Identification of a developmentally regulated iron superoxide dismutase of *Trypanosoma brucei*

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An iron superoxide dismutase (FeSOD) gene of the protozoan parasite *Trypanosoma brucei* has been cloned and its gene product functionally characterized. The gene encodes a protein of 198 residues which shows 80% identity with FeSODs from other trypanosomatids. Inhibitor studies with purified recombinant FeSOD expressed in *Escherichia coli* confirmed that the enzyme is an iron-containing SOD. The FeSOD is developmentally regulated in the parasite, expression being lowest in the cell-cycle-arrested, short stumpy bloodstream forms. Differential expression of the FeSOD protein contrasts with only minor quantitative changes in the FeSOD mRNA, indicating post-transcriptional regulation of the enzyme. As the level of FeSOD increases during differentiation of cell-cycle-arrested short stumpy into dividing procyclic forms, it is suggested that the enzyme is only required in proliferating stages of the parasite for the elimination of superoxide radicals which are released during the generation of the iron-tyrosyl free-radical centre in the small subunit of ribonucleotide reductase.

Key words: differentiation, life cycle, ribonucleotide reductase, superoxide radical.

INTRODUCTION

Superoxide dismutases (SODs; EC 1.15.1.1) are a group of metalloenzymes that eliminate superoxide radicals ($O_2^{\cdot-}$) by decomposing them into hydrogen peroxide and oxygen [1]. In concert with catalase, SODs have strong anti-oxidant properties and have been shown to protect normal cells as well as a number of pathogens from reactive oxygen species. According to their metal cofactor, SODs can be classified into three isoform types: copper/zinc-, manganese- and iron-containing enzymes (Cu,ZnSOD, MnSOD and FeSOD) [2].

Trypanosoma brucei is an extracellularly living protozoan flagellate that causes sleeping sickness in humans in many countries of sub-Saharan Africa. During its life cycle, as the parasite shuttles between the insect vector (tsetse fly) and mammalian host, it differentiates into a number of distinct developmental stages [3]. In the blood and tissue fluids of the mammalian host *T. brucei* occurs as a proliferating long slender form that differentiates into a cell-cycle-arrested short stumpy form at the peak of parasitaemia. The short stumpy trypanosomes are pre-adapted to life in the insect vector. After ingestion by the tsetse fly, short stumpy forms differentiate into the next life-cycle stage, the proliferating procyclic form which colonizes the insect midgut. In culture, differentiation into procyclic insect forms can be easily achieved by incubating short stumpy trypanosomes at 27 °C in the presence of the tricarboxylic-acid-cycle intermediates citrate and *cis*-aconitate [4–6].

In *T. brucei*, SOD activity has been detected in long slender forms; however, whether it is present in other developmental stages of the parasite is not known [7]. Since trypanosomes lack catalase activity, the functional role of SOD remains to be defined. In this paper, we report on the cloning of a gene encoding SOD from *T. brucei* and on the functional characterization of the gene product. As shown here, the *T. brucei* enzyme is an iron-containing SOD that is regulated during the life cycle of the parasite.

MATERIALS AND METHODS

Trypanosomes

Bloodstream forms of the pleomorphic *T. brucei* clone AnTat 1.1 [8] were grown in NMRI mice and purified by DEAE-cellulose chromatography [9]. Long slender and short stumpy trypanosomes were harvested 3 and 5 days post-infection, respectively. Differentiation of short stumpy bloodstream forms into procyclic insect forms was performed as described previously [4–6]. Established procyclic cultures were maintained in SDM-79 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum at 27 °C in closed culture flasks [10]. Culture-adapted bloodstream forms of *T. brucei* cell line TC221 [11] were propagated in Baltz medium plus 16.7% (v/v) heat-inactivated fetal bovine serum at 37 °C in a humidified atmosphere containing 5% CO_2 [12].

