Glyoxalase II of African Trypanosomes Is Trypanothione-dependent*

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The glyoxalase system is a ubiquitous pathway catalyzing the glutathione-dependent detoxication of ketoaldehydes such as methylglyoxal, which is mainly formed as a by-product of glycolysis. The gene encoding a glyoxalase II has been cloned from Trypanosoma brucei, the causative agent of African sleeping sickness. The deduced protein sequence contains the highly conserved metal binding motif HXXHXXXH but lacks three basic residues shown to fix the glutathione-thioester substrate in the crystal structure of human glyoxalase II. Recombinant T. brucei glyoxalase II hydrolyzes lactoylglutathione, but does not show saturation kinetics up to 5 mM with the classical substrate of glyoxalases II. Instead, the parasite enzyme strongly prefers thioesters of trypanothione (bis(glutathionyl)spermidine), which were prepared from methylglyoxal and trypanothione and analyzed by high performance liquid chromatography and mass spectrometry. Mono- (lactoyl)trypanothione and bis- (lactoyl)trypanothione are hydrolyzed by T. brucei glyoxalase II with $k_{cat}/K_m$ values of $5 \times 10^3$ M$^{-1}$ s$^{-1}$ and $7 \times 10^5$ M$^{-1}$ s$^{-1}$, respectively, yielding $\beta$-lactate and regenerating trypanothione. Glyoxalase II occurs in the mammalian bloodstream and insect procyclic form as well as the homeostasis of ascorbate (5).

The pathogenic form of T. brucei multiplying in the blood of the mammalian host depends on glycolysis as the sole energy source and has a very high glucose turnover, which is about 200–300-fold higher than in erythrocytes (8). In E. coli and human red blood cells, the glycolytic rate has been shown to quantitatively correlate with the formation of methylglyoxal (2, 3). These findings together with the fact that trypanothione instead of glutathione is the main low molecular mass thiol prompted us to characterize the glyoxalase system in African trypanosomes.

The T. brucei genome contains two probable glyoxalase II sequences. Here we report on the cloning and overexpression of a glyoxalase II gene encoded on chromosome VI. The recombinant T. brucei protein slowly hydrolyzes lactoylglutathione, the substrate of classical glyoxalases II, but strongly prefers trypanothione thioesters. We provide strong evidence that glutathione is replaced by trypanothione also in the glyoxalase system of trypanosomatid parasites.

EXPERIMENTAL PROCEDURES

Materials

S-Lactoylglutathione, methylglyoxal, yeast glyoxalase I (400–800 units/mg), bovine liver glyoxalase II (10 units/mg), and Leucosarcina mesenteroides $\beta$-lactate dehydrogenase were purchased from Sigma.

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Hog muscle t-lactate dehydrogenase was from Boehr. T. cruzi trypane-
thione reductase was prepared as described (9). Trypanothione disul-
hide was obtained from Bachem. Restriction enzymes and Pfu/Turbo
DNA polymerase were from MBI Fermentas. Primer synthesis and
dNA sequencing were performed by MWG Biotech. All other chemicals
were commercially available reagents of the highest quality.

Cloning and Overexpression of T. brucei Glyoxalase II

T. brucei HisGLX II—The coding region of the glxII gene was
amplified from T. brucei genomic DNA (strain TREU 927/4 (10)) by PCR
using sequence-specific primers derived from the data base. The 5’
primer (5’-gccgcagatggctgtaagctagcagc-3’) contained a BamHI
site (underlined) and gag encoding glutamate 2 of the protein. The 3’
primer (5’-ggctgaagctatgtaagctagcagc-3’) was placed in the 3’-transun-
lated region directly after the stop codon. The gene was amplified
from genomic DNA by PCR (95 °C for 2 min; 95 °C for 30 s; 55 °C for 30 s;
72 °C for 2 min; 30 cycles; 72 °C for 10 min; Pfu). The PCR product was
digested with BamHI and cloned into the pQE-30 vector (Qiagen),
digested with BamHI and Smal. E. coli NovaBlue cells (Novagen) were
transformed with the pQE-30his-glxII plasmid. The plasmid was iso-
lated using the NucleoBond® plasmid purification kit (Macherey-Na-
gel) and the insert was completely sequenced in both directions. For
overexpression of T. brucei HisGLX II, a 1-liter culture of recombinant
NovaBlue cells was incubated at 37 °C in LB-medium containing 100
µg/ml carbenicillin. At an A600 of about 0.5, expression was induced by
adding 300 µM isopropyl-β-D-thiogalactoside and the cells were
allowed to grow overnight at 15 °C.

