EFFECTS OF TRYPANOCIDAL DRUGS ON THE FUNCTION OF TRYPANOSOMES

ANNUAL/FINAL REPORT

GEORGE C. HILL

JUNE 30, 1983

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-81-C-1038

Colorado State University
College of Veterinary Medicine
and Biomedical Sciences
Fort Collins, Colorado 80523

Approved for public release; distribution unlimited

The findings in this report are not be construed as an official Department of the Army position unless so designated by other authorized documents.
The glycerophosphate oxidase system has been solubilized and partially purified using ubiquinol as an alternate electron donor and octyl glucoside as detergent. Some properties of the solubilized ubiquinol oxidase were investigated. 1) Once the holoenzyme had been in contact with the detergent, 2) glycerol-3-phosphate could no longer be used as substrate, 3) no matter whether the detergent was added during the assay or in the solubilization procedure. However, activity of the oxidase component could then be measured with ubiquinol analogs such as CoQ1, with its isoprenoic side chain, 2,3-dimethoxy-5-methyl-6-nonyl-1,4-benzoquinone (NB) or 2,3-dimethoxy-5-methyl-6-decyl-1, 4-benzoquinone (DB) with their saturated straight chain alkyl groups. NB was preferentially used because it is more stable than CoQ1, and can be synthesized easily. /Probably due to their poor solubility, CoQ7, 9 and 10 showed negligible activity, even after the addition of 0.1%
deoxycholate, 0.05% Triton X-100 or 0.05% Tween 20 and 80. To investigate whether the ubinuqinol oxidase activity is indeed part of the glycerol-3-phosphate oxidase complex, cross reactions with the two substrates were performed. The addition of ubinuqinol analogs to the assay of the not enzyme with glycerol-3-phosphate as substrate did not affect the rate of oxygen consumption, whereas the addition of glycerol-3-phosphate to the same assay with ubinuqinol analogs as substrate increased the rate. This indicates that the oxidation of glycerol-3-phosphate and ubinuqinol analogs are not two independent process but are reactions of the same enzyme complex. The relative insolubility and autoxidation of the artificial substrates at pH values above 8.0 prevented an accurate determination of the Km and the pH optimum. The activity of the solubilized oxidase could be stimulated 2- to 3-fold with BSA and this stimulation had the same optimum of 3.0 mg/ml as the holoenzyme. The solubilized oxidase was, as expected, fully inhibited by SHAM (0.5 mM), but not by cyanide (5 mM), whereas suramin (0.1 mM) completely inhibited the holoenzyme but did not prevent the oxidation of the artificial substrates. The oxidase activity disappeared completely after treatment with perchloric acid (10%), acetone (25%, 30 min) or trypsin (1.5 mg), and also after heating to 100 for 2 min. This strongly suggests that a protein is involved and that the oxidase activity can not be solely explained by a fatty acid peroxy radical scheme as was proposed for the cyanide-insensitive pathway of plant mitochondria.

Significant progress has been made on the identification of the apocytochrome b gene in Trypanosoma rhodesiense. The region of the maxicircle of Trypanosoma brucei kinetoplast DNA which hybridizes at low stringency with the apocytochrome b gene of Saccharomyces cerevisiae has been identified and cloned. The nucleotide sequence of a 1.7 kb segment of this region has been determined. This segment contains a single long open reading frame capable of coding for a 350 amino acid protein with substantial homology to apocytochromes b of other species. The trypanosome protein is considerably more distantly related to other apocytochromes b than they are to each other. Several short unassigned open reading frames (300 nucleotides or shorter) have been described. If polypeptide are synthesized from these regions, they are more hydrophilic than known mitochondrially coded proteins.
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Approach to the Problem</td>
<td>2</td>
</tr>
<tr>
<td>Background</td>
<td>5</td>
</tr>
<tr>
<td>Results and Discussion</td>
<td>12</td>
</tr>
<tr>
<td>A. Purification of a-glycerophosphate oxidase</td>
<td>12</td>
</tr>
<tr>
<td>B. Identification of Apocytochrome b</td>
<td>17</td>
</tr>
<tr>
<td>Conclusions</td>
<td>21</td>
</tr>
<tr>
<td>Literature Cited</td>
<td>22</td>
</tr>
</tbody>
</table>
APPROACH TO THE PROBLEM