PCR amplification of a *T. brucei SOD* gene

Total RNA (5 μg) of TC221 trypanosomes was reverse-transcribed into cDNA using reverse transcriptase (Qiagen) and an oligo(dT) primer (5′-CGCATTCTTTTTTTTTTTTTTVNN-3′). PCR amplication with a primer derived from consensus sequences of *FeSOD* genes (SODR1, 5′-TACGCATGCTCCC-ATACATC-3′) and the mini-exon-specific primer (5′-ACAGT-TTCTGTACTATATTG-3′) yielded the 5′-end of the gene. One Ready-To-Go PCR bead (Pharmacia) was used and conditions were as follows: 42 °C for 30 s, 95 °C for 1 min, then 30 cycles of 94 °C for 1 min, 62.5 °C for 1 min and 72 °C for 1 min, followed by 72 °C for 5 min. The PCR fragment was cloned into the pGEM-T Easy vector (Promega) and sequenced.

Abbreviations used: Ni^{2+}-NTA, Ni^{2+}-nitrilotriacetic acid; 3′-RACE, rapid amplification of 3′ cDNA ends; SOD, superoxide dismutase; Cu,ZnSOD, copper/zinc-containing SOD; MnSOD, manganese-containing SOD; FeSOD, iron-containing SOD; DTPA, diethylenetriaminepenta-acetic acid.

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The complete coding sequence was obtained by rapid amplification of 3′ cDNA ends (3′-RACE). First, total RNA (5 μg) was reverse-transcribed into cDNA using an adapter primer (5′-GGCCACGCGTCGACTAGTACTTTTTTTTTTTTTTT-3′; Life Technologies). Then, PCR amplification was performed with an abridged universal amplification primer (5′-GGCCACGCGTCGACTAGTAC-3′; Life Technologies) and a sequence-specific primer (SODRACEF1, 5′-TGGAATACGACCTCAAAGTG-3′) using one Ready-To-Go PCR bead. Conditions were: 95 °C for 1 min; 30 cycles of 94 °C for 1 min, 42.5–67.5 °C (gradient) for 1 min, 72 °C for 1 min, followed by 72 °C for 10 min. Size fractionation of the PCR products on a 2 % agarose gel gave two fragments, of 450 bp and 1000 bp. The 1000 bp fragment was isolated, cloned into the pGEM-T Easy vector and sequenced. Positive clones contained the complete open reading frame including the 3′-end of the gene. All sequencing was performed by GATC Biotech AG (Konstanz, Germany) and both strands were sequenced completely.

Expression of a *T. brucei SOD* gene and purification of recombinant protein

By PCR using the vector pGEM-T/*SOD* and primers 5′-GACGGATCCACCTTCAGCATTCCA-3′ and 5′-CTG-AAGCTTCTAGCTTTTCAGCAG-3′ (with the restriction sites underlined), a *Bam*HI and a *Hin*dIII site were introduced immediately after the start and stop codons of the open reading frame, respectively (one Ready-To-Go PCR bead; 95 °C for 1 min, then 94 °C for 1 min, 45 °C for 1 min and 72 °C for 1 min for 35 cycles, followed by 72 °C for 1 min). The PCR fragment was size-fractionated on a 2 % agarose gel, isolated and digested with *Bam*HI and *Hin*dIII. The purified *Bam*HI–*Hin*dIII *SOD* fragment was ligated with *Bam*HI/*Hin*dIII-cut pQE 30 vector (Qiagen). Competent *Escherichia coli* XL_1-Blue (Stratagene) were transformed with the pQE 30/*SOD* vector by electroporation. A 10 ml overnight culture was diluted with 10 ml of Luria–Bertani medium and expression induced with 60 μg/ml isopropyl β-D-thiogalactoside for 5 h. After centrifugation the cells were suspended in 1 ml of buffer A (100 mM NaH_2PO_4/10 mM Tris/HCl/8 M urea, pH 8.0) and sonicated in a water bath until clear. The lysate was centifuged for 25 min at 700 ***g*** and 900 μl of supernatant applied to a Ni^{2+}-nitrilotriacetic acid (Ni^{2+}-NTA) spin column (Qiagen) pre-equilibrated with buffer A. The column was centrifuged for 2 min at 300 ***g*** and washed twice with 600 μl of buffer A adjusted to pH 6.3. Then, His-tagged *T. brucei* SOD was eluted with 120 μl of buffer A adjusted to pH 4.5. For purification of active recombinant *T. brucei* SOD, *E. coli* cells were suspended in buffer B (50 mM NaH_2PO_4/300 mM NaCl, pH 8.0) containing 10 mM imidazole and disintegrated by sonication. After centrifugation the supernatant was applied to a Ni^{2+}-NTA spin column. The column was washed with buffer B containing 20 mM imidazole and the bound His-tagged *T. brucei* SOD eluted with buffer B containing 250 mM imidazole.