T. brucei GLX II—For overexpression of tag-free T. brucei glyoxalase II,
the 5’ primer (5’-gccgcagatggctgtaagctagcagc-3’) contained an Ncol
site (underlined) and the start ATG (italic). The 3’ primer (5’-
gcgcagatggctgtaagctagcagc-3’) was placed in the 3’-transun-
lated region directly after the stop codon. The gene was amplified from
genomic DNA (95 °C for 2 min; 95 °C for 30 s; 64 °C for 30 s; 72 °C for 2 min; 30 cycles;
72 °C for 10 min; Pfu) and the PCR product was cloned using the Ncol and BamHI restriction sites into the pQE-60 vector, resulting in the
pQE-60his-glxII plasmid. The plasmid was isolated and sequenced.
NovaBlue cells were transformed with the plasmid and the gene was
overexpressed as described above.

Purification of T. brucei HisGLX II

The fusion protein, carrying a 14-residue long N-terminal extension
with six histidine residues, was purified at 4 °C by chromatography on
a TALON® metal affinity resin column (Clontech). Cells from a 1-liter
bacterial culture were harvested by centrifugation, resuspended in 50
mM sodium phosphate, 300 mM NaCl, 1 mM imidazol, pH 7.0, 150 mM
peptatin, 4 mM cystatin, and 100 µM phenylmethylsulfonyl fluoride and
lysed by sonification, and the cell debris was removed by centrifugation
at 33,000 × g. The supernatant was applied onto a 5-ml resin pre-
equilibrated in 50 mM sodium phosphate, 300 mM NaCl, 1 mM imidazol,
pH 7.0. After washing the column with the equilibration buffer, fol-
lowed by 50 mM sodium phosphate, 300 mM NaCl, 5 mM imidazol, pH
7.0, the protein was eluted with 250 mM imidazol in 50 mM sodium
phosphate, 300 mM NaCl, pH 7.0. The recombinant T. brucei HisGLX
glyoxalase II was ≥95% pure as judged by SDS-polyacrylamide gel
electrophoresis. The protein concentration was determined using the
bicinchoninic acid kit (BCA, Pierce).

Purification of Tag-free GLX II

The recombinant protein without tag was purified on a Q-Sepharose
fast flow cation exchanger (Amersham Biosciences). The column (10 ml)
was washed at a flow rate of 0.3 ml/min for 5 min with solvent A (0.25% (w/v)
N-camphor sulfonate Li-salt, pH 2.64) at 40 °C. Then the theosides
were eluted by 19-min isocratic step of 90% solvent A and 10% solvent
B (25% 1-propanol in solvent A). Before applying a new sample, the
column was washed with 100% solvent B for 10 min and equilibrated for
23 min with 100% solvent A. The S-lactoyltrypanothione conjugates
were detected by the thioester absorption at 240 nm. To show the
theioside composition, matrix-assisted laser desorption/ionization-time of
flight) analyses of the thio-
esters were carried out by Drs. Jens Pfannstiel and Johannes Lechner
(Biochemie-Zentrum der Universität Heidelberg) using a 2.5-dihy-
droxybenzoic acid matrix.

