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Trypanosome alternative oxidase: from molecule to function

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Trypanosome alternative oxidase (TAO) is the cytochrome-independent terminal oxidase of the mitochondrial electron transport chain. TAO is a diiron protein that transfers electrons from ubiquinol to oxygen, reducing the oxygen to water. The mammalian bloodstream forms of *Trypanosoma brucei* depend solely on TAO for respiration. The inhibition of TAO by salicylhydroxamic acid (SHAM) or ascofuranone is trypanocidal. TAO is present at a reduced level in the procyclic form of *T. brucei*, where it is engaged in respiration and is also needed for developmental processes. Alternative oxidases similar to TAO have been found in a wide variety of organisms but not in mammals, thus rendering TAO an important chemotherapeutic target for African trypanosomiasis.

Introduction

There are two terminal oxidases in the mitochondrial electron transport chain. One is the usual cytochrome oxidase (COX), which is a cyanide-sensitive oxidase, and the other is the cytochrome-independent, salicylhydroxamic acid (SHAM)-sensitive alternative oxidase (AOX) [1–3]. COX is a multisubunit protein complex, whereas all the catalytic activity of AOX is contained in a single protein [1–3]. The AOXs were identified in fungi and in several thermogenic plants initially, and then in African trypanosomes such as *Trypanosoma brucei* [2–4]. Electron transport through AOX produces heat instead of ATP. In plants and fungi, AOX is involved in respiration when the normal cytochrome mediated electron transport pathway is blocked by specific inhibitors or saturated by electrons. The bloodstream forms of the African trypanosomes are unique in that they have only the AOX, which is known as trypanosome alternative oxidase (TAO) [5]. It has long been known that a similar terminal oxidase is absent from the mammalian host of trypanosomes; thus, TAO is a potential target for chemotherapy. However, it has been controversial whether TAO is essential for the survival of the bloodstream forms. Recent findings highlighted several new aspects of the structure and function of TAO, further validating TAO as an ideal chemotherapeutic target for trypanosomiasis. Here, we summarize the initial discoveries of TAO in the trypanosome system, including its molecular and biochemical properties, regulation of its expression in the parasite life cycle, and its role as a potential chemotherapeutic target.

Historical background

Identifying molecular and biochemical differences between parasitic pathogens and their hosts is crucial for developing novel chemotherapies for parasitic infections. Several such differential metabolic targets were discovered in *T. brucei*, a group of hemoflagellated parasitic protozoa that are the causative agent of African sleeping sickness [6]. The forms of the parasite residing in mammalian blood do not have any detectable level of cytochromes, yet they respire at a high rate [4,7]. The enzyme system responsible for this respiration was discovered in 1960 as the glycerol-3-phosphate oxidase system (GPO) [4]. GPO was found to consist of at least two enzymes: glycerol-3-phosphate dehydrogenase and glycerol-3-phosphate oxidase [4,7]. α-Glycerol-3-phosphate is the substrate for the glycerolphosphate dehydrogenase component, which transfers electrons to the oxidase component and subsequently to oxygen. Earlier studies revealed that GPO is present in a particulate fraction of the trypanosomes that consists of some type of microbody [8]. Opperdoes *et al.* [9] demonstrated for the first time, using isopycnic sucrose gradient centrifugation, that GPO is located in the same fraction as all other mitochondrial enzymes, such as isocitrate dehydrogenase, malate dehydrogenase and oligomycin-sensitive ATPase [9]. The holoenzyme complex was partially purified from *T. brucei* by Fairlamb and Bowman [10], who showed that FAD is the cofactor for the glycerol-3-phosphate dehydrogenase component. Subsequently, Clarkson *et al.* [5] demonstrated that the dehydrogenase component of the GPO system is linked to the terminal oxidase through the coenzyme Q9. Thus, the oxidase component of GPO is a ubiquinol oxidase and transfers electrons to molecular oxygen [4,5]. The electron transfer through this terminal oxidase is not coupled with proton translocation and ATP production in the mitochondria [5]. The specific inhibitors of GPO are salicylhydroxamic acid (SHAM) or benzohydroxamic acid, and ascofuranone [5,11,12]. AOXs that have similar characteristics to those of GPO have been found in many higher plants and fungi [13,14]. Thus, from these similarities, and because ubiquinol not α-glycerol-3-phosphate is the substrate, the trypanosome GPO was renamed trypanosome alternative oxidase (TAO) by Clarkson's group [5]; this terminology has been accepted by others.

