ANTIOXIDANTS & REDOX SIGNALING
Volume 4, Number 1, 2002
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Mini Forum Review

Trypanosomal Antioxidants and Emerging Aspects of Redox Regulation in the Trypanosomatids

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ABSTRACT

***Leishmania* and *Trypanosoma* are two genera of the protozoal Order Kinetoplastida that cause widespread diseases of humans and their lifestock. The production of reactive oxygen and nitrogen intermediates by the host plays an important role in the control of infections by these organisms. Signal transduction and its redox regulation have not been studied in any depth in trypanosomatids, but homologs of the redox-sensitive signal transduction machinery of other eukaryotes have been recognized. These include homologs of activator protein-1, human apurinic endonuclease 1 (Ref-1) endonuclease, iron-responsive protein, protein kinases, and phosphatases. The detoxification of peroxide is catalyzed by a trypanothione-dependent system that has no counterpart in mammals, and thus ranks as one of the biochemical peculiarities of trypanosomatids. There is substantial evidence that trypanothione is essential for the survival of *Trypanosoma brucei* and for the virulence of *Leishmania* spp. Apart from trypanothione and its precursors, trypanosomatids also possess significant amounts of N^1-methyl-4-mercaptohistidine or ovothiol A, but its function in the trypanosomatids is not presently understood. The biosynthesis of ovothiol A in *Crithidia fasciculata* proceeds by addition of sulfur from cysteine to histidine to form 4-mercaptohistidine. S-(4′-L-Histidyl)-L-cysteine sulfoxide is the transsulfuration intermediate. 4-Mercaptohistidine is subsequently methylated with S-adenosylmethionine as the likely methyl donor.** Antioxid. Redox Signal. 4, 105–121.

INTRODUCTION

TWO GENERA OF PROTOZOA belonging to the Order Kinetoplastida, *Trypanosoma* and *Leishmania,* are pathogenic for mammals and cause widespread diseases of man and his lifestock in developing countries. The Order Kinetoplastida is thought to have diverged from the main line of evolution of the eukaryotes at least 500 million years ago, before the differentiation into the Plant and Animal Kingdoms occurred, and has since adopted an alternate lifestyle that has fascinated generations of biochemists and parasitologists.

Pathogenic trypanosomatids have digenic life cycles that alternate between their mammalian and insect hosts. At least nine different *Leishmania* species have been identified as causative agents of the cutaneous, mucocutaneous, and visceral forms of leishmaniases. *Leishmania* parasites are transmitted by female sandflies as motile, flagellated promastigotes. Once injected into the mammalian host, the promastigotes are phagocytized by macrophages and transform into nonmotile, oval amastigotes with retracted flagella. These are obligate intracellular parasites that proliferate within the macrophage phagolysozomes, but

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can also invade nonphagocytic cells (77). Both life cycle stages can be cultured axenically, and their interaction with their host cells has been intensively studied to gain an insight into the mechanisms whereby they evade the microbicidal mechanisms of the macrophage. Whereas promastigotes grow optimally at ~28°C and at neutral pH, amastigotes are adapted for survival within the phagolysozome; they multiply at 37°C and are acidophiles that use the differential in pH between their intracellular domains and the phagolysozome for the uptake of nutrients (28). These characteristics can be used to achieve an *in vitro* transformation of the organisms between the two principal life cycle stages (107, 111).

The most intensively studied members of the genus *Trypanosoma* are *Trypanosoma brucei brucei,* the causative agent of nagana in cattle, the human pathogens *Trypanosoma brucei rhodesiense* and *T. brucei gambiense,* responsible for African trypanosomiases or sleeping sickness, and *Trypanosoma cruzi,* responsible for South American trypanosomiases or Chagas' disease. Although these diseases are all insect-borne, the insect vector and mode of transmission of the *T. brucei* subspecies differ from those of *T. cruzi.* The African trypanosomes have a salivarian mode of transmission and are transferred to the host as infective metacyclic forms in the saliva of tsetse flies (*Glossina* spp.) while taking a blood meal. The mammalian life cycle stage occurs extracellularly in the bloodstream and, in the late stages of the disease, in the cerebrospinal fluid of the victim. The principal mechanism of evasion of the host immune response is by antigenic variation of the variable surface glycoprotein, which covers the surface of the metacyclic and bloodstream forms. The bloodstream form, however, seems more susceptible to oxidant stress, at least in terms of its lower content of low molecular mass thiols, than several other trypanosomatids (see Tables 1 and 2 in ref. 4 and Table 1 in ref. 25). It is perhaps as a consequence of this fact that African wildlife use the imposition of oxidant stress on invading trypanosomes as a mechanism of innate immunity against a parasitic disease that has rendered large parts of the African continent off limits for most breeds of cattle [reviewed by S.J. Black and co-workers in this issue (106)].

T. cruzi is transmitted in a stercorarian mode by reduviid bugs (*Triatoma* spp.). These insects harbor two stages of the parasite in the lumen of the gut: the epimastigotes and metacyclic trypomastigotes. In the stercorarian mode of transmission, the parasites gain entry into the host when the insect inadvertently rubs feces or urine contaminated with infective metacyclic parasites into the bite marks from which it has fed. When trypomastigotes are phagocytized by macrophages, they are able to escape from the phagolysozome into the cytoplasm using lytic enzymes that disrupt the membrane, whereas epimastigotes are unable to perform this feat and are killed. Trypomastigotes can also invade nonphagocytic cells, including muscle cells of the heart and the reticuloendothelial system. Here they transform into rounded nonmotile amastigotes with a retracted flagella and begin to multiply. Thus, the mammalian life cycle stage can exist either as replicating intracellular amastigotes or as nondividing flagellated trypomastigotes that circulate in the blood (7, 15).

As the production of reactive oxygen intermediates (ROI) is an important microbicidal strategy used by the mammalian host against invading pathogens, the localization of the parasites within the host and the biochemical mechanisms used to detoxify ROI is a fundamental aspect of the host–parasite interaction (70, 71). Trypanosomatids also undergo major morphological and biochemical transformations when they adapt to environments as diverse as the alimentary tract of the insect vector as opposed to the bloodstream or intracellular milieu of the mammalian host. These transformations rely on the sensing of external stimuli and their transduction into an appropriate response.

