Millipore), dialyzed against 20 mM sodium phosphate, pH 7.0, additionally loaded onto a Resource PHE Hydrophobic interaction column (Amersham Biosciences) that 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 sul-

fate 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 HiPrep 16/10 DEAE ion exchange column (Amersham Biosciences) that had been equilibrated with the same buffer. The elution was performed with an increasing linear gradient of 0–400 mM NaCl in the same buffer. The active fraction was additionally purified by gel filtration on a Hiload 16/60 Superdex 200 pg column.

Protein concentration was determined by the use of bicinchoninic acid reagent (Pierce Chemical Co.) with BSA as a standard according to 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 sypro orange (Bio-Rad Laboratories) or Coomassie Brilliant Blue (Daiichi Pure Chemicals).

Absorbance spectra of oxidized and reduced TcOYE were measured on a DU® 640 spectrophotometer (Beckman Coulter) by using 80 μl TcOYE (4 μg/μl) and 100 μM NADPH (reduced TcOYE) in 0.1 M sodium phosphate buffer, pH 7.0. The spectra of the oxidized or reduced flavin were read at 37°C between 300–600 nm.

In-Gel Protein Digestion and Partial Amino Acid Sequence Analysis. 20 μg pure TcOYE was separated by SDS-PAGE on a 12.5% acrylamide gel and stained with Coomassie Brilliant Blue. The stained protein band was in-gel digested with lysyl-endopeptidase in Tris/Cl buffer, pH 8.5, at 35°C for 20 h according to Rosenfeld et al. (21). Peptides were purified on a C_{18} reverse phase HPLC column as described by Rosenfeld et al. (21) and the amino acid sequence analysis was performed on a Shimadzu PSQ-1 system protein sequencer (Shimadzu Scientific Instruments).

PCR Amplification, cDNA Cloning, and Sequencing. Total RNA was extracted from *T. cruzi* epimastigote cells ($2–4 \times 10^9$ cells) with ISOGEN (a guanidine HCl/phenol procedure; Nippon Gene). First strand cDNA was synthesized by RT-PCR using avian myeloblastosis virus reverse transcriptase after annealing 1 μg of *T. cruzi* total RNA with Oligo dT adaptor primer (Takara Shuzo). TcOYE open reading frame (ORF) was amplified with gene-specific primers from *T. cruzi* dehydrogenase ORF, i.e., sense primer 5′-CGGAATTCATGGCGACGTTCCCT-GAACTTC-3′ and antisense primer 5′-CCGCTCGAGT-TATTTGTTGTACGTCGGGTA-3′ that carried EcoRI and XhoI restriction sites, respectively, at their 5′ end and cDNA first strand as template by using the following program: 95°C for 5 min, 94°C for 1 min, 56°C for 30 s, and 72°C for 1 min at 30 PCR cycles. Nested PCR amplification was performed with the first PCR amplification product as a template. The amplified fragments were cloned into pGEX-4T-1 vector (Amersham Biosciences). At Takara Co., DNA sequences were determined from both strands by use of a dye terminator system (BigDye Terminator; Applied Biosystems).

Alignment of Primary Structures and Phylogenetic Analysis. The sequences of nine members of flavin-dependent oxidoreductase family were retrieved from public database. Protein sequences were aligned by using the CLUSTAL W algorithm and BLOSUM Clustered Scoring Matrix (22) available at the National Institute of Genetics, Mishima, Japan. Phylogenetic analyses were performed with the PHYLIP package (23).

Heterologous Expression of the Recombinant Enzyme. The PCR product encoding TcOYE ORF was digested with EcoRI and XhoI restriction enzymes and then cloned into the corresponding sites of the pGEX-4T-1 expression vector (Amersham Biosciences). The resultant expression vector was used for transformation of *Escherichia coli* BL21. Transformed cells were cultured

for 6–7 h in the presence of 0.5 mM isopropyl-β-D-thiogalacto-pyranoside (IPTG) and then harvested and sonicated. The soluble recombinant protein was produced in *E. coli* BL21 and purified by affinity chromatography on glutathione (GSH)–Sepharose 4B resin (Amersham Biosciences), ion exchange chromatography, and gel filtration according to the manufacturer's protocol.

Electron Spin Resonance (ESR) Experiments. ESR experiments were performed as described by Moreno et al. (24). For hydrogen peroxide, 4-nitroquinoline-*N*-oxide, nifurtimox, and β-lapachone, the reaction mixture (200 μl each) contained 10 mM NADPH, a reaction buffer (24), 200 μg TcOYE, and 2 mM substrate. For menadione, the mixture contained 0.7 mM NADPH, 5 mM Tris/Cl, pH 7.0, 200 μg TcOYE, and 0.6 mM substrate. The mixtures were gassed with argon for 2 min in a small glass bottle tightly closed with septum. The reaction was started by the addition of enzyme and substrate and was kept for 3 min at 37°C or 25°C (for menadione) before recording the spectra of 100 μl mixture in an ESR cell. ESR measurements were performed on a JEOL X-band spectrometer (JES-RE1XE) and ESR spectra were recorded under nonsaturating microwave power conditions. The magnitude of modulation was chosen to optimize the resolution and the signal to noise ratio of the spectra. The *g* values were calibrated with an Mn^{2+} marker. Computer simulation was performed on a Macintosh personal computer using Calleo ESR version 1.2 (Calleo Scientific Publisher).

Western Blotting and Immunoprecipitation. Polyclonal antibody against TcOYE was generated as previously described (25) with 300 μg recombinant TcOYE in 5 mM Tris/Cl buffer, pH 8.0. Rabbits were immunized subcutaneously and boosted four times every 2 wk with the same preparation for 1 mo. Total epimastigote lysates or pure TcOYE was resolved on 14% SDS-PAGE gels. Proteins were transferred onto polyvinylidene difluoride membranes (Millipore), blocked with BlockAce (Dainippon Seiyaku), and cross reacted with rabbit anti–TcOYE polyclonal antibody before being developed with horseradish peroxidase–labeled secondary antibody (ECL kit; Amersham Biosciences). For immunoblotting, total epimastigote lysates were diluted to 1 μg/μl with PBS and 250 μl aliquots were incubated overnight at 4°C with various concentrations of anti-TcOYE polyclonal antibody. The immunocomplex was captured by recombinant protein A agarose beads (Upstate Biotechnology). After centrifugation, the supernatants were tested for their different reductase activities.