Differentiation occurs during the life cycle of African trypanosomes. It is a complex process involving the regulation of the expression of numerous genes, the expression of which is important in controlling the life cycle of these organisms. For example, in bloodstream trypanosomes, nuclear genes for the expression of the surface coat antigen are functioning. However, after differentiation to procyclic trypomastigotes, these genes no longer are expressed. In contrast, it is very clear that while no cytochromes are present in bloodstream trypomastigotes, they are essential during respiration of procyclic trypomastigotes. As we have noted previously, our approach to the development of new trypanocides is to study two specific and related areas of the molecular biology of these organisms, these areas being the expression of nuclear and mitochondrial genes.

One potential target for these parasites is the cyanide-insensitive terminal oxidase, the a-glycerophosphate oxidase system, present in bloodstream trypomastigotes. More biochemical information is needed on the properties of this unique terminal oxidase, and if one could alter the functioning of the electron transport systems in trypanosomes, perhaps it would be possible to inhibit their life cycle. In addition, in order to identify new targets for potential trypanocides, it would be extremely helpful to learn more about the properties of the mitochondria
in trypanosomes, including the replication and transcription of kinetoplast DNA and the repression and synthesis of the mitochondrial electron transport systems.

We are also interested in the inhibition of novel enzymes on processes which are under control of the nuclear genome. These include processes such as antigenic variation or synthesis of a-glycerophosphate oxidase on cytoplasmic ribosomes. Our direction in the development of new trypanocidal drugs is to gain additional knowledge on the control mechanisms involved in the transcription of nuclear and mitochondrial genes in these organisms. In this way, we hope to identify potential targets for trypanocides that so far have not been investigated. The proper establishment of the primary mode of action of a drug requires systematic study of its effect on the various metabolic processes of the cell at the lowest concentrations that inhibit growth. Only when such a survey is complete can it be concluded confidently that a particular pathway or reaction is most sensitive to inhibition by a drug and is therefore the primary target of that drug. We are carrying out this type of systemic study with several trypanocidal drugs including berenil, suramin, antrycide and ethidium bromide.

The numerous and necessary speculative points raised in any discussion of trypanocidal drug action are now mostly capable of experimental verification with currently available techniques and data from related fields of cell and molecular biology and biochemistry. Given the necessary attention this largely
neglected but important field should yield results of considerable Value, not only for an understanding both of trypanocidal drug action and trypanosomal metabolism, but for cell biology in general. This remains the purpose of this contract. As far as trypanocidal drug design is concerned, we strongly feel that the era of intelligent empiricism is unlikely to be superseded until the balance of effort and expenditure of drug production is adjusted more favorably in the direction of research on the metabolism of trypanosomes and on the mechanisms whereby existing drugs exert their specific effects.

In developing new trypanocidal agents, we are turning our attention to investigating:

1. Application of recombinant DNA technology to a clarification of nuclear and mitochondrial gene function in trypanosomes;

2. Effects of trypanocidal drugs affecting enzyme systems in trypanosomes as well as the host tissues;

3. Comparisons of homologous enzymes in host and trypanosomes;

4. Unique cell components or metabolic pathways in trypanosomes.
African trypanosomiasis is confined to Africa by the distribution of its vectors. *T. gambiense* infection occurs over a broad belt from Senegal in the west to the great lakes Victoria, Albert, Benguelo, Tanganika in the east, extending as far south as northern Angola. *T. rhodesiense* is located in east and central Africa and is scattered over a longitudinal band from the south Sudan to Mozambique. The importance of trypanosomiasis of man and animals in Africa has been summarized recently by Kershaw (1) in the statement:

"The World Health Organization in determining the ten major health problems facing mankind places trypanosomiasis of man and his domestic animals high on the list along with malaria, cancer and heart disease."