Although it has by now become apparent that trypanosomatids possess macromolecules that are likely to play a role in the detection and transduction of such environmental signals, this information is still very fragmentary, and details of the signal transduction pathways that must govern their complex life

cycles are quite unclear. Nonetheless, the sensing of ROI is likely to be an important component of the interaction of trypanosomatids with their environment.

Thus far, it seems that trypanosomatids have elaborated no mechanism analogous to the flavohemoglobin, present in bacteria (16, 17, 33, 52) and fungi (48), for the detoxification of reactive nitrogen intermediates (RNI). Whether this fact is of relevance in diseases that affect humans is uncertain. The role of the inducible nitric oxide synthase (iNOS) isozyme as a microbicidal mechanism in humans is controversial (24, 51, 55, 58). Although a deficiency in components of the phagocyte NADPH oxidase in humans gives rise to chronic granulomatous disease, a disorder characterized by severe and recurrent infections, a corresponding genetic disorder resulting from a deficiency in iNOS has not been identified. In line with the ability of murine macrophages to produce nitric oxide *in vitro* in response to appropriate stimuli, mice can compensate for a deficiency in components of the phagocyte oxidase by virtue of the presence of a functional iNOS, but do not survive, other than in a pyrogen-free environment, when they lack both iNOS and an essential component of the phagocyte oxidase (62). In a recent study (51), it was shown that the fatal progression of visceral leishmaniasis in hamsters parallels that observed in the case of humans, and this was ascribed to the less pronounced up-regulation of iNOS than in mice, which are able to control a similar infection principally through the production of microbicidal amounts of nitric oxide (30).

PECULIARITIES OF TRYPANOSOMAL METABOLISM

The biochemistry of the trypanosomatids differs from that of other eukaryotes in a number of respects. Probably the most unusual feature is the possession of a kinetoplast, a disk consisting of kinetoplast DNA (kDNA), which is located in the mitochondrion. kDNA is made up of two kinds of circular DNA molecules: there is a network consisting of several thousand interlocked minicircles, each 0.5–2.5 kb in size depending on the species, and 20–30 maxicircles of 20–40 kb. Maxicircles encode ribosomal RNA and the genes for some of the mitochondrial proteins, whereas minicircles encode guide RNAs that direct a remarkable process, known as RNA editing, which results in the insertion and deletion of uridine residues in the maxicircle transcripts to create functional open reading frames. Several of the enzymes involved in the replication of kDNA have been identified, but due to the complexity associated with the assembly of a structure such as the kinetoplast a detailed picture has yet to emerge (54).

The energy metabolism of trypanosomatids undergoes major adaptations between the insect and mammalian stages, yet the most exceptional aspect, which is also peculiar to the Kinetoplastida, is the localization of the first seven enzymes of the glycolytic pathway, the enzymes of fatty acid β-oxidation, two enzymes of glycerol metabolism, ether-lipid and pyrimidine biosynthesis, purine salvage, and several enzymes of the pentose phospate pathway inside a separate organelle, the glycosome (65). Peptide sequences responsible for the targeting of the requisite enzymes to the glycosome have been identified (88), as well as some of the components required for their import (40).

There are also pronounced differences between the antioxidant machinery of trypanosomatids and other eukaryotes. Trypanosomatids lack catalase and glutathione peroxidase, two key antioxidant enzymes. As much as 70% of their glutathione is converted by means of two ATP-dependent ligase reactions to the N^1,N^8-bis(glutathionyl)spermidine adduct, trypanothione (25). Trypanothione is maintained in the reduced state by a trypanothione reductase (TR), a member of the disulfide reductase family, with high sequence homology to glutathione reductase. It was known for some time that trypanosomatids can metabolize peroxide, but an enzyme analogous to glutathione peroxidase could not be isolated. The riddle was eventually solved when the presence of a trypanothione peroxidase activity, consisting of a trypanothione-dependent member of the thioredoxin

family, tryparedoxin, and a peroxiredoxin, tryparedoxin peroxidase, was demonstrated (27).

Regulation at the transcriptional level is an important component of the response to oxidative or nitrosative stimuli in most organisms. In trypanosomatids, however, the capacity for transcriptional regulation is limited. Trypanosomal genes are arranged in compact polycistronic arrays, the transcription of which is directed by a relatively small number of promoters (101). This means that differential expression of the genes is achieved mainly by posttranscriptional control mechanisms, and few transcription factors have thus far been identified. Results from the *Leishmania* genome sequencing program are consistent with these generalizations (60).

Adenylate cyclases play important roles in the regulation of the transition between different life cycle stages of trypanosomatids. Trypanosomal adenylate cyclases differ significantly in structure from the mammalian enzymes. They are also membrane proteins, and the cytoplasmic domain has a single catalytic site in which the amino acid residues required for catalysis are conserved. A single transmembranous helical region connects the cytoplasmic domain to a large extracellular domain. *T. brucei* was reported to possess >100 adenylate cyclase genes (83). The extracellular domains of these vary in sequence, but contain several conserved cysteine residues that would probably result in similar disulfide bonding and folding of the peptide chain. This arrangement bears a structural similarity to the receptor guanylyl cyclases of multicellular organisms, but there is no evidence that trypanosomatids possess heterotrimeric G proteins. Thus, the adenylate cyclase extracellular domains can potentially activate the synthesis of cyclic AMP by interaction with a variety of extracellular effector molecules. The activity of the *T. brucei* enzyme can be inhibited by treatment with the sulfhydryl modifying reagent *p*-chloromercuriphenylsulfonic acid (74). Alterations in activity of adenylate cyclase and cyclic AMP-phosphodiesterase in response to thiol reducing agents in organisms from widely different stages of evolutionary development have been reported (17, 56, 61). There are many unresolved questions concerning the function of trypanosomal adenylate cyclases, not least of which is the presence of an adenylate cyclase in the variable surface glycoprotein bloodstream form expression site as one of the ESAGs (expression site associated gene; ESAG 4). The three-dimensional structures of the catalytic domains of two GRESAG adenylate cyclases (gene related to ESAG; GRESAG 4.1.1 and GRESAG 4.3) were recently reported and show the presence of a stereospecific binding site for (2s,3s)-1,4-dimercapto-2,3-butanediol [dithiothreitol (DTT)] (12).