Results

T. cruzi Produces PGs from AA. We used stationary phase epimastigotes of *T. cruzi* cultured without exogenous AA supplement, except for the trace amounts present in serum. Lysates from these cells produced high levels of PGD_2, PGE_2, and $PGF_{2\alpha}$ when incubated with 1 mM AA in the reaction mixture (Fig. 1). In the absence of the 1 mM AA, epimastigote lysates produced high amounts of PGD_2 and $PGF_{2\alpha}$, whereas PGE_2 production decreased almost 4.5-fold (Fig. 1). These results suggest that a substantial source of intracellular AA may exist in the parasite. In addition, heat treatment (100°C for 20 min) inactivated PG synthesis by the lysates and no PG production was observed after the incubation of 1 mM AA in the absence of lysates (unpublished data). The addition of nonsteroidal antiinflammatory drugs (3 mM aspirin or 42 μM indomethacin) to the reaction mixture had no inhibitory effects on PG
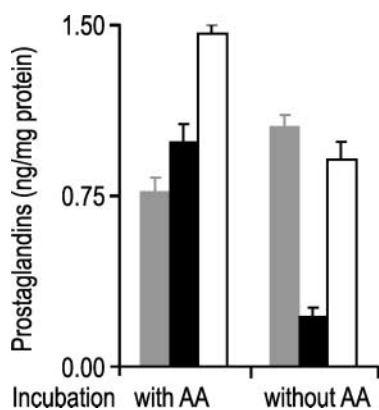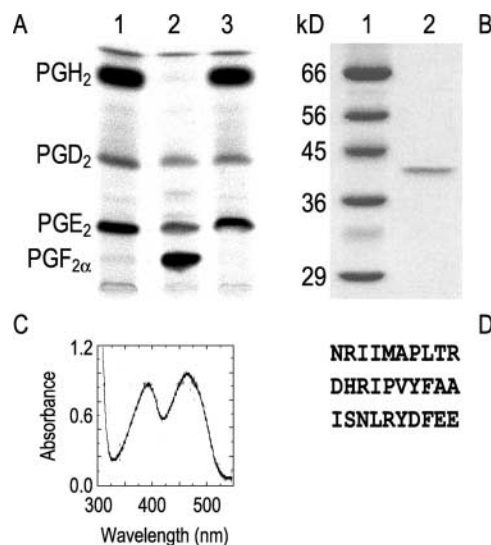
Figure 1. PG production by *T. cruzi* epimastigote lysates. Stationary growth phase epimastigotes were cultured without the addition of AA into the culture medium, whereas lysates from these cells were incubated with or without 1 mM AA. Gray, black, and white bars indicate PGD_2, PGE_2, and $PGF_{2\alpha}$, respectively. PG detection limits were less than 7.8, 7.8, and 3.6 pg/assay for PGD_2, PGE_2, and $PGF_{2\alpha}$, respectively. Values shown are the mean from three independent experiments along with SE.

Figure 2. (A) Reduction of PGH_2 by native TcOYE. Lane 1, substrate incubated in the absence of enzyme; lane 2, with 2 μg pure TcOYE; lane 3, with 20 μg heat-inactivated TcOYE. (B) SDS-PAGE of native TcOYE. 2 μg protein was resolved on a 14% SDS polyacrylamide gel and detected by Coomassie Brilliant Blue staining. Lane 1: molecular weight markers; lane 2, pure TcOYE. (C) Absorbance spectrum of 4 μg/μl oxidized TcOYE in 0.1 M sodium phosphate buffer, pH 7.0, at 37°C. (D) Partial amino acid sequences of the three internal fragments from the in-gel digestion of TcOYE.

synthesis by *T. cruzi* lysates although these concentrations of the drugs are known to inhibit the action of cyclooxygenase from sheep seminal vesicles (16).

Isolation and Cloning of TcOYE Gene. To investigate the de novo synthesis of PGs, we incubated cell lysates with 1-[^{14}C]PGH$_2$ and then monitored PGF$_{2\alpha}$ synthesis. Under aerobic conditions, we detected only one major PG synthase activity in the cytosolic fraction, i.e., PGF$_{2\alpha}$ synthase that depended on NADPH-generating system (Fig. 2 A). No PGF$_{2\alpha}$ synthesis was detected in the absence of protein or the presence of heat-inactivated cytosolic fraction (Fig. 2 A), NADPH, membrane fraction, dithiothreitol, and GSH (unpublished data). However, we detected nonenzymatic conversion of PGH$_2$ to PGE$_2$ but not to PGF$_{2\alpha}$ (Fig. 2 A).

To identify the PGFS gene from *T. cruzi*, we searched the Expressed Sequence Tags database for a gene homologous to *T. brucei* PGF$_{2\alpha}$ synthase (TbPGFS) by using its amino acid sequence (sequence data are available from GenBank/EMBL/DDBJ under accession no. AB034727) and found some homology with an unidentified fragment of the aldo/ketoreductase gene from *T. cruzi* (sequence data are available from GenBank/EMBL/DDBJ under accession no. AF262056). However, we failed to express a protein from this gene, indicating the possibility that a nonfunctional gene might have been amplified. We then decided to purify native PGFS from the cytosolic fraction of *T. cruzi*. After ammonium sulfate fractionation, the active fraction was purified by sequential HPLC. The overall procedure resulted in an ~1,630-fold purification with a recovery of 1%. Pure TcOYE exhibited a specific activity of 700 nmol/min/mg of protein. SDS-PAGE analysis revealed the presence of a single protein band with an apparent molecular mass of 42 kD (Fig. 2 B). Calibration of TcOYE by gel filtration confirmed the molecular mass of 42 kD (unpublished data), indicating that TcOYE was a

monomeric protein. In addition, pure TcOYE was found to bind one FMN cofactor and spectroscopic characterization revealed visible absorbance spectra characteristic of an oxidized flavin, with maxima at 462 and 382 nm (Fig. 2 C). Then, we subjected pure TcOYE to partial amino acid sequencing and determined the amino acid sequences from three internal peptide fragments (Fig. 2 D). Then, we identified a 1,686-bp full-length cDNA (sequence data are available from GenBank/EMBL/DDBJ under accession no. U31282) of the *T. cruzi* amastigote homologue of the yeast OYE gene from the database. The cDNA encoded a 1,140-bp ORF that predicted a protein of 379 amino acid residues with a calculated molecular weight of 42,260 daltons. Cloning and sequencing of the amplified 1,140-bp ORF fragment revealed one nucleotide mutation with substitution of the amino acid residue and seven nucleotide substitutions with the same sense mutations probably due to *T. cruzi* strain polymorphism.