SOD assay

The activity of SOD was determined indirectly by the inhibition of pyrogallol autoxidation in 50 mM Tris/HCl, pH 8.0/1 mM diethylenetriaminepenta-acetic acid (DTPA). The increase in absorbance at 420 nm was followed [13].

Immunoblot analysis

SDS/PAGE, immunoblotting and preparation of whole-cell extracts were performed as described previously [14]. Polyclonal rabbit antiserum against a *T. brucei* SOD peptide (peptide 133/147, TTKKLKVFQTHDAGC) was produced by Eurogentec. Polyclonal antibodies against AnTat 1.1 variant surface glycoprotein were raised in Balb/c mice [14]. Rabbit antisera against the recombinant R2 subunit of *T. brucei* ribonucleotide reductase (αR2) and *T. brucei* aldolase were provided kindly by Professor Luise Krauth-Siegel (Biochemie Zentrum Heidelberg, Heidelberg, Germany) and Professor Christine Clayton (Zentrum für Molekulare Biologie Heidelberg, Heidelberg, Germany), respectively. Monoclonal antibodies against procyclin (clone TBRP1/247) were purchased from Cedarlane.

Semi-quantitative reverse transcriptase PCR

Total RNA was purified from long slender, short stumpy and procyclic trypanosomes by the single-step guanidine method, treated with RNase-free DNase for 1 h at 37 °C, and the mRNA reverse-transcribed into cDNA as described above. Internal fragments of *SOD* and actin genes were amplified from cDNA with primers SODR1 and SODRACEF1, and with primers ACTINF1 and ACTINR1 [15], respectively, at 95 °C for 60 s followed by 35 cycles of 60 s at 94 °C, 30 s at 60 °C and 50 s at 72 °C, and a final extension at 72 °C for 7 min using one Ready-To-Go-PCR bead. Finally, 10 μl of the PCR mixtures were size-fractionated on a 2 % agarose gel and stained with ethidium bromide.

RESULTS

Cloning and sequencing of a *SOD* gene from *T. brucei*

The gene encoding SOD was cloned from cDNA of culture-adapted bloodstream forms of *T. brucei* (GenBank® accession number AF364812). PCR using a primer deduced from consensus sequences of FeSODs and a mini-exon primer, which is added to the very 5′-end of all trypanosomal mRNAs by a process called trans-splicing [16], led to amplification of a fragment containing an open reading frame coding for amino acid residues that are known for FeSODs. The complete coding region of the gene together with the 3′-untranslated region was amplified by 3′-RACE PCR using an abridged universal amplification primer and a sequence-specific primer. An open reading frame of 594 bp corresponds to a deduced protein sequence of 198 amino acid residues with a molecular mass of 22137 Da and a calculated pI of 6.15.

Sequence comparison of *T. brucei* SOD with other SODs

The *T. brucei* SOD exhibits significant homology with FeSODs from other eukaryotes. The highest identity was scored with FeSODs from the two trypanosomatids *T. cruzi* (82 %) and *Leishmania donovani* (80 %), which reflects the close relationship of these organisms. A high degree of amino acid identity was also obtained with FeSODs from other parasitic protozoa (*Toxoplasma gondii*, 57 % identity; *Plasmodium falciparum*, 55 %; *Entamoeba histolytica*, 53 %) and from prokaryotes (*E. coli*, 56 %). In contrast, FeSODs from plants (*Arabidopsis thaliana*, 42 %; *Nicotiana plumbaginofolia*, 42 %; soybean, 40 %) and MnSODs from mammals (human, 47 %) are more distant. Additional sequence analysis showed that all amino acid residues which are considered distinctive for FeSODs [17] (Ala-71, Gln-72, Tyr-79, Ala-145 and Gly-146) and that all metal-binding residues (His-28, His-76, Asp-161 and His-165) are conserved in the *T. brucei* protein.