Trypanosomatids also share with other parasitic protozoa biochemical characteristics that differ from those of their mammalian hosts. All parasitic protozoa are deficient in the biosynthesis of purines and rely on salvage pathways. The salvage pathway enzymes, such as hypoxanthine guanine phosphoribosyl transferase and the enzymes that convert inosine monophosphate to AMP, have a relaxed substrate specificity, and this raised the possibility of introducing modified purine bases as prodrugs (46, 63, 75, 89). Some enzymes of the pyrimidine biosynthetic pathway are multifunctional in mammals, but present as individual proteins in the parasites, and conversely, dihydrofolate reductase occurs as part of a bifunctional enzyme in the parasitic protozoa, being fused with thymidylate synthase (31).

Although also not unique to the African trypanosomes, the antigenic variation observed in these organisms is perhaps the most vivid demonstration of such a phenomenon; it is of tremendous fundamental and medical importance and has been the subject of intense investigation. Several informative reviews describing progress in this area have recently appeared (14, 69, 102, 103).

REDOX SIGNALING IN THE TRYPANOSOMATIDS: SOME PIECES OF THE PUZZLE

Only fragmentary information on signal transduction in trypanosomes is presently available. A major incentive in studies of the

response of trypanosomatids to ROI and RNI had been to explore ways in which their defense mechanisms against toxic levels of these chemicals can be disrupted, as part of the search for potential chemotherapeutic targets, rather than to gain an insight into the utilization of such species, when present at nontoxic concentrations, as effector molecules.

Trypanosomatids do, however, possess homologs of proteins that play a role in redox signaling in other eukaryotes, and it is likely that these homologs are responsive to the same stimuli. Extracts of *Trypanosoma cruzi* were recently found to contain proteins that bind to activator protein-1 (AP-1) oligonucleotide recognition sequences, and the components of the AP-1 heterodimer, c-Jun and c-Fos, were identified by western blotting using antibodies to conserved regions of these proteins (23). Moreover, AP-1 binding to the recognition sequences was increased by prior exposure of *T. cruzi* epimastigotes to peroxide, in agreement with the known redox sensitivity of AP-1. Genes encoding enzymes known to be involved in regulation of the DNA binding activity of AP-1 are present in trypanosomatids. Thus, open reading frames with sequence homology to class II apurinic/apyrimidinic (AP) endonucleases have been cloned from *T. cruzi* and *L. major* and were found to complement *E. coli* mutants deficient in these DNA repair enzymes (73). Class II AP endonucleases had been shown to regulate the activity of AP-1 in a thioredoxin dependent manner (34). The eukaryotic enzyme known as HAP1 (human apurinic endonuclease 1) or Ref-1 (redox factor 1) has a 61 amino acid terminal domain absent from the bacterial homologs such as exonuclease III, and the redox-active cysteines required for interaction with a specific cysteine residue in c-Jun are located in this region (105). The protozoal enzymes have an even longer N-terminal extension than is the case for HAP1, but there is an element of uncertainty here, because the protozoal enzymes have poor homology to a region that was identified as crucial for the redox regulation of c-Jun, and in particular, a cysteine corresponding to Cys^{65} of the HAP1 protein is missing. On the other hand, there is excellent homology in the protozoal enzymes to a region around Cys^{93} of HAP1, which was proposed to provide the second cysteine required for formation of a disulfide bridge to Cys^{65}, and both protozoal enzymes have a highly homologous region (residues 189–197) that has a cysteine (Cys^{194} in the *L. major* protein), but which differs completely from the corresponding sequence of HAP1. The *Arabidopsis thaliana* AP endonuclease, which has been shown to reduce human Fos and Jun, also lacks the cysteine residue corresponding to Cys^{65} of HAP1, but has a cysteine that aligns with the Cys^{194} of *L. major* AP endonuclease (8). Only further studies could establish whether the AP endonucleases of trypanosomatids are redox-active in a manner similar to HAP1 (Ref-1) in regulating the binding of AP-1 to DNA.

A protein that interacts with the mammalian iron-responsive element (IRE) was identified in *L. tarentolae* (50). The interaction of the leishmanial iron-responsive protein (IRP) with mammalian IRE (K_d = 0.7 ± 0.3 μM) was, however, over two orders of magnitude weaker than the interaction of mammalian IRP with mammalian IRE (K_D = 5 ± 2 nM). The interaction of the *L. tarentolae* IRP with the mammalian IRE was sensitive to similar nucleotide substitutions in the IRE as was the case for mammalian IRP and was also abolished by diamide. The *L. tarentolae* IRP, however, differs from the mammalian protein in that it is inactivated by reducing agents such as mercaptoethanol, and it is not known whether *L. tarentolae* IRP possesses an iron-sulfur cluster. The cytoplasmic aconitase/IRP of mammals (11) is an enzyme with ~30% sequence identity to the mitochondrial aconitase and, like the mitochondrial enzyme, it contains a Fe_4S_4 cluster. In hydratases, including aconitase, one iron atom in the Fe_4S_4 cluster is exposed to the solvent and can accept hydroxyl groups or water as a ligand. Such clusters are sensitive to ROI and RNI and undergo facile conversion of the Fe_4S_4 cluster to a Fe_3S_4 cluster, or complete disassembly of the cluster to form IRP in the cytoplasm. The level of IRP and its binding to IREs on mRNAs are consequently determined by the disassembly and resynthesis of the active-site Fe_4S_4 cluster, disassembly being promoted by

oxidant or nitrosative stress and resynthesis requiring iron and sulfur, the latter by transsulfuration reactions from cysteine (82).