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Molecular and biochemical properties of TAO

TAO was identified as a 33 kDa mitochondrial protein by George Hill's laboratory in 1995 [15], using a monoclonal antibody developed against the *Sauromatum guttatum* (voodoo lily) AOX. The size of the protein is comparable to the size of the AOXs identified in plants and fungi, which range from 32 to 38 kDa [13–16]. The native protein was difficult to purify to homogeneity [15,17], which delayed its further characterization. Later, a monoclonal antibody was developed using the purified denatured protein from the mitochondria of the bloodstream forms by Chaudhuri *et al.* [16].

The cDNA for TAO was initially cloned from *T. brucei* EATRO 110 by PCR amplification using degenerate primers raised against the conserved regions found in the plant and fungal AOXs [18]. Later, it was cloned from *T. brucei* TC221 and from *T. vivax* [19,20]. TAO has also been identified in the genome of *T. rhodesiense* and cloned from different strains of *T. congolense* [21]. Because of a sequencing error, some regions near the carboxy-terminal showed significant sequence dissimilarities between *T. brucei* strains EATRO 110 and TC221 [19], but it was subsequently shown that these two strains share 100% sequence identity for TAO. The amino acid sequence of *T. vivax* TAO showed 76% identity with that from *T. brucei*. Interestingly, another TAO-like sequence (Tb10.6k15.0550, termed TbAOX2 here to distinguish it from TAO) has been identified in the *T. brucei* genome database (Gene DB) (www.genedb.org) [22]. The predicted protein sequence of this gene does not have the characteristic iron-binding motifs, and showed only 19% identity and 18% similarity with that of TAO. It is unclear whether this gene is expressed or the protein has any biological activity. There are no homologs for TAO in similar trypanosomatids such as *Leishmania* or *T. cruzi*. However, the homologs for TbAOX2 are present in both of these parasites (Figure 1) [22].

Unlike other respiratory complexes, TAO contains all its catalytic activity in a single polypeptide [16]. Thus, TAO was able to complement the heme mutant strains of *Escherichia coli* GE1387 and SASX41B by conferring on them the SHAM-sensitive alternative pathway of respiration (Table 1) [16,18]. This complementation system became very useful for the structure–function analysis of TAO.