Characterization of the TcOYE Gene. A database search and alignment of the amino acid sequence (Fig. 3) revealed that TcOYE is a member of the family of flavin-dependent oxidoreductases including NADPH dehydrogenase, xenobiotic reductase, morphine reductase, *N*-ethylmaleimide reductase, 2-cyclohexane-one reductase, 12-oxo phytodienoate reductase from bacteria, yeast, fungi, and protozoa. The TcOYE amino acid sequence showed 16–28% identities to related proteins from a variety of organisms. Among them, TcOYE showed 24–28% identity to oxidoreductase from *Agrobacterium tumefaciens*, morphine reductase and *N*-ethylmaleimide reductase from *Leishmania major*, xeno-

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TcOYE       -------------------MATFPELLRPLKLGRYTLHNRIIMAPLTRCQATEDDHVPRTESMLKYYEDRASAG--LIIARATMVQPNYTGFLTEPGIYSDAQIEEWRKIVDAVHKKG
LmMR        ----------------MSAASKSIDAMLKPLLVGGRWMSNRFVMAPLTRCRAD-DNHVPTAAMVKHYSDRASMG---LLLTEATQIHKGYSTFAHEGGIYGKEQVDGWKKVTDAVHDKG
Agroba      ----------------MTDKTLFEPYALGAISLSNRIVMAPLTRNRAG-AGFVPGDLTAEYYTQRASAG---LIISEATQISQQGGYQDTPGIYTRAQIDGWRKVTAAVHEKG
Methorizo   ----------MTTLFDPLRAGDLTLANRIVMAPLTRNRS--PNAVPGDLTVYYYSQRATAG---LIVTEATAISHQGQGYANVPGLYGADQLAGWRRVTDAVHRGG
Ec          ----------------MSSEKLYSPLKVGAITAANRIFMAPLTRLRSISPGDIPTPLMAEYYRQRASAG---LIISEATQISAQAKGYAGAPGIHSPEQIAAWKKITAGVHAEN
Paer        ----------------MATLFDPIVLGDLELPNRIVMAPLTRCRAD-EGRVPNALMAEYYAQRADAG---LILSEATAVTPMGVPDTPGIWSDDQVRGWSNVTKAVHAAG
Avinel      ----------------MSTLFDPIBIGDLQLPNRIVMAPLTRCRAE-PGRVPGALMAEYYVQRASAG---LIISEATSVCPTGVGYPDTPGIWSDEQVAGWKNVTQAVHAAG
Tomato      ------------MENKVVEEKQVDKIPLMSPCKMGKFELCHRVVLAPLTRQRSY--GYIPQPHAILHYSQRSTNGG--LLIGRATVISETGIGYDVPGIYTKEQVHAAWKPIVDAVHAKG
Sc          ----------MSFVKDFKPQALGDTNLFKPIKIGNNELLHRAVIPPLTRMRALHPGNIPNRDWAVEYYYTQRAQRPGTMIITBGAFISPQAGGYDNAPGVWSEEQMVEWTKITPNAIHEKK
Candida     MTIESTNSFVVPSDTKLIDVTPLGSTKLFQPIKVGNNVLPQRIAYVPTTRFRASKDHIP--SDLQLNYYNARSQYPGTLIITEATFASERGGIDLHVPGIYNDAQAKSWKKINBAIHGNG

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TcOYE       GLIFLQLIHAGRAGIPEKILQQSKSDQDPLAGRLLAASAIPYLHDHRIPVYFAASGEKE'TYGVPBELLTDDEVRDGIIPLFVEGAKNAIFKAGFDGVEIHGANGYLLDAFFRESSNKRQSGP
LmMR        GIIFCQIHNGGRSTVPS--------NVDEDVRIVAPSAVAITGHKCAGSFARNGKTQPYPVPDHAMAAEEIASYVK-LYAAAARNAIAAG-FDGVEVHGANGYLVDQFLKTSSN-QRTDE
Agroba      GRIVLQLHHVGRISHID--------LQPNGGQPVAPSAIRAANTKTFVNN-----APVDVSEPRALEIEELAGIVN-DFRKAAANAIDAG-FDGVEIHGANGYLIDQFLRSGTN-HRNDA
Methorizo   GKIVVQLWHVGRISHNT--------LQPQGGKRVAPSAIRANSKTFLVKPDCSGEFAKTSEPRALDAAHLPGIVD-DFRRAAKAAIHVAGFDGVEIHGANGYLLDQFLRSGTN-HRNDA
Ec          GHMAVQLWHTGRISHAS--------LQPGGQAPVAPSALSAGTRTSLRDENGQAIRVETSMPRALELREIPGIVN-DFRQAIANAREAG-FDLVELHSAHGYLLHQFLSPSSN-HRTDQ
Paer        GRIFLQLWHVGRISDP---------LYLNGELPVAPSATAABGHVSLVRP-----KRPYVTPRALDTEEIADIVE-AYRQGAERAKAAG-FDGVEIHGANGYLLDQFLQDSTN-KRTDR
Avinel      GRIVLQLWHVGRISDP---------LYLNGELPVAPSAIQPKGHVSLVRP-----QKAFETPRALETDEIPGLVE-AYRQGAENARKAG-FDGVEIHAANGYLLDQFLQDSTN-KRTDR
Tomato      GIFFCQIWHVGRVSNKD--------FQPNGEDPISCTDRGLTPQIRSNGID-----IAHFTRPRRLTTDEIPQIVN-EFRVAARNAIEAG-FDGVEIHGAHGYLIDQFMKDQVN-DRSDK
Sc          SFVWVQLWVLGWAAFPD---------NLARDGLRYDSASDNVFMDAEQEAKA----KKANNPQHSLTKDEIKQYIR-EYVQAAKNSIAAG-ADGVEIHSANGYLLNQFLDPHSN-TRTDB
Candida     SFSSVQLWYLGRVANAK---------DLKDSGLPLIAP-SAVYWDENSEKLA----KEAGNBLRALTEEEIDHIVEVEYPNAAKHALEAG-PDYVEIHGAHGYLLDQFLNLASN-KRTDK