A developmentally regulated aconitase with sequence homology to the IRP of mammals was discovered in *T. brucei* (81). *T. brucei* does not seem to possess a homolog of the mitochondrial aconitase present in mammals or yeast, but only the IRP homolog, which is present in both the cytoplasm and mitochondria. Although mRNA for the *T. brucei* aconitase is relatively invariant between the different life cycle stages, posttranscriptional control of expression results in a 30-fold increase in expression of the IRP protein in procyclics, as compared with the bloodstream slender form, with the stumpy form occupying an intermediate position.

During the transformation of the bloodstream form of the organism, first to the stumpy form, which predominates when bloodstream forms reach the stationary phase of growth, and then to the procyclic life cycle stage found in the midgut of the tsetse fly, the parasites undergo an extensive remodeling of their energy metabolism. The slender replicative forms present in the blood of the mammalian host rely on glycolysis for energy production and have only a vestigial mitochondrion. Their transition to stumpy forms is regulated by density sensing, which relies on the accumulation and sensing of a low-molecular-mass stumpy induction factor of unknown structure. The binding of stumpy induction factor to an effector and the ensuing response is relayed via a cyclic AMP-dependent pathway. Stumpy forms induce the enzymes of the Krebs cycle and a functional respiratory chain, as a preadaptation for their life as procyclics in the tsetse fly. The subsequent conversion of stumpy forms to procyclics can be induced *in vitro* by a decrease in temperature to 27°C and by the addition of aconitate or citrate to the growth medium. The mechanism whereby these Krebs cycle intermediates stimulate specifically a transformation to the procyclic life cycle stage is not known. In other systems, however, the transition from anaerobic to aerobic energy metabolism is mediated by a mechanism that senses oxygen, and it seems reasonable to expect that somewhere between life as a slender bloodstream form and life as a procyclic such a mechanism would exert a measure of control. However, although the IRE binding protein of *L. tarentolae* is an interesting piece of the puzzle, experimental evidence for its physiological function is as yet lacking, and it also remains to be seen whether the cytoplasmic aconitase of *T. brucei* plays a regulatory role as an IRP.

Protein phosphorylation and dephosphorylation play critical developmental roles during the life cycle of the trypanosomatids. Whereas redox regulation of protein kinases and phosphatases in eukaryotes is well documented (1, 41), there is unfortunately no corresponding information available on the enzymes of trypanosomatids. With the *Leishmania major* genome sequencing project now about halfway complete, 44 open reading frames with sequence homology to serine/threonine kinases have been identified, with 17 of these being "probable" assignments. At this stage, seven members of this group have been assigned as mitogen-activated protein kinases (MAP kinases), a family of enzymes known to be sensitive to redox regulation. Some trypanosomatid MAP kinases have been studied in more detail (37, 108). The *T. brucei* MAP kinase, an enzyme related to the extracellular signal-regulated MAP kinases (ERK), was found to be induced by interferon-γ and seems to be essential for the survival of the parasite, because disruption of the gene on both alleles could not be achieved (37). The presence of a similar enzyme in *L. mexicana* was demonstrated, and although not required for the growth or infectivity of promastigotes, development of the intracellular amastigote life cycle stage was arrested in null mutants of *lmpk* (ERK homolog) gene (108). Reintroduction of the gene on a *Leishmania* expression vector restored the wild phenotype. Trypanosomatids also possess a number of other protein kinases, including MAP kinase kinases (47), cdc2-related kinases (56), serine/threonine kinases such as protein kinases A and C (29, 64), and tyrosine kinases (109). In other eukaryotes, the molecular properties of these enzymes are such that they are sensitive to redox regulation (80).

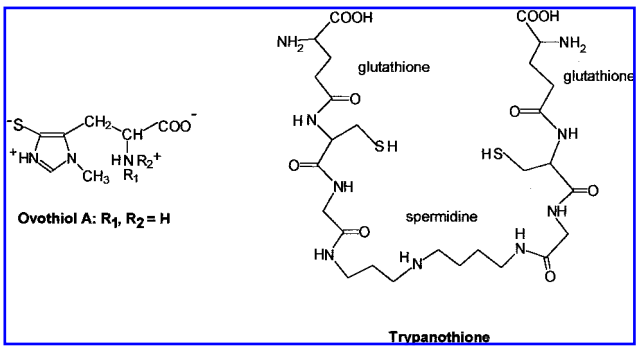

FIG. 1. Structures of major trypanosomatid thiols.

The dephosphorylation of proteins by protein tyrosyl phosphatases proceeds via phosphorylthioesters of an active-site cysteine. Oxidants inactivate these enzymes by reversible oxidation of the active-site cysteine to a sulfenic acid. Dephosphorylation by protein serine/threonine phosphatases, on the other hand, is dependent on the redox state of the metal cofactors in their active sites.

Many studies have placed an emphasis on the importance of nitric oxide production by macrophages in the killing of pathogenic trypanosomatids. The discovery of a *T. cruzi* nitric oxide synthase (NOS) related in its activity to the neuronal NOS of mammals was, however, unanticipated (68), because NOSs were previously thought to be present only in multicellular organisms. A nitric oxide-activated guanylyl cyclase activity was also demonstrated, but neither of these enzymes has to date been purified to homogeneity and characterized. A trypanosomal NOS was proposed to play a role in motility of the parasites (72) and to rescue the organisms from apoptosis induced by fresh human serum (74).

THE ANTIOXIDANT SYSTEMS OF TRYPANOSOMATIDS

Trypanosomatids possess significant amounts of four major low-molecular-mass thiols

The antioxidant enzymes of trypanosomatids differ sufficiently from those of other eukaryotes to have made this aspect of their metabolism an attractive target for the development of new antitrypanosomal drugs. This view was, moreover, strengthened by elucidation of the mechanisms whereby African wildlife acquire innate immunity against the African trypanosomes (59). Trypanosomatids were found to lack both catalase and glutathione peroxidase, and early work showed that treatment of *T. brucei*-infected mice with an irreversible inhibitor of glutathione biosynthesis, buthionine sulfoximine, resulted in lysis of the parasites (5). During attempts to isolate a glutathione reductase, it became apparent that the reduction of glutathione in cell-free fractions from the insect parasite, *Crithidia fasciculata*, was dependent on the presence of a low-molecular-mass thiol subsequently identified as N^1,N^8-bis(glutathionyl)spermidine and assigned the trivial name trypanothione (26) (Fig. 1). Trypanothione was shown to be present in all trypanosomatids and is maintained in the reduced form by an NADPH-dependent disulfide reductase closely related in sequence and structure to glutathione reductase. In fact, by site-directed mutagenesis of active-site residues, glutathione reductase and TR could, with some degree of success, be engineered to display the activities of each other (95, 94). It was subsequently found that most trypanosomatids also possess ovothiol A (N^1-methyl-4-mercaptohistidine) (92). The trivial name ovothiol derives from the fact that these small aromatic thiols (Fig. 1) were first discovered in the egg cells and ovaries of marine invertebrates, where they seem to function as bulk antioxidants (67, 99).