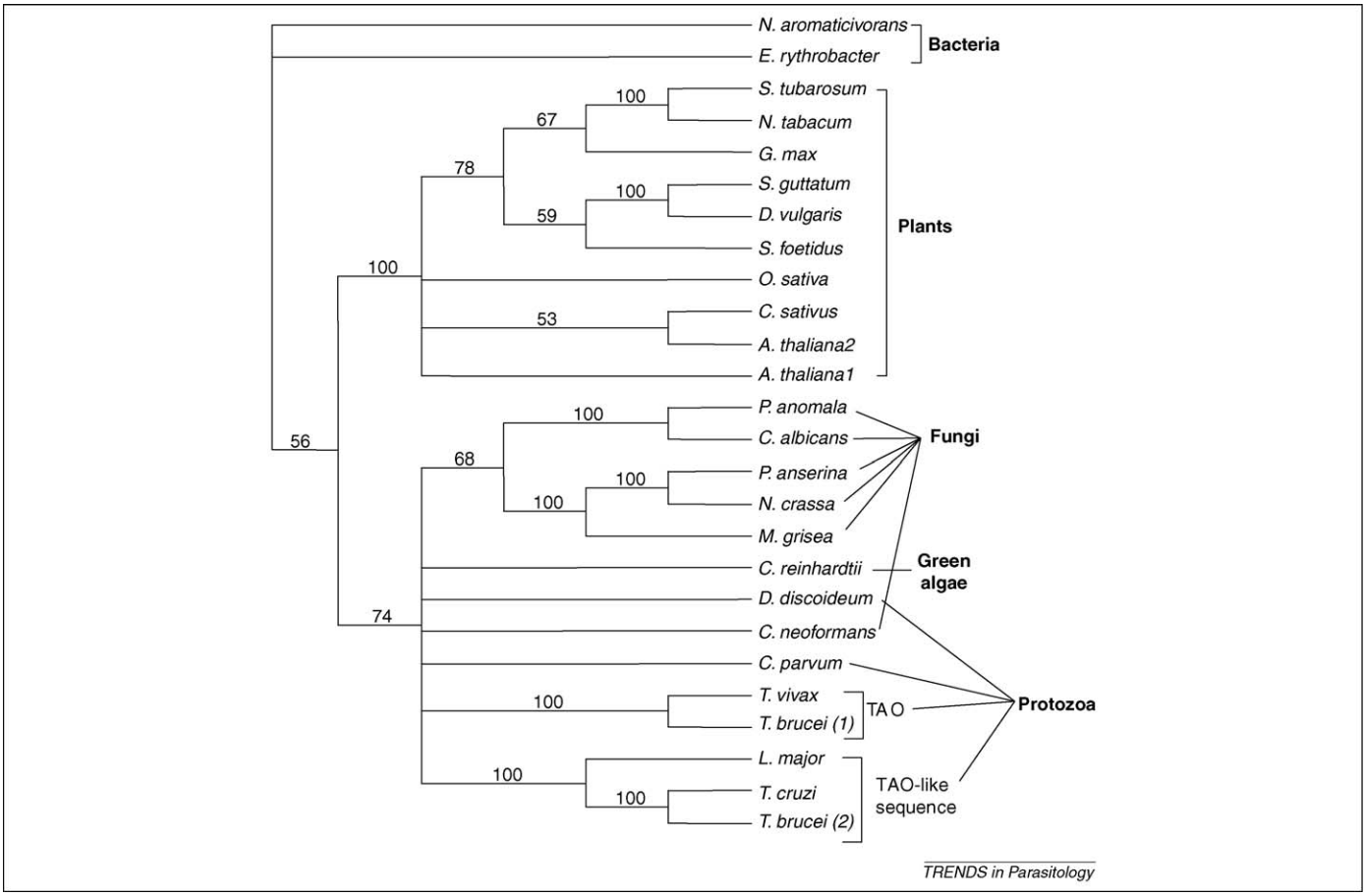

Figure 1. The phylogenetic relationship between alternative oxidases from bacteria, plants, fungi, protozoa and green algae. Different AOX sequences were retrieved from GenBank and aligned using Clustal as implemented in the MacVector program [58]. This is an unrooted 50% majority rule consensus tree (cladogram) that was generated from a bootstrapped analysis on p-distances using the neighbor-joining method of phylogenetic reconstruction, as implemented in PAUP* 4.0610 [59]. Numbers indicate bootstrap proportions supporting each node. The Accession number of the sequences used for these studies are: *Novospingobium aromaticivorans* (ABD26016), *Erythrobacter sp.* (ZP01041525), *Solanum tubarosum* (BAE92716), *Nicitiana tabacum* (CAA56163), *Glycin max* (CAA48653), *Sauromattam guttatum* (CAA78823), *Desulfovibrio vulgaris* (BAD51465), *Symplocarpus foetidus* (BAD83866), *Oryza sativa* (BAA86963), *Cucumis sativus* (AAP35170), *Arabidopsis thaliana* AOX2 (NP564395), *Arabidopsis thaliana* AOX1 (AAB49302), *Pichia anomala* (BAA90763), *Candida albicans* (AAC98914), *Podospora anserina* (CAC27396), *Neurospora crassa* (AAN39882), *Magnaporthe grisea* (BAA34672), *Chlamidomonus reinhardtii* (AAC05743), *Dictylostelium discoideum* (BAB82989), *Cryptococcus neoformans* (AAM22475), *Cryptosporidium parvum* (BAD06177), *Trypanosoma vivax* (BAD11307), *Trypanosoma brucei* (1) TAO (Q8WPA4), *Leishmania major* (LmjF36.4380), *Trypanosoma cruzi* (TC00.1047053504147.180), *Trypanosoma brucei* (2) (Tb10.6k15.0550). The last three sequences are referred as TAO-like sequences because of a lack of characteristic motifs.