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TcOYE       YAGTTIDTRCQLIYDVTKSVCDAVGSDRVGLRISPLNGVHGMIDS-NPEALTKHLCKKIEPLS-------LAYLHYLRGDMVNQQIGDVVA-----------WVRGSYSGVRTISNLRYDF
LmMR        YGG-SIENRCRFLFEVLDAVIBAVGRERVGLRISPLNSFNDQSDE-NPEALTRYICSQLNCRR------IAFLDVVRGDFFSPARGAD------------KWAREEYBGVLFTGMGFEI
Agroba      YGG-SVENRARLMLEVTAAVAQEIGPARTGIRISPVSPANGISCS-DAQAQYDYIVDQLDALG-------VAYIHVVRGATGGPRDVA-----PFDYGSLRRRFSKT------YIANNGYDL
Methorizo   YGG-SIENRTRFLFEVVDAVTGAVGAGRTGIRLSPVTPANDASDS-DPQPLFNHVVAGLGGRG-------LAYIHIVEGATGGARDFSQGDRPFDYEBLKATYRKAGGAGAWLVNNGYDK
Ec          YGG-SVENRARLVLEVVDAGIEEWGADRIGIRVSPIGTFQNTDNGPNEEADALYLIEQLGKRG-------IAYLHMSEPDWAGGEPYT--------DAFREKVRARFHGPIIGAGAYTV
Paer        YGG-SIENRARLLLEVTDAAISVWGAQRVGVHLAPRADSHDMGDS-NRLETFSHVARELGKRG------IAPICARRAQADDSIGVA-------------LKKAFGGPYIANEQFTL
Avinel      YGG-SIENRARLLLEVTDAAISVWGAGRVGVHLAPRADLHDMGDS-NRABTFSHVARELGKRG------IAPICTREKEDADSLTPQ-----------IKQAFGGAVIANERFTK
Tomato      YGG-SIENRCRFALEVVEAVANBIGSDRVGIRISSPPAHYNBAGDT-NPTALGLYMVESLANKYD-------LAYCHVVBPRMKTAWBKIBCT------ESLVPMRKAVKGTFIVAGGYDR
Sc          YGG-SIENRARFTLEVVDALVEAIGHEKVGLRLSPYGVFNSMSGGAETGIVAQYAVVAGELEKRAKAGKRLAPVHLVEPRVTNPFLTBGEG---EYBGGSNDFVYSIWKGPVIRAGNFAL
Candida     YGCGSIENRARLLLRVVDKLIEVVGANRLALRLSPWASFQGMEIBGEE----IHSYILQQLQQRADNGQQLAYISLVEPRVTGIYDVSLK----DQQGRSNEFAYKIWKGNFIRAGNYTY

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TcOYE       EE----ADQQIREGKVDAVAFGAKFIANPDLVERAQQNWPLNEPRPETYYTRT-AVGYNDYPTYNK----------------
LmMR        EE----AANTVESGAADAVVFGTKTLANPDLIARAVAGAPLNKPDFATFYTTG-EAGYNDYPFMECSSCLPASPSGAQEAPPQQ
Agroba      EL----AATHIAEGKADLIAFGRPFIANPDLVKRLQSGAPLAEIDPATIYGGG-AEGYTDYPRFNEASGS-------------
Methorizo   EL----AEKAVGDGYAELVAFGKLFIANPDLVSRLKRNABLNAPDKATFYGGD-AEGYTDYPTAA----------------
Ec          EK----AETLIGKGLIDAVAFGRDWIANPDLVARLQRKAELNPQRAESFYGGG-AEGYTDYPTL-----------------
Paer        DS----ANAILAKGDADAVAFGVPFIANPDLVERLRQGAELNPPETFYTGG-TEGYLDYPTLA----------------
Avinel      EQ----ADRWLAESKADAVAFGVPFIANPDLPARLAADAPLNEAHPETYYSSG-PQGYLDYPTL----------------
Tomato      ED----GNRALIEDRADLVAYGRLFISNPDLPKRFELNAPLNKYVRPTDPIVGYTDYPFLETMT---------------
Sc          HP----BVVREEVKDKRTLIGYGRFFISNPDLVDRLEKGLPLNKYDRDTFYQMS-AHGYIDYPTYEEALKLGWDKK---------
Candida     DAPEFKTLINDLKNDRSIIGFSRFFTSNPDLVEKLKLGKPLNTYNREEFYKYY-NYGYNSYDESEKQVIGKPLA----------
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Figure 3. Multiple sequence alignment of deduced TcOYE amino acid sequence with representative members of the flavin-dependent oxidoreductases family. The amino acid sequences were taken from the public database. TcOYE is aligned with: LmMR, *L. major* morphine reductase (sequence data are available from GenBank/EMBL/DDBJ under accession no. AL390114); Agroba, *Agrobacter tumefaciens* oxidoreductase (accession no. NP_535816); Methorizo, *Mesorhizobium loti* morphinone reductase (accession no. NP_103610); Ec, *E. coli* N-ethylmaleimide reductase (accession no. NP_416167); Paer, *P. aeruginosa* xenobiotic reductase (accession no. NP_253046); Avinel, *Azotobacter vinelandi* 2-cyclohexaneone reductase (accession no. AB025798); Tomato, *Lycopersicon esculentum* 12-oxo phytodienoate reductase (accession no. AJ242551); Sc, *Saccharomyces cerevisiae* NADPH dehydrogenase 1 (accession no. Q02899); Candida, *C. albicans* NADPH dehydrogenase estrogen binding protein (accession no. P43084). * and : indicate identical amino acids and conserved amino acid substitutions, respectively. Boxes indicate the peptide sequences identified from purified native TcOYE.

biotic reductase from *Pseudomonas aeruginosa*, and N-ethylmaleimide reductase from *E. coli*. TcOYE also showed 17–19% identities to 12-oxo phytodienoate reductase from tomato and NADPH oxidoreductase from *Saccharomyces cerevisiae* and *Candida albicans*. In addition, phylogenetic analysis supported a premise that *Leishmania* morphine reductase is the closest associate of TcOYE (bootstrap value 95%, unpublished data).

Expression of Recombinant TcOYE. We cloned TcOYE ORF into a pGEX expression vector and produced the recombinant TcOYE as a soluble GSH–transferase fusion protein in *E. coli* after IPTG induction. We purified the recombinant TcOYE by chromatography on GSH-Sepharose affinity and ion exchange columns (Fig. 4 A). Pure recombinant TcOYE was found to bind one FMN cofactor and exhibited visible absorbance spectra characteristic of an oxidized flavin identical to the native TcOYE. SDS-PAGE showed a single protein band of 42 kD (Fig. 4 A). Crude proteins from *E. coli* BL21 expressing pGEX-TcOYE, but not lysates from *E. coli* host or *E. coli* transformed with empty pGEX vector, showed 9,11-endoperoxide PGH_2 reductase activity and pure recombinant TcOYE exhibited a specific activity of 760 nmol/min/mg of protein. These data indicate that the detected 9,11-endoperoxide PGH_2 reductase activity depended on *T. cruzi* protein and not on any contaminating activity from the host or vector. We used the recombinant TcOYE as an antigen to generate rabbit anti–TcOYE polyclonal antibody. We incubated lysates from *T. cruzi* and *T. brucei* with anti-TbPGFS polyclonal serum and anti-TcOYE polyclonal antibody, respectively, to investigate the specificity of both antibodies. Western blot analysis revealed no cross reaction between the two parasite lysate proteins, indicating the high speci-