The trypanosomatids are, therefore, more complex in the composition of their major

low-molecular-mass thiol species than is the case for most eukaryotes and bacteria, which tend to rely on a single major low-molecular-mass thiol, such as glutathione, mycothiol (90), or coenzyme A (18), for the maintenance of thiol homeostasis and redox regulation. The presence of appreciable quantities of four thiol species (glutathione, glutathionylspermidine, trypanothione, and ovothiol A) is unparalleled in the majority of eukaryotes and bacteria and raises questions concerning the significance of variations in the relative amounts in which they occur. It should be seen as a major challenge to establish the functional significance of this compartmentalization of the major antioxidant thiol into several species. In *Crithidia fasciculata,* changes in the composition of the four major thiols occur in an almost programmed manner during growth in culture (87) (Fig. 2) and are quite reproducible. The decrease in trypanothione and corresponding increase in glutathionylspermidine in late log phase was rationalized as achieving a sequestration of spermidine in preparation for stationary phase (25). In *C. fasciculata,* this behavior could also be brought about by exposure of the cells to low pH. The pathogenic trypanosomatids, especially *Leishmania* species, however, are faced with even more drastic changes in the pH of their environment. The *in vitro* conversion of promastigotes to amastigotes is achieved by lowering the pH of the medium to 5.5 and eventually increasing the temperature to 37°C (107). Nonetheless, a similar sequestration of spermidine as a conjugate with glutathione, by elevation of the proportion of glutathionylspermidine relative to trypanothione, was not observed when *L. donovani* promastigotes were grown to stationary phase. Literature reports on the thiol content of various trypanosomatids differ widely (4, 25, 53, 107), and comparisons of the thiol content of promastigotes and amastigotes are complicated by the difference in size of amastigotes as compared with promastigotes (32) when expressed in terms of numbers of cells. From the thiol levels reported in a recent study of several trypanosomatids, it is difficult to draw any definitive conclusions concerning the roles of the different thiol species (4). Thus, ovothiol A represents 58% of the total low-molecular-mass thiol content in both the amastigote and promastigote life cycle stages of *L. major,* but in *L. donovani* promastigotes only 35% and <3% in the amastigotes. In *T. cruzi* insect stage epimastigotes, cultured in the presence of putrescine, ovothiol A amounts to only 7% of the total thiol content, compared with 28% in bloodstream trypomastigotes. It is difficult to exclude the possibility that the analytical values for the trypomastigote or amastigote stages of the parasites could be influenced by procedures required for separation of the trypanosomatids from host cells prior to the actual thiol analysis. An important conclusion from the recent study (4) seems to be that ovothiol A is more prominent in the infectious stationary phase of the *Leishmania* promastigotes, which is also considered to be more resistant to oxidant stress, despite a decline in the total thiol content of the cells. The study also indicated that trypanosomatids do not possess a NADPH-dependent ovothiol reductase activity, but that an ovothiol A-dependent nonenzymatic peroxidase activity

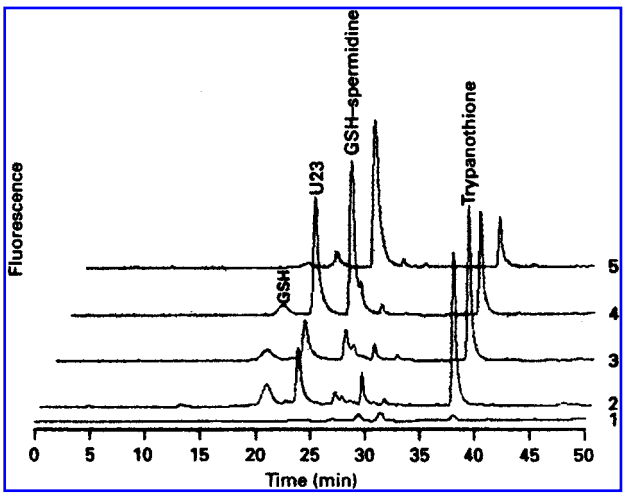

FIG. 2. Analysis of changes in the thiol composition of *Crithidia fasciculata* during growth in culture. Trace 1 represents the HPLC elution profile of an aliquot of the growth medium after derivatization with the fluorogenic thiol reagent 7-diethylamino-3-(4′-maleimidylphenyl)-4-methylcoumarin. Traces 2, 3, 4, and 5 represent thiol analysis of *C. fasciculata* harvested at absorbance readings (A_{600}) of 1.11, 1.25, 1.76 (late log phase), and 1.85 (stationary phase), respectively. U23 was subsequently identified as ovothiol A. Taken from Steenkamp (91) with permission.

can contribute significantly to the overall rate of reduction of peroxide.

As regards the reactivity of the different thiol species with ROI, other than peroxide, and with nitric oxide, there is very little information at our disposal. It is perhaps this limited perspective that makes it difficult for us to rationalize the observed multiplicity of major thiol species and the changes in their concentrations in the trypanosomatids. At this time, nothing is known about the intracellular distribution of the different thiols species, and studies on the interaction with other cellular components have focused predominantly on trypanothione. A major impediment to such studies is the limited commercial availability of trypanothione and glutathionylspermidine, and there is no commercial source of ovothiol A.