Table 1. The molecular and biochemical properties of TAO[a]

Characteristics of TAO	References
TAO is a cytochrome-independent ubiquinol oxidase that is sensitive to SHAM and ascofuranone	[4,5,11,12]
TAO is located in mitochondria	[9,15,16]
TAO showed significant similarities with other AOXs found primarily in plants and fungi	[5,15,16]
TAO has two predicted hydrophobic stretches centrally located in the protein and it also has two iron binding motifs (ExxH), properties that are similar to other diiron proteins	[18,29]
TAO could complement the heme-deficient mutants of *E. coli* by conferring on them SHAM-sensitive respiration	[16,18,29]
TAO expression is developmentally regulated	[15,34]
There is no counterpart of TAO in the mammalian system	[11,16,19,43,55]

[a]Abbreviation: SHAM, salicylhydroxamic acid.

The TAO protein sequence showed 20–30% overall identity with other AOXs from different species. AOX genes have been identified in more than 100 different species of fungi, plants and protozoa. Recent evidence showed that AOX homologs are even present in α-proteobacteria such as *Novosphingobium aromaticivorans* and in several cyanobacteria [23]. A sequence homology search also revealed that the *Arabidopsis thaliana IMMUTANS* gene that encodes the plastid terminal oxidase (Ptox) belongs to the same family as mitochondrial AOXs [24]. Thus, mitochondrial AOX or plastid Ptox possibly originated from the α-proteobacterial and cyanobacterial AOXs, respectively. AOXs have also been found in some animal systems, including the phyla Mollusca, Nematoda and in some species of Chordata, such as the sea squirt *Ciona intestinalis* [25]. However, it is not found in the mammalian system. Recently it has been shown that the expression of the *Ciona intestinalis* AOX conferred cyanide-resistant respiration in cultured human cells, indicating that the heterologous enzyme is functional in a mammalian cell system [26].

Phylogenetic analysis of AOXs from different species revealed that TAO and AOXs from other protozoa are more closely related to fungal AOXs than to those of plants and bacteria (Figure 1). This is supported by the high bootstrap values in our analysis. Because we do not know *a priori* which taxon was the ancestral source of these genes, we could not root it. Thus, its topology can only be interpreted as indicating patterns of relationships not of historical directionality.

All AOXs have two conserved hydrophobic stretches and two to three iron binding ExxH motifs [27,28]. Initially it was thought that the two hydrophobic stretches were membrane-spanning α helices and thus AOXs were integral membrane proteins with both the amino and carboxyl termini located on the matrix side [27]. In 1995, Siedow *et al.* [27] first proposed a model postulating that the active site of this enzyme consists of a non-heme diiron center, which is located at the carboxy-terminal hydrophilic region of the protein. Anderson and Nordlund [28] revised this model, which depicted AOX as an interfacial inner membrane diiron protein. The revised model consists of four α helices with two highly conserved ExxH motifs on helices 2 and 4, respectively. One of the two conserved hydrophobic regions is located between helices 1 and 2, and the other is located between helices 2 and 3 (Figure 2). The length and the arrangement of the helices in this new model fit more closely with the R2-type diiron protein family [28].

TAO has two conserved ExxH motifs at residues 162–165 and 266–269 and it also has two hydrophobic stretches, similar to other AOXs [16]. Thus, TAO fits well with the second model (Figure 2). Furthermore, site-directed mutagenesis provided evidence that H165 and H269, the two proposed ligands within the two conserved ExxH motifs, are crucial for TAO activity [29]. It was also observed that mutating two glutamic acid residues E214 and E215, which are located on the conserved LEEE region in the third helix (Figure 2), abolished TAO activity [16,29]. Mutating the corresponding amino acid E215 in AOX from