Kinetic Studies

The activities of T. brucei and bovine liver glyoxalase II were deter-
mined by measuring the hydrolysis of the thiosides of the (lactoyl)
hydrolysis of the thiosides of the (lactoyl)glyoxalase II and bis-(lactoyl)glyoxalase II directly at 240 nm or in a coupled assay follow-
ing the reaction of the liberated thior with DTNB at 412 nm (13). The assays were performed in a total volume of 1 ml of 100 mM MOPS, pH 7.2, at 25 °C in a Hitachi
150-20 spectrophotometer in 300-µl microcuvettes using a 330-ml DUB-85 spectrophotometer. The lactoylglutathione concentration was
varied between 0.01 and 5 mM in the coupled and between 0.01 and 0.6
mM in the direct assay. Hydrolysis of the lactoylglutathione theosides
was followed only in the direct assay at 240 nm at concentrations
between 0.02 and 0.15 mM. The theioside stock solutions were freshly
prepared from a dried aliquot stored at –20 °C (see above). The extinc-
tion coefficients used are DTNB, ε240 = 13.6 mM–1 cm–1 (11), SLG,
ε240 = 3.3 mM–1 cm–1 (14, 15), MLT, ε240 = 3.3 mM–1 cm–1, and BLT,
ε240 = 6.5 mM–1 cm–1, respectively. The ε values of the thioside theiosides were derived from measuring the thio concentration with
DTNB after complete hydrolysis.

pH and Ionic Strength Dependence of Glyoxalase II

The pH dependence of T. brucei glyoxalase II was determined by
following the activity of the enzyme in the presence of 82 µM bis-
lactoylglutathione in a total volume of 200 µl of 100 mM MOPS
between pH 6.0 and 9.2 at 25 °C. To elucidate the optimum of ionic
strength, assays were performed at 25 °C with 88 µM bis-(lactoyl)
trypanothione in a total volume of 200 μl of 10–250 mM MOPS buffers at a constant pH of 7.2.

**Determination of β-Lactate**

Production of β-lactate was shown by coupling the T. brucei glyoxalase II reaction to the reaction of β- and γ-lactate dehydrogenase, respectively, following formation of NADH. After the glyoxalase II reaction with 87.5 μl bis-[lactoyl]trypanothione in 800 μl of 100 mM Tris-HCl, pH 8.5, had run to completion, 5 mM NAD and 2.75 units of β- or γ-lactate dehydrogenase were added and the absorption increase at 340 nm (εγH = 6.22 mm⁻¹ cm⁻¹) was followed at 25 °C (16).

**Metal Analysis of Recombinant T. brucei Glyoxalase II**

The metal content of His- and tag-free T. brucei glyoxalase II was analyzed by Dr. Peter Schramel (GSF-Forschungszentrum, Neuherberg) by ICP-OES (inductively coupled-plasma optical emission spectrometry). For comparison, the concentration of different metals in the human liver (13), and A. thaliana (6.0) (18) determined by isoelectric focusing. The putative other kinetoplastid glyoxalases II (Fig. 1) also have theoretical acidic pI values. In general, plant glyoxalases II are acidic proteins with pI values ranging from 4.7 to 6.2, whereas animal enzymes have basic pI values. Isoelectric focusing of recombinant human glyoxalase II and the enzyme isolated from erythrocytes yielded isoelectric points of 8.5 (13) and 8.3 (19), respectively. Several glyoxalases II such as the enzyme from Aloe vera (20), spinach leaves (21), and bovine liver mitochondria (22) show multiple protein bands when subjected to isoelectric focusing. At least some of the bands may be explained by a varying content of their metal cofactors that obviously exchange or get lost easily (see below). Because the molecular and kinetic properties of the known glyoxalases II are very similar, the diversities of their isoelectric points probably reflect the evolutionary distance rather than functional differences of the enzymes.