Expression, purification and characterization of *T. brucei* SOD

For overexpression the *T. brucei SOD* gene was cloned into the pQE expression vector. The resulting construct coded for the

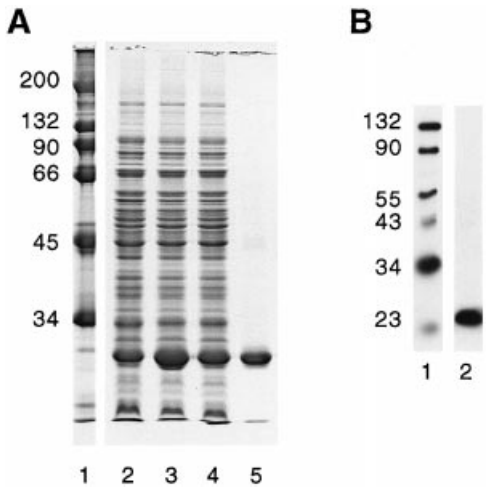

Figure 1 Expression of *T. brucei* SOD in *E. coli*

(**A**) Purification of recombinant *T. brucei* SOD. *T. brucei* SOD was expressed from the vector pQE 30/*SOD* in *E. coli* and purified using Ni^{2+}-NTA spin columns. Proteins were separated by electrophoresis on a 12% reducing SDS/polyacrylamide gel and stained with Coomassie Brilliant Blue. Lane 1, molecular-mass standard; lane 2, non-induced cells; lane 3, cells induced with isopropyl β-D-thiogalactoside; lane 4, flow-through; lane 5, eluate. (**B**) Immunoblot analysis of purified recombinant *T. brucei* SOD. Lane 1, molecular-mass standard; lane 2, purified recombinant *T. brucei* SOD analysed by immunoblotting using antibodies against the synthetic peptide 133/147.

SOD protein with an 11-residue-long N-terminal extension containing a His_6 sequence. After induction of pQE 30/*SOD*-transformed *E. coli* cells the recombinant protein was purified with Ni^{2+}-NTA spin columns. SDS/PAGE revealed a single protein band with a molecular mass of approx. 23 kDa (Figure 1A), which is in accordance with the calculated molecular mass of 23404 Da for the His-tagged protein. To confirm the identity of the recombinant protein, immunoblotting using antibodies prepared against a synthetic *T. brucei* SOD peptide (residues 133–147) was performed. The selected sequence distinguishes the *T. brucei* SOD from FeSOD and MnSOD present in *E. coli*. The purified fusion protein reacted strongly with the anti-peptide antibodies (Figure 1B).

To characterize the isoform type of the cloned *T. brucei* SOD the activity of the purified recombinant enzyme was determined indirectly by the inhibiton of pyrogallol autoxidation in the presence of inhibitors [13]. Cu,ZnSODs are cyanide- and peroxide-sensitive, FeSODs are cyanide-insensitive but peroxide-sensitive, and MnSODs are cyanide- and peroxide-insensitive [2]. Recombinant *T. brucei* SOD as well as Cu,ZnSOD and MnSOD prevented the autoxidation of pyrogallol (Figure 2A). In the presence of hydrogen peroxide the autoxidation of pyrogallol was not inhibited by the *T. brucei* SOD but it was reduced by 90% by MnSOD (Figure 2B). On the other hand, in the presence of cyanide the autoxidation of pyrogallol was prevented by the trypanosome enzyme but not by Cu,ZnSOD (Figure 2C). This analysis showed that the recombinant *T. brucei* enzyme is insensitive to cyanide, yet sensitive to peroxide, confirming that it is an iron-containing SOD.