ficity of each antibody. Instead, we detected only bands corresponding to TcOYE and TbPGFS (Fig. 4 B). These results demonstrate that *T. cruzi* lacks a 33-kD aldo/keto reductase despite the presence of the gene and *T. brucei* does not have the 42-kD OYE homologue. Moreover, attempts to identify TcOYE-generated $PGF_{2\alpha}$ revealed that the reaction product resulting from the reduction of 9,11-endoperoxide PGH_2 displayed a retention time corresponding to that of the authentic $PGF_{2\alpha}$ (Fig. 4 C, top and bottom) on LC-MS. The [M–H]⁻ ions of TcOYE-generated product and that of the authentic $PGF_{2\alpha}$ were identical and observed at m/z 353.2 (Fig. 4 D, top and bottom). Under these experimental conditions, PGD_2, PGE_2, 11-β-PGE_2, $PGF_{2\alpha}$, and PGJ_2 each eluted at different retention times (Fig. 4 C, middle). These LC-MS results demonstrate that TcOYE-generated product is $PGF_{2\alpha}$ and confirm the fact that TcOYE catalyzes specifically the reduction of 9,11-endoperoxide PGH_2 into $PGF_{2\alpha}$.

TcOYE Reduces Peroxides and Trypanocidal Agents. OYEs can oxidize both NADPH and NADH (2, 5, 26) in the presence of various substrates. Because these enzymes catalyze the NAD(P)H-dependent reduction of quinones and nitroderivatives (3, 5, 27), we hypothesized that TcOYE may reduce naphthoquinone and nitroheterocyclic trypanocidal agents thus contributing to the metabolism of antitrypanosomal drugs. We tested this idea by assessing TcOYE reductase activity under aerobic or anaerobic conditions. In the presence of oxygen, TcOYE completely reduced PGH_2 to $PGF_{2\alpha}$ with NADPH-generating system (Fig. 2 A). No $PGF_{2\alpha}$ formation was observed with NADPH or NADH (unpublished data) probably due to the depletion of the cofactors as a result of preincubation of the enzyme with cofactors in the absence of PGH_2 be-
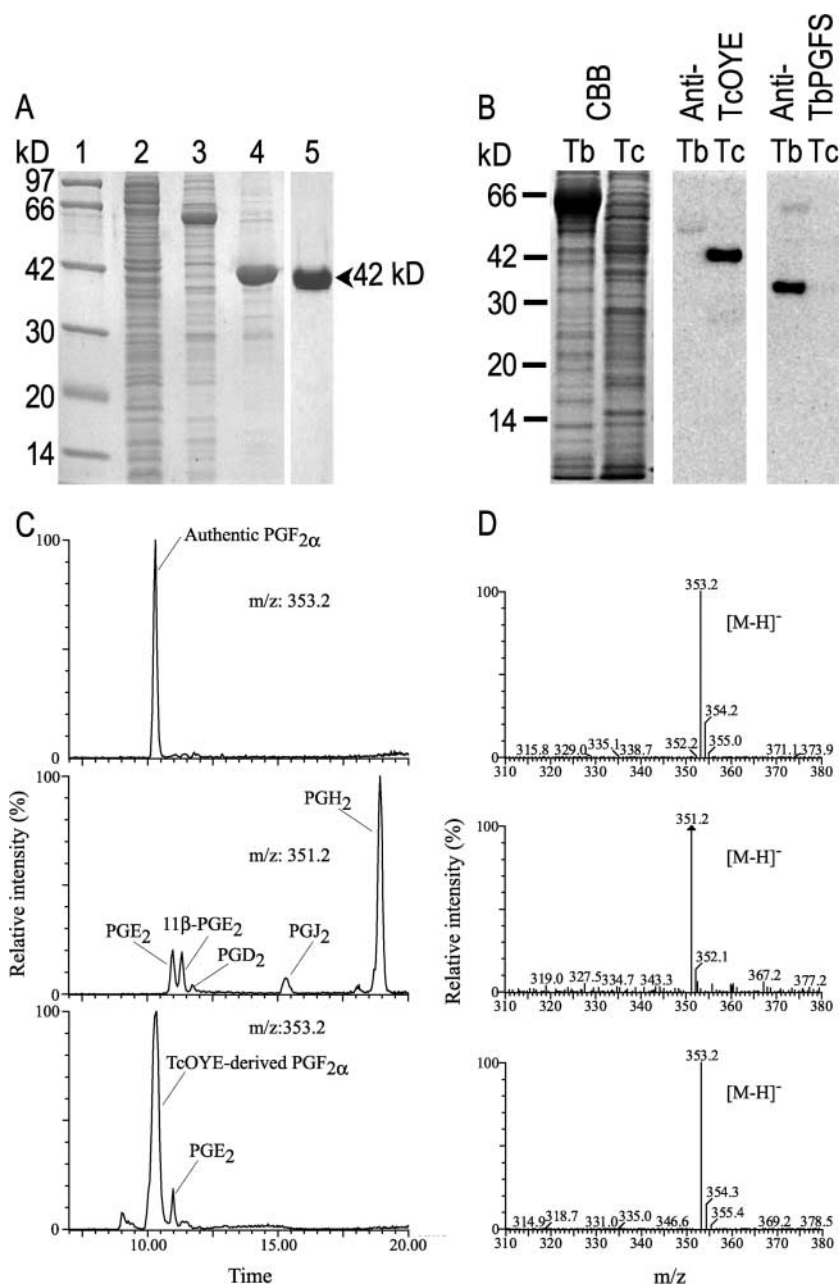
Figure 4. (A) Purification of recombinant TcOYE. TcOYE was expressed as a fusion protein with GSH in *E. coli* BL21. Lane 1, molecular weight markers; lane 2, lysates from noninduced *E. coli* BL21 cells expressing pGEX-TcOYE; lane 3, lysates from IPTG-induced *E. coli* BL21 cells expressing pGEX-TcOYE; lane 4, after affinity chromatography; lane 5, pure recombinant TcOYE. (B) Immunoblot analyses of cell extracts from *T. cruzi* epimastigote and *T. brucei* bloodstream form. After blotting, the membranes were incubated with anti-TcOYE or anti-TbPGFS polyclonal antibodies. On the left, SDS-PAGE of the lysates corresponding to *T. brucei* (Tb) and *T. cruzi* (Tc) is shown. The proteins were detected with Coomassie Brilliant Blue (CBB). The middle shows a Western blot for proteins incubated with anti-TcOYE polyclonal antibody. The right shows a Western blot for proteins incubated with anti-TbPGFS polyclonal antibody. (C) LC-MS chromatograms of the relative ion intensity for TcOYE-generated $PGF_{2\alpha}$. Top, standard $PGF_{2\alpha}$; left, substrate (9,11-endoperoxide PGH_2) showing traces of nonenzymatic degradation products (PGE_2, 11β-PGE_2, PGD_2, and PGJ_2); bottom, $PGF_{2\alpha}$ resulting from the reduction of the substrate by TcOYE. The chromatograms for PGE_2, 11β-PGE_2, PGD_2, and PGH_2 were visualized at m/z = 351.2, whereas that of $PGF_{2\alpha}$ was monitored at m/z = 353.2. (D) Mass spectra showing the $[M-H]^-$ ions of authentic $PGF_{2\alpha}$ (top), PGH_2 (middle), and TcOYE-generated $PGF_{2\alpha}$ (bottom).