Glyoxalases II contain the highly conserved metal binding motif THXIXD (23). In total, five His and two Asp residues interact directly with two metal ions as shown in the three-dimensional structure of human glyoxalase II (24). All these residues are conserved in the trypanosomatid proteins, suggesting that the parasite proteins also possess metal cofactors (Fig. 1). The structure of human glyoxalase II in complex with a substrate analogue revealed three conserved basic residues that are involved in the fixation of the thioester in the active site. Arg-249, Lys-252, and Lys-143 (numbering of human glyoxalase II) are in close proximity to the glycine carboxylate of the glutathione moiety of the substrate analogue (24). These residues, present in all glyoxalases II studied so far, are not conserved in the proteins from T. brucei and the other kinetoplastid organisms. This was the first indication that glutathione thioesters are probably not the physiological substrates of the parasite glyoxalases II.

**Overexpression and Purification of T. brucei Glyoxalase II—**

The glyoxalase II gene was overexpressed from pQE vectors with and without the N-terminal His tag. Purification of His-tagged T. brucei glyoxalase II by metal affinity chromatography yielded 10 mg of pure protein from a 1-liter bacterial culture. The tag-free recombinant protein was purified also in E. coli shows 30% identical residues (Fig. 1). The isoelectric point (pI) of T. brucei glyoxalase II calculated from the protein sequence is 6.5, which is comparable with those of the proteins from A. thaliana (6.2) (17) and Candida albicans (6.0) (18) determined by isoelectric focusing. The putative other kinetoplastid glyoxalases II (Fig. 1) also have theoretical acidic pI values. In general, plant glyoxalases II are acidic proteins with pI values ranging from 4.7 to 6.2, whereas animal enzymes have basic pI values. Isoelectric focusing of recombinant human glyoxalase II and the enzyme isolated from erythrocytes yielded isoelectric points of 8.5 (13) and 8.3 (19), respectively. Several glyoxalases II such as the enzyme from Aloe vera (20), spinach leaves (21), and bovine liver mitochondria (22) show multiple protein bands when subjected to isoelectric focusing. At least some of the bands may be explained by a varying content of their metal cofactors that obviously exchange or get lost easily (see below). Because the molecular and kinetic properties of the known glyoxalases II are very similar, the diversities of their isoelectric points probably reflect the evolutionary distance rather than functional differences of the enzymes.

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**RESULTS**

Cloning and Structural Comparison of T. brucei Glyoxalase II with Glyoxalases II from Other Species—Blast searches with human glyoxalase II as template revealed two sets of putative glyoxalase II sequences in the T. brucei genome. The deduced protein sequences encoded on chromosomes IV and VI were only 25% identical to each other but showed 30 and 36% identity with human glyoxalase II, respectively. Based on this information, the gene on chromosome VI was cloned from genomic DNA of strain TREU 9274 (10). The complete coding region was amplified with two gene-specific primers and sequenced in both directions. PCR on cDNA from bloodstream T. brucei with a sequence-specific primer and a poly(dT) primer amplified a fragment containing the 3′ end of the coding sequence followed by a 390-bp long 3′-untranslated region (data not shown). The deduced protein sequence consists of 296 amino acid residues and clearly classifies the T. brucei protein as glyoxalase II. The highest degree of similarity is found with putative proteins from T. cruzi and L. major where 66 and 51%, respectively, of all residues are identical. In the functionally characterized human and Arabidopsis thaliana glyoxalases II 36 and 31%, respectively, of all residues are conserved. The sequence of a probable hydroxyacylglutathione hydrolase from E. coli shows 30% identical residues (Fig. 1).
Synthesis and Structural Analysis of S-Lactoyltrypanothione—Mono- and bis-(lactoyl)trypanothione thioesters were obtained by reacting reduced trypanothione with methylglyoxal. The spontaneous reaction resulted in a hemithioacetal (14, 25), which is isomerized into the lactoyltrypanothione thioester by yeast glyoxalase I. The method takes advantage of the

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fact that glyoxalase I, in contrast to glyoxalase II, is not highly specific for glutathione but also accepts derivatives of glutathione modified at the glycine carboxylate (26, 27). The thioesters were freed from the other reaction components by chromatography on an Oasis MCX cation exchanger cartridge that retains the positively charged trypanothione derivatives. HPLC analysis of bis-(lactoyl)trypanothione showed two peaks with retention times of 17.5 and 19.5 min. The first, minor peak represents mono-(lactoyl)trypanothione and the second, major peak is bis-(lactoyl)trypanothione (Fig. 2A). Mass spectrometry confirmed bis-(lactoyl)trypanothione as the main reaction product and the presence of a small amount of mono-(lactoyl)trypanothione (Fig. 2B). Mono-(lactoyl)trypanothione (90% pure) was obtained by applying a 10-fold excess of reduced trypanothione over methylglyoxal (data not shown).