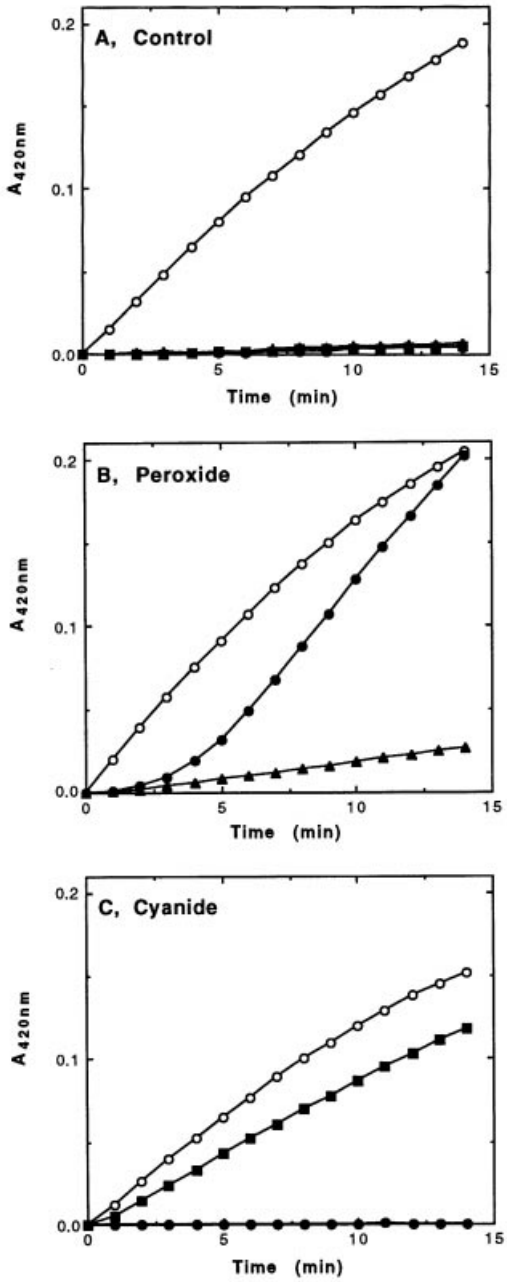

Figure 2 Activity assay of SODs

The activity of SODs was determined by the inhibition of autoxidation of 0.2 mM pyrogallol in the absence (**A**, control) or presence of 0.5 mM H_2O_2 (**B**, peroxide) or 0.5 mM KCN (**C**, cyanide). ○, Control lacking SOD; ●, 5 μg of purified recombinant *T. brucei* SOD; ■, 5 μg of Cu,ZnSOD from bovine erythrocytes; ▲, 5 μg of *E. coli* MnSOD. The autoxidation of pyrogallol was measured photometrically at 420 nm in 50 mM Tris/HCl, pH 8.0/1 mM DTPA. Note that in the presence of cyanide the autoxidation of pyrogallol is inhibited by 20% [13]. Representative experiments are shown out of two performed in each case.

Developmental regulation of SOD in the life cycle

The occurrence of SOD protein during the life cycle was studied by immunoblot analyses using the anti-peptide antibody. A protein band of 22 kDa was detected in whole-cell lysates of long slender and short stumpy bloodstream populations (Figure 3). In lysates of procyclic insect forms two protein bands, of 22 kDa and 24 kDa, were recognized by the antibody (Figure 3). The level of SOD protein was 6- and 4-fold lower in the cell-cycle-arrested short stumpy trypanosomes than in proliferating long slender and procyclic parasites, respectively, as determined by scanning densitometry of the immunoblot. The homogeneity of

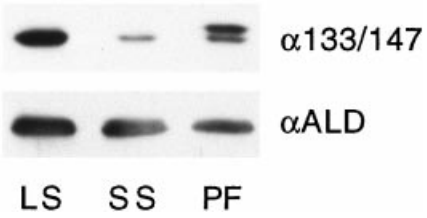

Figure 3 Occurrence of SOD during the life cycle of *T. brucei*

Long slender (LS), short stumpy (SS) and procyclic form (PF) parasites were lysed and analysed by immunoblotting using antibodies against the synthetic peptide 133/147 (α133/147; 1:700) and aldolase (αALD; 1:1000).