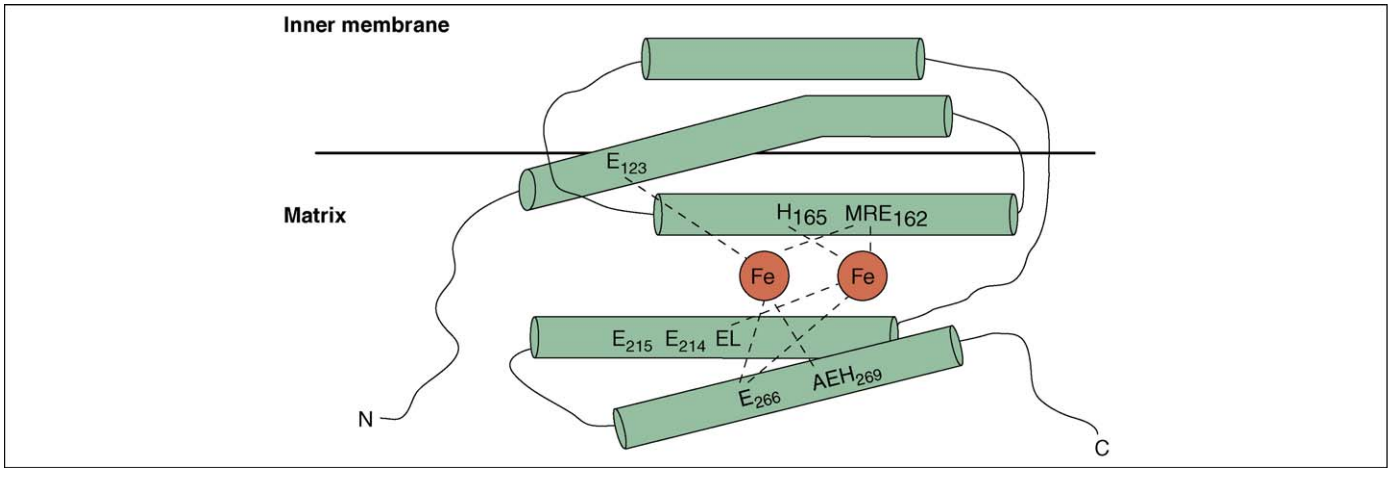

Figure 2. A schematic structure of TAO based on the model proposed by Andersson and Nordlund [28] and revised by Berthold *et al.* [31]. Four α helices containing the amino acid residues that ligand with two iron atoms are shown. E162 and E266 are two bidentate ligands within two ExxH motifs. E123, E213, H165 and H269 are monodentate ligands. The two iron atoms are further connected by two hydroxo bridges (not shown). Single-letter amino-acid code is used. The figure is modified from Affourtit, C. *et al.* [13].

S. guttatum also showed similar results [30]. From the alignment of the amino acid sequences from plant AOXs and that from Ptox, Berthold *et al.* [31] suggested that the two glutamic acid residues E214 and E215 are not involved in iron binding. Thus, the essentiality of these two residues might be because of other crucial functions. In fact, the conserved LEEE region is very close to the site of the proposed ubiquinol binding [31]; any mutation in this region might alter the conformation of the protein, which could be detrimental to the enzyme activity.

Several reports have shown that iron is needed for the activity of TAO and other AOXs [10,29,32]. Further direct evidence for the presence of diiron in AOX came from the detection of a characteristic electron paramagnetic resonance (EPR) signal for this protein by Berthold *et al.* [33]. Diiron carboxylate proteins are EPR silent because two iron atoms are anti-ferromagnetically coupled either in the reduced or in the oxidized state of the enzyme. Berthold *et al.* [33] identified a distinct EPR signal for the mixed valent diiron center as a reaction intermediate in the membrane preparation isolated from *E. coli* that overexpressed the *Arabidopsis* AOX; the signal was absent from a similar preparation from a strain that overexpressed a catalytically inactive AOX [33]. These results suggest that AOXs are diiron proteins that transfer four electrons from two molecules of ubiquinol to reduce one molecule of oxygen to water. TAO, which has similar molecular and biochemical properties to those of AOXs, is thus also a diiron protein (Table 1). Further mutational studies and X-ray crystallographic analysis are necessary to reveal more characteristics of TAO and other AOXs.

TAO gene expression is developmentally regulated in the *T. brucei* life cycle. In the bloodstream form, TAO activity and the steady-state level of TAO transcript are about fivefold greater and the TAO protein level is ~100-fold greater than in the procyclic form [34]. Like many other trypanosomatid proteins, TAO expression is regulated post-transcriptionally. It has been shown that the half-life of the TAO transcript is more than threefold decreased in the procyclic form compared with the bloodstream form; labile protein factor(s) are responsible for the degradation of TAO RNA in the procyclic form [34]. TAO is also the only terminal oxidase in the stumpy bloodstream forms [35] that are found in the later stage of the infection process. It has been reported that TAO activity is increased upon differentiation of the procyclic to the metacyclic form [36], the preadaptive form to transfer to the mammalian hosts. Unlike plant AOXs, TAO activity is not allosterically