The trypanothione system

Several excellent reviews describing the role of trypanothione in trypanosomal metabolism, depicted in Fig. 3, have appeared (25, 27, 44). From these it is evident that the trypanothione system is the best understood aspect of the redox biochemistry of the trypanosomatids. Reduction of cellular thiols in Fig. 3 include thioltransferase-catalyzed reduction of glutathione disulfide (57) and maintenance of protein thiol homeostasis, possibly shared with a trypanothione-independent thioredoxin system (76). Three-dimensional structures for TR (9, 13), tryparedoxin (2) and tryparedoxin peroxidase (3) have been reported. Moreover, the results of disruption of the TR gene in *L. donovani* has confirmed its absolute necessity for the survival of the parasites in macrophages that are capable of the respiratory burst (22). Attempts to disrupt both TR alleles in *L. donovani* were unsuccessful, and consequently studies on the survival of the parasites within macrophages were undertaken with organisms that retained a single wild-type TR allele in which the TR activity, relative to an internal control of alanine aminotransferase, was reduced to 44% of that of normal cells. Such cells maintained a normal level of reduced thiols and were viable in culture.

In a different approach (97), an expression vector bearing the gene for an inactive mutant of *T. cruzi* TR, in which an essential cysteine and histidine had been replaced with an alanine and glutamate, respectively, was introduced into *L. donovani*. Although the C53A/H461Q mutants are inactive, they are

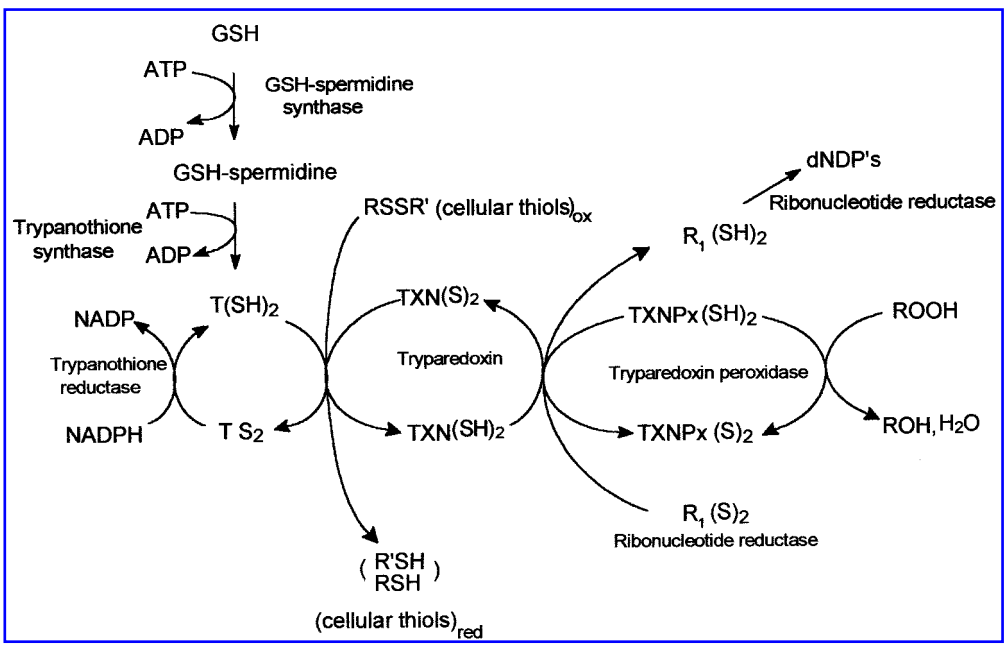

FIG. 3. Schematic presentation of the role of trypanothione in the metabolism of the trypanosomatids.

capable of forming TR dimers. In the resultant recombinants, a large proportion of the TR consisted of inactive heterodimers with the result that TR activity was reduced to ~15% of normal levels, yet the thiol pool sizes of control and recombinant cells were similar, and recombinant cells were only marginally less competent in the detoxification of peroxide. A major difference, however, was apparent in the rate and extent of the regeneration of reduced trypanothione following diamide-induced oxidative stress. Recombinant *L. donovani* was less capable than wild-type *L. donovani* of surviving in interferon-γ-activated murine macrophages. This result suggests that *L. donovani* with much suppressed active TR was more vulnerable to nitrosative stress. Moreover, the effect of nitric oxide on TR is not known, but as the enzyme has an active-site sulfhydryl group, this question also seems to be relevant. In a very informative study (45), *T. brucei* cells containing only a single TR gene under the control of a tetracycline-inducible promoter was generated. In such cells, the expression of TR could be regulated between 1% and 400% of wild-type levels. As cysteine and 2-mercaptoethanol are required in the medium for the growth of *T. brucei* bloodstream forms, cells containing <10% of wild-type levels of TR were found to contain normal levels of reduced low molecular mass thiols, including trypanothione. Nonetheless, such cells were no longer viable. This paradox implies that a depletion of TR itself, rather than of the products of its catalytic activity, was responsible for growth arrest and death of the cells, which do not seem to be merely "reduced-trypanothione auxotrophs" in which chemical reductants replace TR.

Recent studies have indicated that the resistance of trypanosomatids to oxidant stress is probably multifactorial and that the trypanothione system is unlikely to be the only factor that determines the ability of *L. donovani* to tolerate oxidant stress. Thus, *L. donovani* amastigotes can be cultured in the presence of 20 m*M* buthionine sulfoximine, conditions under which they synthesize no glutathione or trypanothione (107) as judged by the incorporation of radiolabel from [^{35}S]cysteine and the absence of detectable trypanothione or glutathione peaks when analyzed as the intensely fluorescent 7-diethylamino-3-(4′-maleimidylphenyl)-4-methylcoumarin derivatives. Buthionine sulfoximine was cytostatic for promastigotes, which remained fully viable as judged by their motility and erythrosin B exclusion. As pointed out by Krieger *et al.* (45), the ratio of reduced to oxidized trypanothione is an important unknown in all these studies, and it could be a vital parameter that compromises the survival of TR-deficient parasites. In the absence of the disulfide reductase, the redox state of trypanothione could be poised at a potential that results in apoptosis.