Hydrolysis of S-Lactoyltrypanothione by T. brucei Glyoxalase II Yields D-Lactate and Trypanothione—Hydrolysis of lactoylglutathione by classical glyoxalases II generates D-lactate (23, 28). To reveal if hydrolysis of the lactoyltrypanothione thioester by T. brucei glyoxalase II yields also the D-isomer of lactate, the reaction was coupled to that of D- and L-lactate dehydrogenase, and subjected to HPLC analysis as described under “Experimental Procedures.” Bis-(lactoyl)trypanothione (BLT) elutes with a retention time of 19.5 min. The peak eluting after 17.5 min is mono-(lactoyl)trypanothione (MLT), which represents ≤12% of the total thioester content as estimated from the peak areas. A MALDI-TOF analysis of the bis-(lactoyl)trypanothione reaction mixture. MLT; appears as free mono-(lactoyl)trypanothione (796.3 m/z (1)), the sodium adduct (818.2 m/z (2)), and the sulfonic acid (829.5 m/z (3)). The four main peaks represent free BLT (868.3 m/z (4), the mono- (890.3 m/z (5) and bis-sodium (912.3 m/z (6)) and the bis-potassium adduct (947.1 m/z (7)). a.i., absolute intensity; m/z, mass to charge values.

Hydrolysis of bis-(lactoyl)trypanothione by T. brucei glyoxalase II was synthesized, freed from the other reaction products, and subjected to HPLC analysis as described under “Experimental Procedures.” Bis-(lactoyl)trypanothione and a small amount of mono-(lactoyl)trypanothione (Fig. 4A). Incubation of the thioester with glyoxalase II for 8 min leads to the decrease of bis-(lactoyl)trypanothione with the concomitant increase of mono-(lactoyl)trypanothione. Two new peaks appear that were identified as trypanothione and trypanothione disulfide (Fig. 4B). After 72 min, bis-(lactoyl)trypanothione is completely hydrolyzed. The reaction mixture still contains a small amount of mono-(lactoyl)trypanothione.
enzyme yielded a $V_{\text{max}}$ of 12 units/mg in accordance with literature data (14). The $K_m$ of 191 $\mu$M corresponded to the values reported for the mitochondrial bovine liver (22) and human liver glyoxalases II (1). $T. brucei$ glyoxalase II hydrolyzed lactoylglutathione but did not show saturation kinetics up to 5 mM. The activities with the trypanothione thioesters were measured in the direct assay following hydrolysis of the thioester at 240 mM. Under the conditions used, bovine liver glyoxalase II did not have any activity with the trypanothione thioesters. In contrast, $T. brucei$ glyoxalase II hydrolyzed bis-(lactoyl)trypanothione and mono-(lactoyl)trypanothione with $V_{\text{max}}$ values of about 100 units/mg and $K_m$ values of 86 and 108 $\mu$M, respectively (Table I). The catalytic efficiencies ($k_{\text{cat}}/K_m$) are in the order of $6 \times 10^6$ M$^{-1}$ s$^{-1}$. The preference of $T. brucei$ glyoxalase II for trypanothione instead of glutathione-based thioesters is probably because of the replacement of basic residues shown to locate the glycine carboxylate of glutathione in the mammalian enzyme (24, 29).