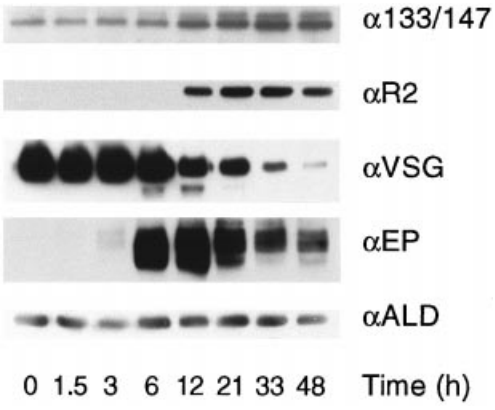

Figure 4 Occurence of SOD during the differentiation from short stumpy into procyclic forms

Short stumpy parasites were purified from the blood of infected mice and differentiation was initiated with 3 mM citrate and 3 mM *cis*-aconitate at 27 °C. At the indicated times, parasites were harvested, lysed and analysed by immunoblotting using antibodies against the synthetic peptide 133/147 (α133/147, 1:700), the small subunit of ribonucleotide reductase (αR2; 1:5000), the AnTat 1.1 variant surface glycoprotein (αVSG; 1:2000), procyclin (αEP; 1:2000) and aldolase (αALD; 1:1000).

the parasite populations was confirmed using antibodies against stage-specific proteins (results not shown, but see [14]). Immunoblot analysis with anti-aldolase antibodies confirmed that equal amounts of proteins were loaded on to the gels [14] (Figure 3).

The occurrence of SOD during the differentiation of short stumpy into procyclic forms was studied *in vitro* (Figure 4). Differentiation was induced by culturing stumpy trypanosomes at 27 °C in the presence of citrate and *cis*-aconitate [4–6]. After 12 h, the level of SOD protein started to increase and after 48 h it reached the level of established procyclic trypanosomes. Concomitant with the increase of the SOD protein, the expression of the small subunit (R2) of ribonucleotide reductase, which is down-regulated in cell-cycle-arrested short stumpy trypanosomes [14], was also increased. The high levels of SOD and R2 proteins were accompanied by proliferation of the cells (results not shown), and by loss of variant surface glycoprotein and appearance of procyclin (Figure 4), which are surface-coat proteins of the bloodstream and insect trypanosome forms, respectively.

To distinguish between transcriptional and post-transcriptional regulation, the level of SOD mRNA in the three life-cycle stages of *T. brucei* was studied by reverse transcriptase PCR. No difference in the amount of the amplified *SOD* fragment (684 bp) was observed between long slender and short stumpy

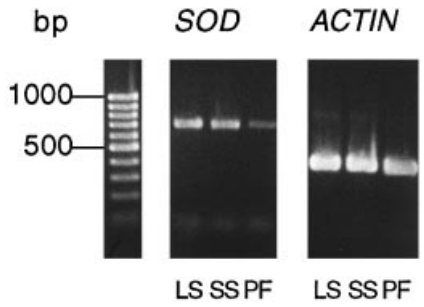

Figure 5 Transcription of the *SOD* gene during the life cycle of *T. brucei*

Total RNA was purified from long slender (LS), short stumpy (SS) and procyclic form (PF) trypanosomes and the mRNA reverse-transcribed into cDNA. Internal fragments of *SOD* (684 bp) and actin (392 bp) genes were amplified from cDNA with respective gene-specific primers. Equal amounts of the PCR mixtures were separated on a 2% agarose gel and stained with ethidium bromide.

trypanosomes whereas some differences were detected between the bloodstream stages and the procyclic insect form (Figure 5). However, the amount of amplified actin fragment, used as an internal control, was also lower in procyclic forms than in the two bloodstream stages (Figure 5). Nevertheless, this result clearly shows that the SOD mRNA is not down-regulated in the non-dividing short stumpy trypanosomes.