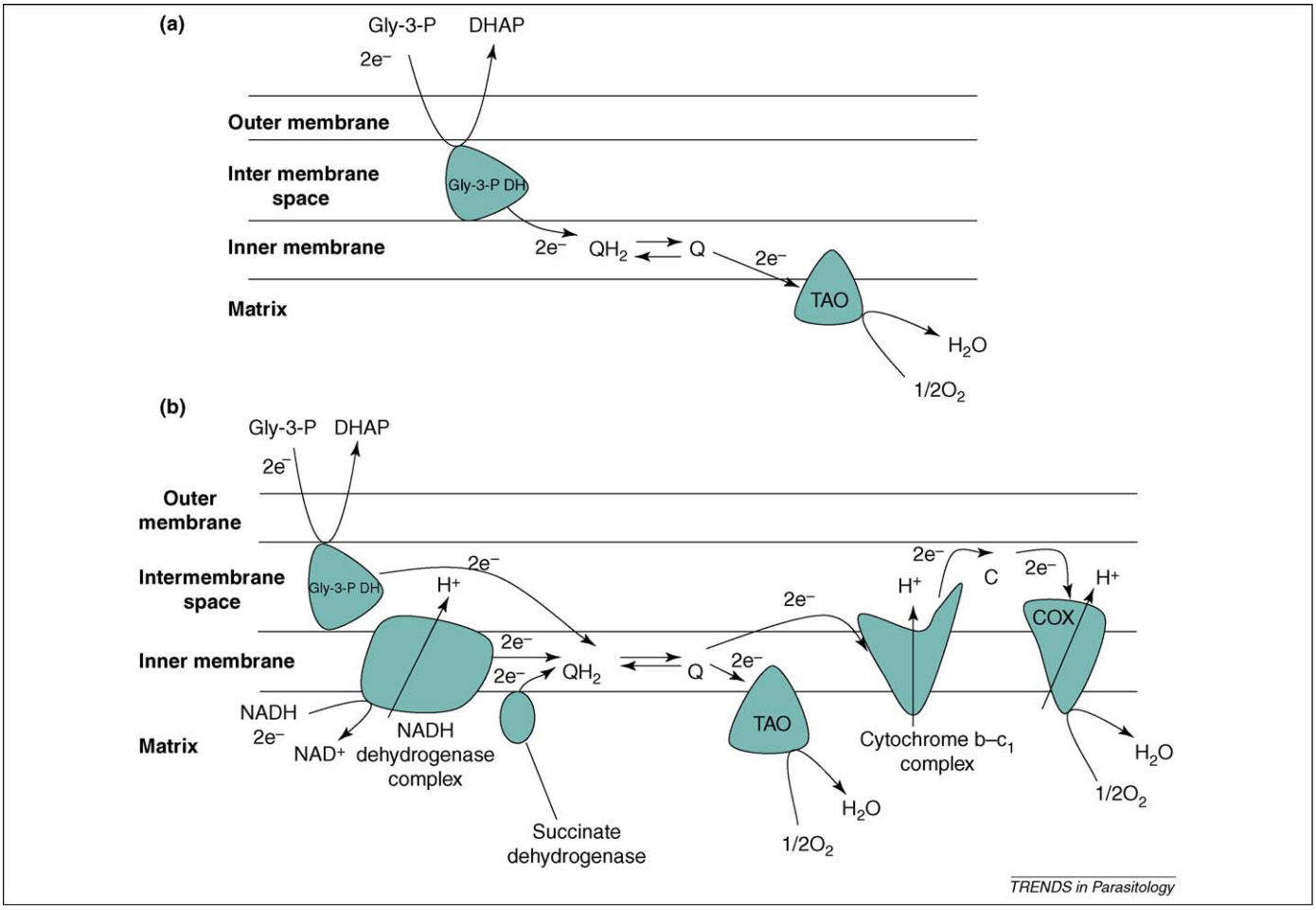

Figure 3. The mitochondrial electron transport system in the bloodstream and procyclic forms of *T. brucei*. **(a)** The bloodstream form of *T. brucei* depends exclusively on TAO for respiration. **(b)** In the procyclic form TAO branches from the primary cytochrome-dependent pathway at the site of ubiquinol. Sub-mitochondrial localization of the enzymes and enzyme complexes are represented by their position in the outer mitochondrial membrane, inner mitochondrial membrane, intermembrane space, and mitochondrial matrix. Abbreviations: C, cytochrome c; COX, cytochrome oxidase; DHAP, dihydroxyacetone phosphate; Gly-3-P DH, glycerol-3-phosphate dehydrogenase; QH_2, ubiquinol; Q, ubiquinone.

regulated by pyruvic acid or by succinate and oxaloacetate [37].