$T. brucei$ glyoxalase II shows a rather broad pH-optimum at pH 7.0–8.0 (Fig. 6A). A corresponding behavior has been reported for human liver glyoxalase II (pH optimum 6.8–7.5) (30), whereby the optimum of the $T. brucei$ enzyme is slightly shifted to more alkaline pH values. Because the conductivity of the 100 mM MOPS buffer increased from 0.3 to 3.4 mS when changing the pH between 6.1 and 9.2 we measured the activity of the enzyme in 10–250 mM MOPS buffer at a constant pH of 7.2 (corresponding to 0.22–4 mS) (Fig. 6B). $T. brucei$ glyoxalase II did not show any dependence on the ionic strength within this range. These findings contrast with glyoxalase II from rat erythrocytes, which has been reported to be highly sensitive to the ionic strength of the buffer, yielding the highest activity at the lowest ionic strength (31).

**Recombinant $T. brucei$ Glyoxalase II Contains Metal Ions**—The metal content of recombinant His$_6$ and tag-free glyoxalase II was determined in several enzyme preparations by inductively coupled-plasma optical emission spectrometry (Dr. P. Schramel). The analyses resulted in quite varying compositions with about 1.5 and 0.7–0.9 mol of total metal ions per mole of His$_6$ and tag-free glyoxalase II, respectively. A typical analysis of the fusion protein yielded 0.45 mol of zinc, 0.42 mol of iron, and 0.6 mol of cobalt ions per mol of protein. Some preparations also contained small amounts of manganese. In the tag-free protein, zinc was the main metal with some iron. The high concentration of cobalt in the fusion protein purified by TALON metal affinity chromatography but not in the tag-free protein obtained by ion exchange chromatography indicates that $T. brucei$ glyoxalase II easily exchanges its metal cofactors. Despite the varying metal compositions, fresh enzyme preparations had very similar specific activities suggesting that $T. brucei$ glyoxalase II is not specific for its metal ligands.

**Glyoxalase II Is Expressed in Bloodstream and Procyclic $T. brucei$**—Western blot analyses revealed the presence of glyoxalase II in bloodstream as well as procyclic $T. brucei$ (Fig. 7A). The cellular concentration of the enzyme was estimated from a standard line derived from blots with different amounts of recombinant glyoxalase II and cell extracts (Fig. 7B). With a cell volume of 58 femtoliters (32, 33), a concentration of glyoxalase II of about 7 $\mu$M is obtained for bloodstream $T. brucei$. When identical cell numbers are applied, procyclic parasites yield slightly weaker signals for glyoxalase II (Fig. 7A). Together with the larger cell volume of procyclic parasites, one can estimate that the glyoxalase II concentration is about 50% of that of bloodstream parasites. The mammalian forms of $T. brucei$ have a very high glucose turnover (8), which probably correlates with formation of large amounts of methylglyoxal and the need for an efficient glyoxalase system (2, 3). On the other hand, cultured procyclic $T. brucei$ contain also significant amounts of glyoxalase II. Because these cells are grown in a proline-rich medium and glycolysis should not play a major role for their energy supply the physiological role(s) of the glyoxalase system in $T. brucei$ remains to be elucidated.

**DISCUSSION**

Kinetoplastid organisms possess a unique thiol metabolism based on the dithiol trypanothione and trypanothione reductase instead of the ubiquitous glutathione system. The trypanothione system is the electron donor for the synthesis of DNA precursors and is involved in the antioxidative defense in these parasites (5, 34, 35). As shown here, trypanothione replaces glutathione also in the glyoxalase system of African trypanosomes. $T. brucei$ glyoxalase II strongly prefers thioesters of trypanothione instead of glutathione as substrates. The glutathione-dependent glyoxalase system is found constitutively in a wide variety of organisms. The pathway has been analyzed in bacteria (36), yeast (37), plants (17, 20), and mammals (13, 19, 26, 38). The $T. brucei$ protein is the first glyoxalase II characterized so far that does not use glutathione as cofactor.