DISCUSSION

The SOD of *T. brucei* clearly belongs to the class of FeSODs. This is evident from its amino acid sequence homology to other FeSODs and the presence of amino acid residues that are distinctive for FeSODs. In addition, the recombinant *T. brucei* SOD has inhibitor sensitivities that are charasteristic of FeSODs. Our findings are in agreement with previous studies in which the SOD activity in crude extracts of bloodstream forms of *T. brucei* were ascribed exclusively to the iron-containing class of SOD [7].

SOD is considered the first line of defence against the toxicity of reactive oxygen intermediates by virtue of its capability to degrade $O_2^{\cdot-}$, which are generated by several oxidative enzymes and during the autoxidation of various biomolecules [2]. In *T. brucei*, the main function of SOD seems to be the elimination of $O_2^{\cdot-}$ that are formed during the generation of the tyrosyl radical in the small R2 subunit of ribonucleotide reductase [18] and which have been shown to irreversibly inactivate *E. coli* ribonucleotide reductase [19]. This suggestion is supported by the finding that both the SOD (this study) and the R2 subunit [14] are down-regulated in cell-cycle-arrested short stumpy trypanosomes. Furthermore, during the differentiation of short stumpy forms into dividing procyclic forms the level of SOD increases concomitantly with the reappearance of the R2 subunit and the proliferation of the cells.

In bloodstream forms, regulation of SOD is probably determined by a post-transcriptional mechanism since no change in SOD mRNA was observed between long slender and short stumpy trypanosomes. In procyclic insect forms, however, the expression level of SOD mRNA was lower although the SOD protein was abundant. The diminished SOD mRNA level in procyclic trypanosomes may be attributed to a cell density effect. Previously it was shown that mRNA levels are markedly reduced in late-logarithmic-phase procyclic trypanosomes [20].

In lysates of procyclic forms two SOD protein bands, of 22 kDa and 24 kDa, were detected by immunoblotting. These

two bands may represent two distinct isoenzymes or they may be partially degraded forms of one enzyme. The latter possibility is unlikely since a cocktail of proteinase inhibitors was present during the preparation of the cell lysates. In addition, during the differentiation of short stumpy into procyclic forms the 24 kDa protein band appeared when the level of the 22 kDa SOD protein started to increase. Moreover, precedents exist for FeSOD isoenzymes [21,22]. For example, two SODs, FeSODA and FeSODB, have been cloned and characterized from *T. cruzi* [21], a species that is closely related to *T. brucei*. *T. cruzi* FeSODA consists of 212 amino acids whereas *T. cruzi* FeSODB has 196 amino acids. It is reasonable to assume that the 22 kDa and 24 kDa *T. brucei* SOD protein bands represent the corresponding counterparts of the two *T. cruzi* SODs. An explanation for the occurrence of two SOD isoenzymes in the same organism may be that one enzyme is produced constitutively whereas the other is induced by external oxidative stress. For instance, in *E. coli* a FeSOD is produced constitutively under anaerobic conditions while a MnSOD is synthesized in response to the presence of oxygen and redox-active compounds [23]. Another explanation for the presence of two isoenzymes may be compartmentalization. Whereas bloodstream-form trypanosomes have an underdeveloped mitochodrion which lacks cytochromes, procyclic insect trypanosomes have an active mitochondrion performing oxidative phosphorylation. Since active mitochondria produce $O_2^{\cdot-}$ [24], the second FeSOD isoenzyme of procyclic trypanosomes may have a function within the mitochodrion like MnSOD of higher eukaryotes [25,26].

SODs of parasitic protozoa have been considered as virulence factors that protect parasites from attack of host effector cells by acting as both antioxidants and anti-inflammatory agents [21,22,27–31]. With the exception of *Acanthamoeba castelanii*, which possesses two isoforms of SOD, FeSOD and Cu,ZnSOD [32], all other protozoan parasites investigated to date only have FeSODs [21,22,27–31]. Since mammals have Cu,ZnSOD and MnSOD but no FeSOD, FeSOD may be a potential target for the design of parasite-specific chemotherapeutic agents.

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