cause NADPH and NADH are readily oxidized by OYEs in the presence of oxygen, which is an opportunistic substrate of these enzymes (2). In addition, TcOYE showed only a very low reductase activity toward menadione, β-lapachone, and 4-nitroquinoline-*N*-oxide, and no activity toward nifurtimox in the presence of oxygen. On the other hand, anaerobic reduction of 9,11-endoperoxide PGH_2 by TcOYE led to a specific activity of the same order of magnitude to that of reduction under aerobic conditions and TcOYE used either NADPH or NADH as cofactor. TcOYE V_{max} and/or specific activity and K_m values for peroxides and trypanocidal agents are shown in Table I. Under anaerobic conditions, TcOYE also reduced peroxides (hydrogen peroxide and butyl hydroperoxide) and trypano-

cidal agents (menadione, β-lapachone, nifurtimox, and 4-nitroquinoline-*N*-oxide). Except for 4-nitroquinoline-*N*-oxide, TcOYE showed specific activity values of the same order of magnitude (Table I). Under aerobic conditions, TcOYE exhibited only 4.5, 14, and 4% of the anaerobic reductase activity toward menadione, β-lapachone, and 4-nitroquinoline-*N*-oxide, respectively. No reductase activity could be observed toward benznidazole, crystal violet, etc. under both conditions (Table I). In the presence of NADH as cofactor, TcOYE exhibited almost 1.5-, 2.2-, and 1.8-fold higher enzymatic activity toward β-lapachone, mevinolin, and econazole, respectively, as compared with the reduction of these substrates in the presence of NADPH. In contrast, all other drugs were reduced with

Table I. *Substrate Specificity of the Recombinant TcOYE*

Substrate	Cofactor	K_m	V_{max}/specific activity
		(100 μM) (μM)	(nmol/min/mg)
9,11-endoperoxide PGH$_2$	NADH	—	554
	NADPH	5.0	766
Hydrogen peroxide	NADPH	2.3	99
BHP[a]	NADPH	ND	282
Menadione	NADH	—	499
	NADPH	0.82	700
β-lapachone	NADH	0.17	650
	NADPH	—	433
4-nitroquinoline–N-oxide	NADH	—	759
	NADPH	9.5	1,110
Nifurtimox	NADH	—	290
	NADPH	19.0	353
Phenazine methosulfate[b]	NADPH	10.4	235
Mevinolin[c]	NADH	ND	555
12-oxo phytodienoic acid[d]	NADPH	ND	152
9-oxo ODE[e]	NADPH	ND	54
Econazole[f]	NADH	ND	43
Benznidazole	—	ND	n.d.
Miconazole[g]	—	ND	n.d.
Ketoconazole[h]	—	ND	n.d.
Crystal violet[i]	—	ND	n.d.
BHT[j]	—	ND	n.d.
BHA[k]	—	ND	n.d.

n.d., not detected.
[a]t-Butyl hydroperoxide
[b]5-Methyl–phenazinium methyl sulfate
[c]2β,6α-dimethyl-8α-(2-methyl-1-oxobutoxy)-mevinic acid lactone
[d]4-oxo-5β-(2Z-pentenyl)-2-cyclopentene-1β-octanoic acid
[e]9-oxo-10E, 12Z-octadecadienoic acid
[f]1-[2-([4-Chlorophenyl]methoxy)-2-(2,4-dichlorophenyl)ethyl-1H-imidazole
[g]1-[2,4-Dichloro-β-([2,4-dichlorobenzyl]-oxy)phenethyl]imidazole
[h]cis-1-Acetyl-4-[4-[[2-(2,4-dichlorophenyl)-2-(1H-imidazol-1-ylmethyl)-1,3-dioxolan-4-yl-methoxy]phenyl]piperazine
[i]N-[4-[Bis[4-(dimethylamino)-phenyl]methylene]-2,5-cyclohexadien-1-ylidene]-N-methyl-methanaminium chloride
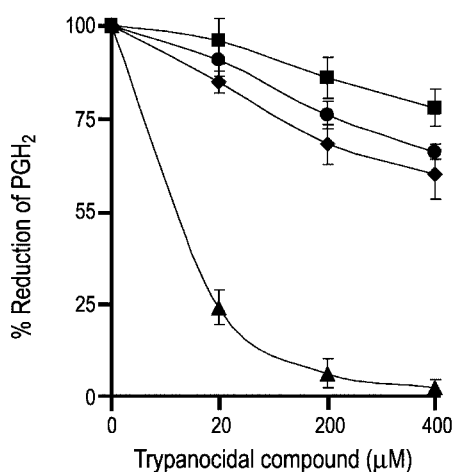[j](2,6-Di-tert-butyl-p-cresol)
[k][2(3)-tert-butyl-4-hydroxianisole]

Figure 5. Semiquinone and superoxide radical anion spectra obtained from menadione and β-lapachone. On the top, ESR spectrum of the semiquinone radical anion generated by the action of TcOYE on menadione after anaerobic incubation is shown. The bottom shows a computer simulation spectrum of the semiquinone radical anion with a g value of 2.0044 and hyperfine coupling constants a(4 H) = 3.0 G, a(3 H) = 0.66 G, and a maximum slope of line width ΔH_{msl} = 0.15 G. (B) On the top, ESR spectrum of the semiquinone radical anion generated by the action of TcOYE on β-lapachone after anaerobic incubation is shown. The bottom shows a computer simulation spectrum of the semiquinone radical anion with a g value of 2.0046 and hyperfine coupling constants a(1 H) = 3.3 G, a(2 H) = 1.7 G, and ΔH_{msl} = 0.7 G. (C) ESR spectrum of $O_2^{\cdot-}$ $g_{//}$ = 2.09 and $g_\perp$ = 2.005 formed in the reaction of semiquinone radical anion with O_2 in 9 mM Tris/Cl, pH 7.4, at −155°C. $g_{//}$ component is magnified five times.