The $T. brucei$ glyoxalase II analyzed here, the gene of which is located on chromosome VI, shows 25% overall identity with a protein encoded on chromosome IV that has also been annotated as glyoxalase II. When comparing the four putative glyoxalase II sequences available in trypanosomatid data bases, 66 and 51% of all residues are conserved between the $T. brucei$ glyoxalase II described here and a $T. cruzi$ and $L. major$ protein, respectively, whereas only 26 and 22% are identical in the $T. brucei$ protein encoded in chromosome IV. In addition, the
The activity of His-tagged T. brucei glyoxalase II was followed (A) in 100 mM MOPS buffers between pH 6.0 and 9.2 and (B) at pH 7.2 in MOPS buffers from 10 to 250 mM as described under “Experimental Procedures.” The data are the mean of duplicate measurements, which differed by less than 15%. These series were conducted twice. ▲, conductivity of the buffer.

Histidine motifs of putative T. brucei GLX II were identified by Western and chemical mapping. The GLX II expressed in E. coli is the recombinant His6-GLX II. Western blot of total cell lysates of bloodstream (BF), procyclic (PC), and tag-free recombinant glyoxalase II (His6-GLX II). Purified polyclonal rabbit antibodies against the human enzyme (His6-GLX II). The standard diagram based on 5 amounts of tag-free recombinant glyoxalase II (▲) using the “Quantity One 1-D Analysis Software” (Bio-Rad). ▲, 1 × 10^6 cells of bloodstream, and +, procyclic parasites. INT, intensity.

**FIG. 7.** Quantification of glyoxalase II in bloodstream and procyclic T. brucei. A, Western blot of total cell lysates of 1 × 10^6 and 2 × 10^6 bloodstream (BF) and procyclic (PC) cultured T. brucei and 10, 20, and 30 ng of recombinant tag-free glyoxalase II as well as 5 ng of tagged protein (His6-GLX II). Purified polyclonal rabbit antibodies against the recombinant His6-GLX II together with the SuperSignal West Pico chemiluminescent substrate were used for visualization as described under “Experimental Procedures.” B, standard diagram based on 5 amounts of tag-free recombinant glyoxalase II (▲) using the “Quantity One 1-D Analysis Software” (Bio-Rad). *", 1 × 10^6 cells of bloodstream, and +, procyclic parasites. INT, intensity.

motif THXHXDH, which together with another two His and an Asp residue are present in all glyoxalases II studied so far (23, 24). The metal analysis of recombinant T. brucei glyoxalase II yielded different metals and between 0.7 and 1.5 mol of total metal ions per mol of enzyme. In the tag-free protein zinc was the main metal. The His6-tagged protein contained zinc, cobalt, and iron (and sometimes manganese). The metal content and composition of glyoxalases II is a continuous matter of discussion. Whereas the old literature reported that the enzymes do not need any metal cofactor, more recent studies demonstrated the presence of two metal ions bound to the protein (23, 24, 39). The crystal structure of human glyoxalase II revealed two metals that were assigned as zinc ions although the authors mention that the analysis could not discriminate between zinc and iron (24). Cytosolic A. thaliana glyoxalase II contains an iron-zinc binuclear metal center that is essential for substrate binding and catalysis (23). A most recent report shed some light on the variable metal composition and content of these enzymes. Recombinant A. thaliana glyoxalase II is able to incorporate zinc, iron, and manganese depending on the metal added to the bacterial culture medium resulting in enzyme species with similar catalytic efficiency. The high degree of
structural flexibility within the binuclear active site observed in the plant enzyme is probably the basis for the broad metal selectivity. It appears that the catalytic and metal binding properties of the enzyme allow full functionality with various types and ratios of bound metal ions (39).

The substoichiometric metal content in T. brucei glyoxalase II may be attributed to a loss during purification or to a limited metal availability during overexpression. On the other hand, recombinant A. thaliana glyoxalase II isolated from E. coli grown in the presence of excess metals also contained only 1.4 to 1.76 metals per protein (39). Interestingly, storage of grown in the presence of excess metals also contained only 1.4 metal availability during overexpression. On the other hand, glyoxalase II causes a simultaneous decrease of cei feature medium (42). In contrast, in isolated rat liver mitochondria, specific translocators mediate the import of d-lactate where a putative d-lactate dehydrogenase metabolizes d-lactate to pyruvate (43). The fate of d-lactate produced in T. brucei still needs to be elucidated.