The need for new trypanocides cannot be overemphasized. At present, chemotherapy of African trypanosomiasis is dependent on a relatively small number of synthetic drugs. Suramin and pentamidine are used for prophylaxis and treatment of early
stages of the disease in man. Organic arsenicals such as tryparsamide and melaminyl compounds are used for advanced cases when trypanosomes have invaded the central nervous system. The disease in cattle and other domestic animals is controlled by quaternary ammonium trypanocides (e.g., antrycide, ethidium, prothidium, and related drugs) and by the aromatic diamidine, berenil. As pointed out by Newton (2), resistance has been reported to occur against all these drugs and development of resistance to one compound is often accompanied by cross-resistance to another.

In human trypanosomiasis, there is still an urgent requirement for a cheap, simply administered and well-tolerated, preferably "one-shot" drug which would be as effective a prophylactic as pentamidine and active therapeutically against all stages of the infection in both Gambian and Rhodesian sleeping sickness. It should also be incapable of inducing drug resistance and active also against strains with acquired resistance to other drugs.

Possibly the two requirements of prolonged tissue retention (for prophylaxis) and ability to penetrate into the central nervous system are mutually exclusive, but with increasing knowledge of the structure and function of the so-called "blood-brain barriers," this problem should not be insuperable.

In none of the active drugs is the mode of action precisely known. An excellent review of the mode of action of trypanocidal drugs has been prepared by Williamson (3). More recent studies
have suggested that berenil and ethidium bromide form complexes with DNA. In the case of ethidium, it is clear this drug is a potent and selective inhibitor of DNA synthesis. It has been shown by several investigators that both phenanthridines and acridines combine with DNA by the heterocyclic chromophore of the drug molecules becoming inserted, or intercalated, between the adjacent base pairs in the double-stranded helix of DNA. Such intercalation is achieved by a partial uncoiling of the DNA helix which results in the base pairs above and below the bound drug molecule becoming separated by twice their normal distance (2).

More recently, it has been shown that phenanthridines also bind to supercoiled DNA of the type found in certain tumor viruses, mitochondria of many cell types and kinetoplast of trypanosomes. There is evidence that these drugs bind preferentially to such DNA in vivo and give rise to dyskinetoplastic trypanosomes (4) and "petite mutants" of yeast (5). The molecular basis of this preferential binding is not yet fully understood. The findings that have been observed could adequately explain the growth inhibitory activity of phenanthridine drugs but it remains to be established whether their primary action on bloodstream forms of trypanosomes is to inhibit DNA synthesis.

Berenil, and aromatic diamidine, has been shown to interact with DNA and can selectively block kinetoplast replication (6,7). The earliest reported effect observed of berenil is the localization in the kinetoplast of T. brucei. This has been
detected by ultraviolet microscopy within an hour of curative dose being injected intraperitoneally into infected mice and within seconds of the drug being added to an *in vitro* suspension of trypanosomes (7). Recent work has shown that berenil can form complexes with purified DNA, but in contrast to phenanthridines, there is good evidence that the complexes are not formed by intercalation (8).

A detailed examination of kinetoplast DNA isolated from berenil-treated *T. cruzi* has shown that many of the small circular DNA molecules appear as branched structures (6). These forms, which are thought to be replicative molecules, are rarely seen in control preparations, suggesting that berenil does not block the replication of kinetoplast DNA at initiation but binds preferentially to certain specific points in the circular DNA molecule. As for phenanthridines, it cannot be said what is the primary effect of berenil or other diamidines on trypanosomes.