In plants, AOXs exist as covalent and noncovalent homodimers in the mitochondrial inner membrane [38]. The oxidized covalent form of the enzyme is inactive, whereas reducing the disulfide bond activates the enzyme [38]. A conserved cysteine residue (Cys78 in *Arabidopsis* AOX and Cys122 in *S. guttatum* AOX) in the amino-terminal domain is involved in this regulation process [39]. TAO lacks this conserved cysteine residue and thus would not be expected to form dimers. However, there are several reports showing that TAO often appears as a dimer on denaturing SDS–PAGE and as an oligomer on the native blue-gel and during molecular sieving chromatography [15,16,37]. Further analysis has shown that the dimerization and oligomerization of TAO are artifacts of the solubilization process. Similar conflicting observations have been made for other hydrophobic membrane proteins [40]. Unlike plant AOXs, TAO does not form dimers when mitochondria are treated with chemical cross-linkers

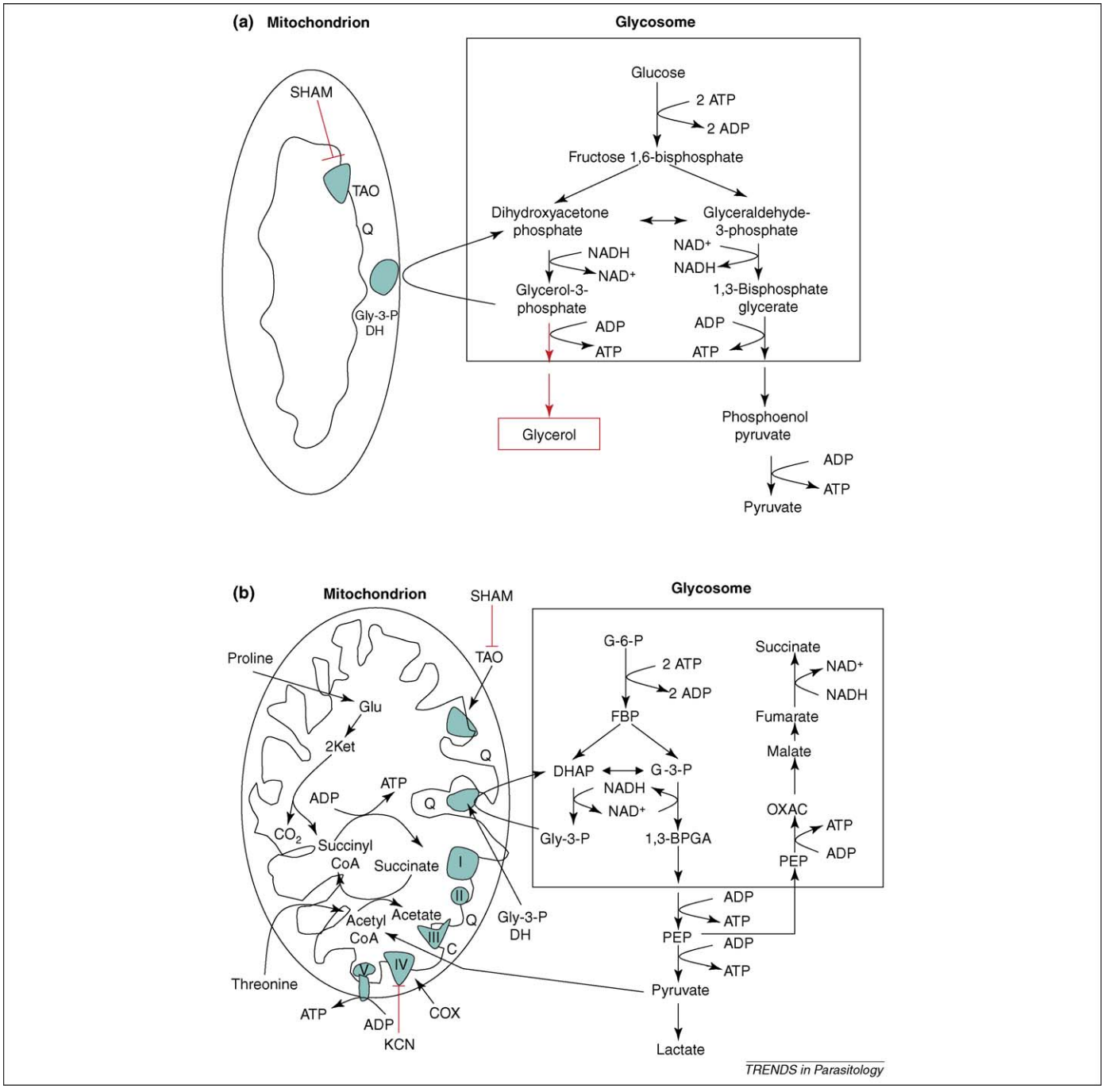

Figure 4. Energy metabolism in the bloodstream and procyclic forms. **(a)** A summary of glucose catabolism in the bloodstream form. Glycerol production is induced upon inhibition of TAO by SHAM as indicated in red. **(b)** A summary of energy metabolism in the procyclic form. Abbreviations: 1,3-BPGA, 1,3-bisphosphoglycerate; C, cytochrome c; COX, cytochrome oxidase; DHAP, dihydroxyacetone phosphate; FBP, fructose 1,6-bisphosphate; G-3-P, glyceraldehyde-3-phosphate; G-6-P, glucose-6-phosphate; Gly-3-P DH, glycerol-3-phosphate dehydrogenase; Glu, glutamate; Gly-3-P, glycerol-3-phosphate; 2Ket, α-ketoglutarate; OXAC, oxaloacetate; PEP, phosphoenol pyruvate; Q, ubiquinol. I, II, III, IV and V represent the respiratory complexes in the cytochrome-mediated pathway. Potassium cyanide (KCN) and salicylhydroxamic acid (SHAM) are the specific inhibitors of COX and TAO, respectively, as indicated in red.

or diamide. Thus, TAO exists as a monomer on the mitochondrial membrane [37]. Like TAO, AOXs from fungi do not have this conserved cysteine residue and do not form dimers upon similar treatment of mitochondria [41], further supporting the observation that TAO has a closer phylogenetic relationship with fungal AOXs than with those of plants.