The two thioester substrates, mono- and bis-(lactoyl) trypanothione, were obtained by reacting different ratios of methylglyoxal and reduced trypanothione. Formation of bis-(lactoyl)trypanothione is favored in the presence of a 3–4-fold excess of methylglyoxal over trypanothione. Considering a cellular concentration of trypanothione of ≥350 μM in bloodstream T. brucei (44) and a methyglyoxal concentration of 1–2 μM reported for human blood (45) and Saccharomyces cerevisiae (46), the monothioester of trypanothione is probably the main physiological substrate of the parasite enzyme.

The catalytic efficiency of T. brucei glyoxalase II with the lactoyltrypanothione thioesters is comparable with that of the C. albicans enzyme with lactoylglutathione (18). The recombinant glyoxalases II from man (13), A. thaliana (17), and yeast (47) have kcat/Km values for lactoylglutathione that are about an order of magnitude higher. This may at least partially be attributed to the higher assay temperature (37 °C instead of 25 °C) and differing buffer conditions. The commercially available bovine liver glyoxalase II used here showed under identical assay conditions with lactoylglutathione a 20-fold lower catalytic efficiency than the T. brucei enzyme with the lactoyltrypanothione thioesters.

The physiological function(s) of the trypanothione-dependent glyoxalase system in T. brucei remains to be elucidated. The enzyme occurs in both bloodstream and procyclic parasites. In yeast (47), spinach (21), as well as bovine (22) and rat liver (38), glyoxalase II is present in the cytoplasm and mitochondria. In yeast, and A. thaliana the cytosolic and mitochondrial forms of the enzyme are the products of two different genes (47, 48). The protein sequence of T. brucei glyoxalase II does not show a sorting signal and preliminary Western blots with cell fractions of bloodstream T. brucei indicate a cytosolic localization (data not shown). If methylglyoxal is indeed the main physiological substrate the question arises where the ketoaldehyde is formed and detoxified. In trypanosomiasis, the first seven enzymes of glycolysis reside in glycosomes, peroxisome-related organelles. Thus methylglyoxal may be generated in the glycosomes and subsequently exported to the cytosol for detoxification. Alternatively and more likely, the ketoaldehyde is formed in the cytosol. In bloodstream parasites, the NAD/NADH balance of glycolysis is maintained by reduction of dihydroxyacetone phosphate to glycerol 3-phosphate within the glycosome. Glycerol 3-phosphate is then reconverted into dihydroxyacetone phosphate by a mitochondrial glycerolphosphate oxidase (49). This shuttle implies the presence of cytosolic dihydroxyacetone phosphate. Triose-phosphate isomerase deficiency is associated with accumulation of dihydroxyacetone phosphate and concomitantly methylglyoxal (50). As shown by Helfert et al. (51) triose-phosphate isomerase is essential for bloodstream T. brucei. Enzyme levels of about 15% of wild-type parasites the growth rate is halved and the total cellular dihydroxyacetone phosphate concentration is increased. Because dihydroxyacetone phosphate spontaneously degrades to methylglyoxal the lethality of the triose-phosphate isomerase knock-out may at least partially be because of increased formation of toxic methylglyoxal.

Inhibitors of glyoxalase I and glyoxalase II are investigated as potential anti-tumor and anti-malarial agents (52–54). The high glucose turnover of T. brucei together with the unique trypanothione dependence renders the glyoxalase system an attractive new target for anti-trypanosomial chemotherapy. Future work will include kinetic studies on other physiological ketoaldehydes such as glyoxal. RNA-interference experiments are in progress to elucidate if glyoxalase II is essential for T. brucei.

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