The mode of action of suramin remains enigmatic even after more than a half century of use. *In vitro* exposure to trypanosomes to suramin at concentrations as low as 10⁻⁵M is known to reduce their infectivity whereas concentrations as high as 10⁻²M do not affect the motility or respiration of cells. As would be expected form its structure, the drug binds avidly to basic proteins and is known to inhibit many isolated enzymes (3). The most sensitive enzymes appear to be hyaluronidase, inhibited at 10⁻⁵-10⁻⁶M, fumarase, inhibited at *ca.* 10⁻⁷M, urease at pH 5 (ca. 10⁻⁴M), hexokinase (10⁻⁴-10⁻⁵M), and RNA polymerase (10⁻⁵m)
Recent studies by our laboratory supported by this contract and other investigations have demonstrated that suramin also inhibits the a-glycerophosphate oxidase in bloodstream trypanosomes in vitro. Whether this is its mode of action in vitro remains to be determined.

The ready absorption of this drug by plasma proteins may well account for the long retention time of the compound in man and animals and contribute to its value as prophylactic agent. The question of how a molecule as large as suramin enters trypanosomes is an interesting one and it seems possible that, when protein bound, suramin actively stimulates pinocytosis. As with the other drugs that we have discussed, there is evidence that suramin becomes localized in lysosomes. Again whether this is important to the trypanocidal action of the drug or whether it is a secondary phenomenon is unknown.

The Importance of African Trypanosomiasis as a Public Health Problem.

The WHO recently published information on the importance of African trypanosomiasis as a public health problem. Human trypanosomiasis, causing sleeping sickness, and animal trypanosomiasis, referred to as nagana, affecting cattle and other domesticated animals, are the two classical notorious plagues of Africa rooted in the continent since time immemorial. Sleeping sickness constitutes a permanent and serious risk to the health and well being of at least 35 million people, and animal trypanosomiasis is the main obstacle to the development of the
vast potential for livestock production in the continent. Any involvement of our military troops in areas endemic with African trypanosomiasis would be health-threatening.

Ten thousand new cases of human trypanosomiasis are known to occur yearly, but this figure does not truly reflect the importance of the disease as a public health problem. As with many tropical diseases, prevalence figures are underestimates due to failure to recognize the disease and to under-reporting. The relatively low prevalence is due to the major control efforts which have been made over the past 50 years. Without these, sleeping sickness would still be a major cause of death, as it was at the turn of the century, with great epidemics raging along the Congo river and the northern shores of Lake Victoria costing the lives of some 750,000 people.

At the present time, some 10 million people at risk are examined annually by mobile teams at an estimated cost of 5 million dollars. Expenditure for control related to animal disease. In view of the potential serious danger of sleeping sickness, national health services accord high priority to control services but efforts are frequently inadequate since sufficient resources in terms of finance, manpower and administrative facilities may not be available.

An outbreak of sleeping sickness is a dramatic event in a community since the disease causes severe symptoms due to lesions of the central nervous system and is fatal if not treated. Outbreaks may cause populations to abandon villages and fertile
farmlands, and the effect is such that even after two generations, fear of re-exposure may prevent the people for returning. Current control measures do not usually eliminate the disease; moreover, they are costly and cumbersome. With the available tools, control is a continuing effort, producing suppression rather than eradication. The experience of the past 50 years has been that whatever control efforts are interrupted, for example due to political or economic circumstances, or out of complacency, a flare-up of the disease will sooner or later occur. A recent example was the resurgence of trypanosomiasis in Zaire in the early 1960's, when after six years of interruption of surveillance, prevalence figures rose form 0.01% to 12%, and even to 18% in some areas. Severe outbreaks are now reported from Angola, Cameroon and Sudan and particularly in the Lake Victoria region of Uganda and Kenya. Some affected villages in East Africa have been estimated to have 60-80% of the villagers infected. Recent estimated from the Ivory Coast suggest 8-10% infection with T. gambiense. It is to be expected that more outbreaks will occur in coming years unless improved control measures can be found. Development of new tools is therefore a matter of urgency, not only as a means to eradicate the disease but to provide measures which are more effective and can be more widely applied than those presently available.
Results and Discussion