In a different study (53), an attempt was made to correlate trypanothione content of *Leishmania chagasi* with increased resistance to oxidant stress. Treatment of *L. chagasi* with difluoromethylornithine (DFMO), a mechanism-based inhibitor of ornithine decarboxylase, limited the amount of spermidine available for the biosynthesis of trypanothione, and consequently DFMO-treated parasites have elevated glutathione, but decreased trypanothione contents. Nonetheless, DFMO-treated parasites were more resistant to oxidant stress, as judged by their viability following exposure to peroxide. This kind of study points to the difficulty of unambiguous interpretation, because the improved ability of DFMO-treated parasites to tolerate doses of peroxide could also have been ascribed to the elevated content of glutathione, and the study ignored any potential contribution of ovothiol A. The increased resistance of stationary-phase promastigotes and amastigotes to oxidant stress did not correlate with an elevated level of antioxidant thiols, because stationary-phase promastigotes and amastigotes, especially, have significantly lower levels (4, 53) than log-phase promastigotes. Other factors that have been proposed to play a role in resistance to oxidants are heat-shock proteins and lipophosphoglycan, although recent studies have cast doubt on the role of lipophosphoglycan as a virulence determinant, at least in *Leishmania mexicana* (38).

An important function of trypanothione is to serve as a reductant for the rate-limiting

enzyme in DNA biosynthesis in the trypanosomatids. It was established in early work on the ribonucleotide reductase that the enzyme can utilize small dithiols such as lipoic acid or DTT *in vitro* as electron donors (96). As a dithiol, trypanothione can also be utilized as a direct donor of reducing equivalents for the B1 subunit of the *T. brucei* ribonucleotide reductase (20). The V_{max} for trypanothione is, however, only a sixth of that obtained with dithioerythritol as the reductant and is increased ~2.5-fold in the presence of tryparedoxin, which indicates that the reaction is catalyzed by tryparedoxin *in vivo.* The tryparedoxin-mediated formation of dGDP by ribonucleotide reductase is inhibited by ~50% when oxidized trypanothione is present in a ratio of 1:20 relative to reduced trypanothione (50 μM trypanothione$_{ox}$/1 mM trypanothione$_{red}$), which suggests a pronounced sensitivity of deoxynucleotide formation to the redox potential poised by trypanothione. The K_m for tryparedoxin in dGDP synthesis (3.7 μM) is, however, quite similar to that for *T. brucei* thioredoxin (76), which can also be utilized by ribonucleotide reductase. The relative contributions of thioredoxin and tryparedoxin toward the synthesis of the deoxynucleotides in trypanosomatids have not been investigated, and it is, therefore, uncertain to what extent trypanosomal ribonucleotide reductase is indeed subject to redox control *in vivo.*

Ovothiol A

Previous reviews on the thiol biochemistry of protozoa devoted little space to ovothiol A, because not much information had been available. The recent survey of the distribution of ovothiol A in a number of different trypanosomatids (4) must, therefore, be seen as a valuable contribution. The ovothiols have a wider distribution in nature than originally anticipated (86). In the egg cells of marine organisms, its role is most likely that of a bulk antioxidant (85). The aromaticity of the imidazole ring in the 4-mercaptohistidines confers unusual reactivity on the thiol group, the reactive form of which is the thiolate anion (35). The model compound, 1,5-dimethyl-4-mercaptoimidazole, was reported to exist as a zwitterion with pK_a values of 2.3 for the protonation of the thiolate anion and 10.3 for deprotonation at N^1 of the imidazole ring. Ovothiols, therefore, exist as the thiolate anion over a wide range of pH values, and this fact contributes to an enhanced reactivity with alkylating agents, ROI, and other free radical species when compared with glutathione, in which the thiol group has a pK_a of 8.6. As ovothiol A has a less negative redox potential than trypanothione or glutathione, it will also be maintained in the reduced form in cells. Studies *in vitro* on the redox chemistry of the ovothiols indicated that they are efficient radical scavengers (36, 49), possess glutathione peroxidase activity (10), and can afford protection against peroxynitrite-induced damage. These properties are all suggestive of a potential physiological function as a bulk antioxidant. Important in this regard is the ability of ovothiols to act as a one-electron donor with the formation of a ovothiol thiyl radical, which is unreactive toward oxygen. This means that ovothiols do not propagate free radical reactions to generate ROI. The ability of a redox-active molecule to participate in one-electron as opposed to two-electron transfers is an important attribute, which can influence its physiological role significantly. Thus, exposure of enteric bacteria to redox cyclers and superoxide results in activation of the *SoxR* transcription factor by one-electron oxidation of its iron-sulfur clusters (19), whereas activation of the bacterial response to peroxide is mediated by two-electron oxidation of thiol groups in the *oxyR* transcription factor (110). In the resting state, *oxyR* is maintained in the reduced form by glutaredoxin, whereas the electron donor for the reduction of *SoxR* has not yet been established.

The physiological functions of the ovothiols seem to be more varied than originally inferred from the high ovothiol content of marine invertebrate ova and their chemical properties. Ovothiol A was recently identified as the male pheromone of the marine polychaete *Platynereis dumerilii* (78). Ovothiols may also be involved in redox regulation in some organisms, because a study aimed at

the identification of the redox regulator of the chloroplast ATP synthase complex in the unicellular green alga, *Dunaliella salina,* led to the isolation of the disulfide form of ovothiol A as the most likely candidate (84).