the highest reduction rate in the presence of NADPH as cofactor rather than NADH. No reduction and oxidation of carbonyl and hydroxyl groups, respectively, were observed after TcOYE incubation with either 1-[^{14}C]PGD$_2$, PGE$_2$, or PGF$_{2\alpha}$ (unpublished data), indicating that TcOYE did not catalyze the reduction of PGD$_2$, PGE$_2$ into PGF$_{2\alpha}$, or the formation of any 15-oxo-derivative of these PGs. However, additional bands observed upon the incubation of *T. cruzi* lysates with 1-[^{14}C]PGH$_2$ suggest the presence of different enzymes in the lysates that produce 15-oxo– or 15-keto–containing structures.

TcOYE Catalyzes the Formation of Radical Anions from Naphthoquinones but Not from Nitroheterocycles. The finding that TcOYE catalyzed the anaerobic reduction of trypanocidal drugs prompted us to explore its involvement in either xenobiotic reduction (parasite defense mechanism) or the redox cycling process (generation of free radicals). Therefore, we studied the interaction of TcOYE with hydrogen peroxide, 4–nitroquinoline-N-oxide, nifurtimox, menadione, β-lapachone, and mevinolin. The incubation of menadione or β–lapachone with recombinant TcOYE generated multiline ESR spectra identified as the respective semiquinone radical metabolites (Fig. 5, A and B, top). The semiquinone radical spectra were additionally analyzed by computer simulation (Fig. 5, A and B, bottom). The mag-

netic parameters of the semiquinone radicals from computer simulation agreed well with those obtained with TcOYE. No ESR signals could be detected in the absence of either the enzyme or substrate or upon the incubation of naphthoquinones with heat-denatured TcOYE. The subsequent reoxidation of both menadione and β-lapachone radical anions by O_2 resulted in superoxide anion-free radical generation (Fig. 5 C). Our findings confirmed the involvement of TcOYE in free radical generation from at least naphthoquinone drugs. No ESR signals related to any other free radicals could be detected with 4-nitroquinoline-N-oxide, nifurtimox, or mevinolin.

Naphthoquinone and Nitroheterocyclic Drugs Inhibit the Endoperoxide Reductase Activity of TcOYE. Several naphthoquinone and nitroheterocyclic compounds are inhibitors and/or redox cycling substrates for flavoprotein enzymes (9, 15). To gain insight into the inhibitory effect of these compounds on 9,11-endoperoxide PGH_2 reductase activity of TcOYE, we investigated the effects of these drugs on 9,11-endoperoxide PGH_2 reduction by TcOYE. In the presence of up to 200 μM menadione, β-lapachone, or 4-nitroquinoline-N-oxide, TcOYE exhibited 68, 86, or 76%, respectively (Fig. 6), of the control (9,11-endoperoxide PGH_2 reductase) activity, indicating that these drugs were weak inhibitors. In contrast, nifurtimox inhibited endoperoxide reduction in a dose-dependent manner, with 200 μM nifurtimox allowing only 6% of the TcOYE activity (Fig. 6).

TcOYE Is the Major Drug Metabolizing Enzyme. To reveal a possible role for TcOYE as a target enzyme in the metabolism of trypanocidal drugs and generation of free radicals, we incubated cell lysates with anti-TcOYE polyclonal antibody and confirmed the immunoprecipitation of TcOYE by Western blot analysis (Fig. 7 A). After capturing the immunocomplex with recombinant protein A aga-

rose beads, the resulting lysate supernatants exhibited only trace reductase activity toward endoperoxide PGH_2 and β-lapachone compared with untreated or anti-TbPGFS polyclonal antibody-treated lysates (Fig. 7 B and Table II). Surprisingly, under our experimental conditions, menadione, nifurtimox, and 4-nitroquinoline-N-oxide reduction was abolished after the immunoprecipitation of TcOYE (Table II), demonstrating that under anaerobic conditions TcOYE might be a major reductase of the parasite for peroxides, naphthoquinones, and nitroheterocyclic compounds and that *T. cruzi* also possesses other minor 9,11-endoperoxide PGH_2– and/or β-lapachone–reductases.

Discussion

Here, we have shown that *T. cruzi* lysates synthesize PGs from AA. This parasite also produces high levels of PGD_2 and $PGF_{2\alpha}$, and a decreased amount of PGE_2 even in the absence of AA addition. These results suggest the existence of a free intracellular AA source in the epimastigotes of *T. cruzi* and probably the presence of a PGE_2-metabolizing system in the parasite. Indeed, the presence of AA in the parasite cells has been demonstrated by several earlier investigations (28, 29). These studies showed around 0.7% AA in phospholipid fraction of *T. cruzi* Y strain epimastigotes (28), whereas fatty acid composition of individual phospholipids revealed 1.3% and trace amount of AA in phosphatydilcholine and phosphatidylethanolamine, respectively. Although de novo synthesis of AA from acetate is not known in trypanosomes, *T. cruzi* needs AA as a component of the building block of its phospholipid structure and AA, along with other fatty acids, is responsible for the fluidity of cell membranes in this organism. It has been demonstrated that to meet its AA needs the uptake of this fatty acid from its environment must occur (29). This intracellular arachidonate source may provide the substrate needed for PG production by the parasite in the absence of exogenously added AA. Ongoing investigations on PG metabolism in *T. cruzi* would probably help to unravel any PGE_2-degrading enzyme in this parasite.
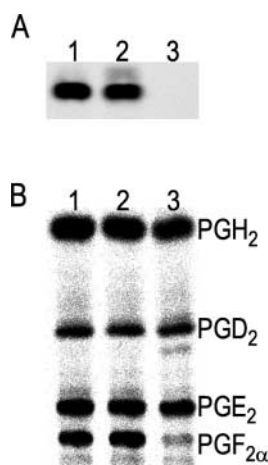
Figure 6. (A) Trypanocidal drug dose-dependence inhibition of 9,11-endoperoxide PGH_2 reduction by TcOYE. The enzymatic activity of the control (0 μM drug) was given a 100% value to calculate the percentage of residual activity. ◆, menadione; ■, β-lapachone; ▲, nifurtimox; ●, 4-nitroquinoline-N-oxide. Data are expressed as the mean along with SE from three independent experiments.