A. Purification of α-glycerophosphate oxidase

The bloodstream forms of African trypanosomes are completely dependent on glycolysis for their energy supply and utilize a unique shuttle, which includes a terminal oxidase, to reoxidize the glycolytically-produced NADH (13). This terminal oxidase, which is located in the mitochondrial membrane (14), is cytochrome independent, not inhibited by classical inhibitors of the mammalian respiratory chain and not coupled to ATP production. This enzyme complex consists of 2 components: a flavin-linked glycerol-3-phosphate dehydrogenase (EC 1.1.99.5) and the oxidase part which are probably linked via ubiquinol (15).

The holoenzyme complex has been partially purified and characterized (16). The oxidase component, however, deserves further investigation as it is the enzyme which is absent in the mammalian host, and is specifically inhibited by salicylhydroxamic acid (SHAM).

Trypanosomes were isolated from blood of male Wistar rats, infected with Trypanosoma brucei brucei EATRO 427, clone 117. A buffy coat was prepared, generously separated and centrifuged for 5 min at 48,000 g in a 7-ml tube (Sorvall SS34 rotor) in 45% (v/v) Percoll. After swelling in Tris (3.3 mM), EDTA (0.7 mM), 2-mercaptoethanol (1.7 mM) and bovine serum albumin (BSA) (0.5%) (pH 8.0), the isolated trypanosomes were lysed by passage twice through a 27-G needle (100 psi). The 20-min, 48,000-g pellet was
treated for 30 min at 0 C with DNAse (160 Kunitz units in 5 ml DNAse buffer) and recentrifuged. The pellet was homogenized, 100 mg octylglucoside was added in 0.5 ml of distilled water and left in ice for 30 min. After centrifugation (20 min; 48,000 g) the supernatant containing the solubilized oxidase was recovered. Enzyme activities of the holoenzyme and the oxidase component were measured with a Clark-type electrode in 1.5ml 40 mM Tris (pH 8.0) containing 3 mg BSA per ml with 10 mM glycerol-3-phosphate or 0.6mM of a ubiquinol analog as substrate.

The above solubilization procedure is the result of several investigations. Both octylglucoside and sodium deoxycholate proved to be effective in solubilizing the oxidase, whereupon octylglucoside was preferred due to ease of removal by dialysis. The optimal concentrations of detergent for approximately 20 mg protein in 5 ml were 2% for octylglucoside and 1% for deoxycholate which resulted in 30-60% recovery of the activity in the supernatant. Solubilization did not result in an increase in specific activity of the preparation, which was form the start about 50 nmoles of oxygen per min per mg protein with the artificial substrates. With glycerol-3-phosphate as a substrate the activity of the crude homogenate after lysis of the trypanosomes was about 2 to 3 fold higher, which is still much lower than the reported values of crude and partially purified particulate preparations (17). The presence of EDTA and 2 mercaptoethanol during the entire procedure increased the specific activity 20% and the addition of NaCl in the last step.
increased the yield 100%. The DNase step made the pellet less sticky and, therefore, more manageable afterwards and remained in the procedure although it only slightly increased the yield. The solubilized oxidase was very unstable after the entire procedure as storage overnight at 4°C resulted in the loss of approximately 80% of the activity.