The biosynthesis of ovothiol A proceeds via the intermediate formation of 4-mercaptohistidine (Fig. 4) (Vogt *et al.,* see 104). In intact *C. fasciculata* or *L. donovani* (107), the radiolabel from [^{3}H-*methyl*]methionine is recovered in ovothiol A, and therefore *S*-adenosylmethionine is the most likely methyl group donor for the final step in ovothiol A synthesis. The transsulfuration reaction uses cysteine as sulfur donor, as is the case in virtually all transsulfurations (Vogt *et al.,* unpublished observations), but follows an unusual route for which there exists only a single literature precedent (39): the conversion of hercynine to ergothionine. The formation of 4-mercaptohistidine involves the addition of sulfur to an unsaturated carbon in histidine, but with the retention of unsaturation. This is only possible if the reaction also involves the removal of a hydride equivalent from the product. In the formation of 4-mercaptohistidine, this is achieved by an unusual monooxygenase reaction, leading to the formation of *S*-(4′-L-histidyl)-L-cysteine sulfoxide (Vogt *et al.,* see 104), in which reducing equivalents for the reduction of one oxygen atom of dioxygen originates from the substrate, rather than from NAD(P)H_2. The reaction, therefore, requires O_2 and also Fe^{2+}. The lyase reaction that results in the formation of 4-mercaptohistidine also gives rise to pyruvate, from the carbon skeleton of cysteine, which would have been an expected product had the precursor been the thioether, *S*-(4′-L-histidyl)-L-cysteine, rather than its sulfoxide. This means that the lyase reaction entails redox chemistry for reduction of the sulfoxide to the oxidation level of a thioether, and effectively then both oxygen atoms in the original dioxygen, which served as substrate in the synthase reaction, must be reduced to generate mercaptohistidine as a product. A plausible alternative would have been a flavoenzyme-catalyzed removal of a hydride equivalent from the adduct of cysteine and histidine, to form *S*-(4′-L-histidyl)-L-cysteine as the transsulfuration intermediate. Consequently, the mechanistic rationale for the circuitous route via the sulfoxide is not at this time apparent. Surprisingly, cysteine was also formed in the lyase reaction, but at the expense of pyruvate as a product. When using some of the enzyme fractions to catalyze the reaction, the recovery of cysteine and 4-mercaptohistidine exceeded the amount of sulfoxide supplied as a substrate, which indicated that there must be a source of sulfur in addition to the sulfoxide. With ^{35}S-labeled sulfoxide as the substrate, the label was recovered only in mercaptohistidine and not in cysteine. The formation of cysteine was found to be dependent on an adequate supply of DTT, which was routinely included in the assay mixtures. When the DTT concentration was lowered from 1 m*M* to 72 μ*M*, no cysteine was recovered. This latter experiment was performed under anaerobic conditions to prevent atmospheric oxidation of the reaction products. Although not yet conclusive, these experiments suggest that DTT can be used to supply sulfur for the formation of cysteine, and that this affected the recovery of pyruvate and not that of mercaptohistidine. This implies that DTT can add on to the pyridoxal phosphate-bound aminoacrylate, which serves as precursor for the formation of pyruvate in β-lyase reactions, to form a thioether intermediate, which is then used to regenerate cysteine. One can speculate that trypanosomatids possess a molecule

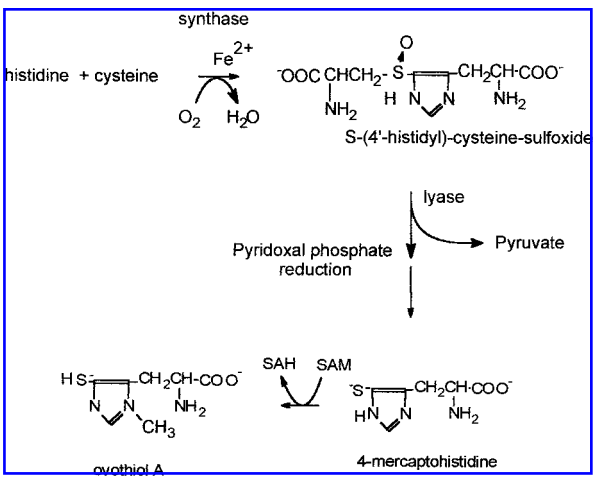

FIG. 4. The biosynthesis of ovothiol A in the trypanosomatids.

closely related to DTT that can be used as sulfur donor. In this regard, the recent discovery of a stereospecific binding site for DTT in a regulatory domain of a trypanosomal adenylate cyclase is of much interest (12). These authors inferred that DTT probably mimics a closely related molecule produced by the parasites.

Only a limited number of sulfoxides are produced as intermediary metabolites in nature, and these have proven to be physiologically active compounds. Sulfoxides possess signaling properties that are reflected in a suppression of hypercholesterolemia (43), stimulation of insulin secretion (6), and priming of neutrophils for the respiratory burst (42). It is, therefore, evident that in considering the biochemical significance of the ovothiol biosynthetic pathway, the possibility that the production of *S*-(4′-histidyl)-L-cysteine sulfoxide itself could be of consequence should not be overlooked.

CONCLUSION

There have been very significant advances during the past two decades in the characterization of the antioxidants and antioxidant enzymes of the trypanosomatids. These advances came about mainly through the discovery of trypanothione and the subsequent studies on its role in the detoxification of metals and peroxides and as an electron donor for the formation of deoxyribonucleotides. It is, however, of interest that significant amounts of the trypanothione precursors, glutathione and glutathionylspermidine, are observed, sometimes exceeding trypanothione itself in quantity. Moreover, trypanosomatids also produce variable amounts of the small aromatic thiol, ovothiol A, which is chemically distinct from the aliphatic thiols. The function of ovothiol A and the physiological significance of quantitative variations in the major thiol species in the trypanosomatids have not as yet been clarified.

Information on redox regulation in the trypanosomatids is at present very limited, but they do possess homologs of regulatory proteins that are known to be redox-sensitive, and exploratory experiments that have thus far been performed on putative transcription factors in the trypanosomatids suggest that the trypanosomal counterparts respond as in other eukaryotes.

ABBREVIATIONS

AP-1, activator protein-1; AP endonuclease, apurinic/apyrimidinic endonuclease; DFMO, difluoromethylornithine; DTT, dithiothreitol; ERK, extracellular signal-regulated kinase; ESAG, expression site associated gene; GRESAG, gene related to expression site associated gene; HAP1, human apurinic endonuclease 1; iNOS, inducible nitric oxide synthase; IRE, iron-responsive element; IRP, iron-responsive protein; kDNA, kinetoplast DNA; MAP kinase, mitogen-activated protein kinase; NOS, nitric oxide synthase; Ref-1, redox factor-1; RNI, reactive nitrogen intermediate; ROI, reactive oxygen intermediate; TR, trypanothione reductase.

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Received for publication July 7, 2001; accepted November 3, 2001.

This article has been cited by:

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