Figure 7. (A) Immunoblot analysis of the supernatant from *T. cruzi* epimastigote cell lysates after immunoprecipitation with anti-TcOYE polyclonal antibody. Lane 1, lysates incubated without primary antibody; lane 2, anti-TbPGFS polyclonal antibody–treated lysates; lane 3, anti-TcOYE polyclonal antibody–treated lysates. After immunoblotting, the membrane was incubated with anti-TcOYE polyclonal antibody. (B) Supernatants from immunoprecipitated lysates were tested for their 9,11-endoperoxide PGH_2 reductase activity. Lanes 1–3 correspond to those in A.

Table II. *Effect of Anti-TcOYE Polyclonal Antibody Treatment of T. cruzi Lysates on Drug Reduction*

Sample	Percent residual reductase activity toward:			
	Menadione	β-lapachone	Nifurtimox	4-nitroquinoline-*N*-oxide
Lysates from treatment with anti-TcOYE polyclonal antibody	n.d.	10 (±2)	n.d.	n.d.
Lysates from treatment with anti-TbPGFS polyclonal antibody	98 (±8)	103 (±3)	100 (±5)	100 (±10)
Lysates from treatment with bovine IgG	100 (±4)	100 (±6)	100 (±10)	100 (±6)

n.d., not detected. Values shown are the mean along with SE from three independent experiments.

In this study, we have also reported on the biochemical isolation, cloning, and molecular characterization of a TcOYE that has limited homology to OYEs from other sources. In addition, we obtained direct evidence for the implication of TcOYE in the synthesis of a biologically active molecule, i.e., $PGF_{2\alpha}$, and in the metabolism of trypanocidal compounds with generation of free radicals from naphthoquinone drugs. The findings that *T. cruzi* produces PGs and that TcOYE catalyzes $PGF_{2\alpha}$ formation raise questions about *T. cruzi* biology and the interaction of this organism with its mammalian host. PGs are potent mediators of physiological and pathological responses (30–32), some of which are observed in Chagas' disease (33). In addition, it has been reported that PGE_2 and $PGF_{2\alpha}$ may play a signal coupling role during phagocytosis in the protozoan parasite *Amoeba proteus*, because they elicit vacuole formation (34), whereas PGD_2 was shown to play an important role in *T. brucei* cell growth regulation by inducing programmed cell death (unpublished data). However, whether or not *T. cruzi*–derived PGs play similar roles remains to be investigated. We have identified a gene whose flavoprotein product TcOYE catalyzes $PGF_{2\alpha}$ synthesis in this parasite. This result is in contrast to previous observations that in other trypanosomatids, i.e., *T. brucei* (17), *Leishmania*, and *Crithidia fasciculata* (unpublished data) $PGF_{2\alpha}$ synthesis is catalyzed by aldo/keto reductases that are markedly different from TcOYE. These findings suggest that for unknown reasons different species of the phylum trypanosomatid have evolved different enzymes to synthesize $PGF_{2\alpha}$ during the course of evolution.

Our results also indicate that TcOYE could reduce naphthoquinone and nitroheterocyclic drugs. These drugs can undergo either one- or two-electron reduction. The one-electron reduction produces semiquinone and nitro radical anions from naphthoquinone and nitroheterocyclic drugs, respectively, with the concomitant formation of superoxide radical anions that cause oxidative damage (35, 36). The two-electron reduction of quinones leads to the formation of hydroquinones without producing semiquinone-free radical intermediates and represents a detoxification pathway for many organisms (37, 38). Alternatively, the successive two-electron reduction of nitroaromatic compounds ultimately produces amino substituents (39). One of the first demonstrations of the enzymatic formation of drug-derived free radicals in eukaryotic organisms was made by incubating β-lapachone (14, 40) and then nifurtimox (12) with intact cells or lysates of *T. cruzi* and identifying the semiquinone or nitro radical anions by ESR. Although β-lapachone and nitro reductase activities have been detected in epimastigote mitochondria (12, 14) and the damaging effect of β-lapachone has been demonstrated (41, 42), the parasite proteins involved in the reduction of both drugs are not well known. Our data support a role for TcOYE in the specific one-electron reduction of menadione and β-lapachone to semiquinone radicals with the concomitant generation of a superoxide anion radical. Because OYEs have been so far isolated from yeasts, plants, and bacteria, and that these enzymes catalyze the two-electron reduction of quinones (2) with no semiquinone radical production (2, 43), the identification of OYE that catalyzes the specific one-electron reduction of menadione and β-lapachone in a parasitic protozoa appears to be unique. On the other hand, TcOYE seems to catalyze the two-electron reduction of nitroheterocyclic drugs with no obvious nitro radical anion production. These findings are in agreement with the study by Karplus et al. (44), who found that bacterial OYE also catalyzed the two-electron reduction of nitro compounds.

Unexpectedly, we also discovered that all reductase activities of epimastigotes were abolished by immunoprecipitation of the TcOYE lysates with anti-TcOYE polyclonal antibody except those toward endoperoxide and β-lapachone. Indeed, several investigators have proposed *T. cruzi* trypanothione reductase (TR) and lipoamide dehydrogenase to be major naphthoquinone and nitroheterocyclic drug–metabolizing enzymes (9, 15, 45, 46). Both enzymes catalyze the one-electron reduction of naphthoquinone and nitroheterocyclic drugs to semiquinone and nitro radical anions, respectively, with the concomitant generation of superoxide radical anions. However, the failure of overexpressed TR to alter sensitivity to agents that induce oxidative stress in *T. cruzi* (47), as well as the lack of a correlation between either nifurtimox sensitivity and the ability to catalyze nifurtimox redox cycling or nifurtimox sensitivity and the amount of TR mRNA (48), argue against any major role for TR in generating oxidative stress. Our observation that anti-TcOYE polyclonal antibody abolished the major naphthoquinone and nitroheterocycle reductase activities means that either TR and lipoamide dehydrogenase are not involved in the redox cycling process of the para-

site, thus supporting previous studies (47, 48), or the enzymes do not catalyze these reactions by an anaerobic pathway. Although the parasite still contains minor endoperoxide and/or β-lapachone reductases other than TcOYE, our results indicate that TcOYE might be the major enzyme used by *T. cruzi* to catalyze the redox cycling of naphthoquinones and the reduction of other trypanocidal drugs. Therefore, this study provides new insight in our understanding of trypanocidal drug metabolism and stresses the need for additional research on TcOYE gene disruption and analysis of its biological relevance.

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