Some properties of the solubilized ubiquinol oxidase were investigated. Glycerol-3-phosphate could no longer be used as substrate, once the holoenzyme had been in contact with the detergent, no matter whether the detergent was added during the assay or in the solubilization procedure. However, activity of the oxidase component could then be measured with ubiquinol analogs such as CoQ 1, with its isoprenoic side chain, 2,3-dimethoxy-5-methyl-6-nonyl-1,4-benzoquinone (NB) or 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone (DB) with their saturated straight chain alkyl groups. NB was preferentially used because it is more stable than CoQ 1, and can be synthesized easily. Probably due to their poor solubility, CoQ 7, 9 and 10 showed negligible activity, even after the addition of 0.1 deoxycholate, 0.05% Triton X-100 or 0.05% Tween 20 and 80. To investigate whether the ubiquinol oxidase activity is indeed part of the glycerol-3-phosphate oxidase complex, cross reactions with the two substrates were performed. The addition of ubiquinol analogs to the assay of the not solubilized enzyme with glycerol-3-phosphate as substrate did not affect the rate of oxygen consumption, whereas the addition of glycerol-3-phosphate to the
same assay ubiquinol analogs as substrate increased the rate. This indicates that the oxidation of glycerol-3-phosphate and ubiquinol analogs are not two independent process but are reactions of the same enzyme complex. The relative insolubility and autoxidation of the artificial substrates at pH values above 8.0 prevented an accurate determination of the Km and the pH optimum. The activity of the solubilized oxidase could be stimulated 2- to 3-fold with BSA and this stimulation had the same optimum of 3.0 mg/ml as the holoenzyme. The solubilized oxidase was, as expected, fully inhibited by SHAM (0.5 mM), but not by cyanide (5 mM), whereas suramin (0.1 mM) completely inhibited the holoenzyme but did not prevent the oxidation of the artifical substrates. The oxidase activity disappeared completely after treatment with perchloric acid (10%), acetone (25%, 30 min) or trypsin (1.5 mg), and also after heating to 100 for 2 min. This strongly suggests that a protein is involved, and that the oxidase activity can not be solely explained by a fatty acid peroxy radical scheme as was proposed for the cyanide-insensitive pathway of plant mitochondria.

In an attempt to purify the isolated oxidase, an affinity-chromatography column was used. 4-Amino-2-hydroxyl benzoic acid was coupled to Affigel-10 (Biorad) and thereafter modified to yield bound SHAM (19). A few percent of the total protein content of the dialyzed supernatant was bound to the column and could be eluted with borate. This approach looked promising although no oxidase activity could be detected in the retarded
protein fractions, probably due to the already mentioned instability of the oxidase.

As shown, the oxidase component of the glycerol-3-phosphate oxidase complex of trypanosomes can be solubilized and studied with artificial substrates. Therefore, a more systematic complex would be ubiquinol: oxygen oxidoreductase (EC number unclassified). Its instability has so far precluded a further purification and thorough study of the intriguing properties of this important enzyme. This research was recently published (20).

In order to identify the role of coenzyme Q in T. brucei and T. gambiense, efforts were taken in collaboration with Dr. Karl Folkers at the University of Texas at Austin in order to identify the ubiquinone present in these organisms. T. brucei LUMP 1026 procyclic trypomastigotes, T. brucei EATRO 110 bloodstream trypomastigotes and T. gambiense TRT -8 procyclic and bloodstream trypomastigotes were analyzed and CoQ 9 was identified. This research was reported in a published paper (21).
B. Identification of Apocytochrome b

Significant progress has been made on the identification of the apocytochrome b gene in Trypanosoma rhodesiense. The region of the maxicircle of Trypanosoma brucei kinetoplast DNA which hybridizes at low stringency with the apocytochrome b gene of Saccharomyces cerevisiae has been identified and cloned. The nucleotide sequence of a 1.7 kb segment of this region has been determined (22). This segment contains a single long open reading frame capable of coding for a 350 amino acid protein with substantial homology to apocytochromes b of other species. The trypanosome protein is considerably more distantly related to other apocytochromes b than they are to each other. Several short unassigned open reading frames (300 nucleotides or shorter) also are described. If polypeptide are synthesized from these regions, they are more hydrophilic than known mitochondrionally coded proteins.

Apocytochrome b is the subunit of the reduced coenzyme Q-cytochrome c reductase complex coded for by mitochondrial DNA and synthesized on mitochondrial ribosomes. The "short" form of the gene for apocytochrome b in S. cerevisiae consists of three exons and two introns containing 1155 and 2150 nucleotides, respectively. Most of this yeast gene has been cloned as a 3.5 kbp Eco RI restriction endonuclease fragment in pMB9 and is
denoted pBLT727 R6. The region of the kinetoplast DNA (kDNA) maxicircle putatively encoding apocytochrome \( b \) was first identified by hybridization at low stringency with the yeast gene carried by pBLT727 R6. A single restriction endonuclease fragment of the maxicircle hybridized with the heterologous mitochondrial DNA probe. This fragment of 3.2 kbp is bounded by Eco RI and Hind III restriction sites.

The 3.2 kbp Eco RI- Hind III maxicircle fragment was cloned by standard techniques into pBR325 which had been digested with Eco RI and Hind III. Colonies of the appropriate antibiotic sensitivity (ampicillin resistant, chloramphenicol-sensitive) were screened for the presence of inserts of approximately 3.0 kbp DNA; plasmids carrying inserts of the desired size were further characterized by Southern hybridization with the yeast probe. The DNA from one of the positive clones, pTBK102, was mapped in detail. The apocytochrome \( b \) probe predominantly hybridized to the central region of the cloned maxicircle fragment which is bounded by Hind III and Eco I restriction endonuclease sites.

The sequence for 1.7 kb of the Eco RI Hind III maxicircle segment has been determined. If TGA is translated as sense (tryptophan), one long open reading frame of 1089 nucleotides is present of 1089 nucleotides is present (position -39 to+1050). This open reading frame exhibits less than 50% homology at the nucleotide level with apocytochrome \( b \) gene of \textit{S. cerevisiae}. Like yeast, the \textit{T. brucei} sequence is particularly AT-rich. The AT
content of the long open reading frame is 77%, compared with 73% for the yeast apocytochrome $b$ gene.

The *T. brucei* sequence is capable of coding for a protein of 350 amino acids beginning with methionine. The apocytochrome $b$ character of this protein is unmistakable when it is compared with the amino acid sequences of other apocytochromes $b$. The maxicircle encoded protein is slightly shorter than other apocytochromes $b$ at both the amino and carboxyl termini. There is 22% homology between the amino acid sequence of *S. cerevisiae* apocytochrome $b$ and the deduced *T. brucei* sequence. In spite of major differences in the organization, coding, and mode of expression of their genes, the protein sequences of apocytochromes $b$ from fungal, bovine, and human mitochondria are highly conserved. The apocytochrome $b$ of *S. cerevisiae*, for example, has 50% homology with the apocytochrome $b$ of *B. taurus* and it has 60% homology with the *A. nidulans* protein. The *T. brucei* apocytochrome $b$ sequence, therefore, is much more distantly related. Many of the amino acid differences present in the *T. brucei* sequence, however, conserve the chemical character of the other polypeptide. About half of the substitutions are arg---lys, glu---asp, phe---tyr, and substitutions within the ile-leu-val-ala group. The *T. brucei* sequence also permits some conservation of the secondary structure of other apocytochromes $b$. Proline, which produces bends in a polypeptide backbone, occurs six times. In five of these six positions, proline is invariant in the bovine, human, and fungal sequences.
In one case, the agreement is with all but the yeast sequence, which has a valine at that position, our of the six prolines occur in regions where the adjacent residues are also highly conserved. Conservation also is evident in the four histidine residues thought to bind the prosthetic groups o apocytochrome b (32,33).

Taken together, these results suggest that the region of the maxicircle sequenced in these studies contains DNA sequences coding for apocytochrome b. These results were published in Molecular and Biochemical Parasitology (22).
CONCLUSIONS

(1) The apocytochrome b gene from *Trypanosoma brucei brucei* maxicircle DNA has been cloned and sequenced;

(2) The ubiquinol oxidase component of the a-glycerophosphate oxidase system has been solubilized and partially purified.

Thus, experiments are now planned to characterize gene expression in mitochondria, and further purify the ubiquinol oxidase.
LITERATURE CITED