The function of TAO in cellular energy metabolism in African trypanosomes

TAO is the only terminal oxidase of the mitochondrial electron transport chain in the bloodstream forms of *T. brucei* (Figure 3a). These forms rely on blood glucose as their sole source of energy and suppress most of their mitochondrial activities [7]. Glucose enters the cell and is metabolized to pyruvate, which is secreted by the parasite. The reducing equivalents generated during glycolysis are reoxidized through a glycerol-3-phosphate/dihydroxyacetone phosphate shuttle between glycosomes, the unique glycolytic compartments of the parasite, and the mitochondrion (Figure 4a) [42]. Because electron transport through TAO does not produce any ATP, under aerobic conditions the net production of ATP is two molecules per molecule of glucose in the bloodstream form. Under anaerobic conditions or when TAO is inhibited by SHAM, ATP production is reduced to half with the production of equimolar pyruvate and glycerol (Figure 4a). Under these conditions, the glycosomal NAD^+/NADH balance is maintained by the conversion of glycerol-3-phosphate to glycerol by glycerol kinase, which is kinetically unfavorable under normal conditions. Thus, it was thought that the bloodstream form of *T. brucei* could survive using an anaerobic pathway while TAO is inhibited. However, a recent finding suggests that this might not be true. The bloodstream forms of *T. brucei* do not survive under anaerobic conditions for long time periods [43]. Clayton's group [43] has shown that SHAM alone kills the *in vitro* culture of the bloodstream form within 24 h and a reduction in the TAO level by RNAi is detrimental for parasite growth. The group has further shown that triosephophate isomerase (TPI), which converts dihydroxyacetone phosphate to glyceraldehyde-3-phosphate and vice versa, is essential for the survival of the bloodstream form [43]. A reduction in the TPI level to less than 15% of the wild type by RNAi reduced the growth of the parasite to half. All these results suggest that the bloodstream form cannot survive under anaerobic conditions by producing equimolar amounts of glycerol and pyruvate, otherwise TPI probably would not be essential. TAO is therefore considered to be an essential enzyme for the survival of the bloodstream trypanosomes so is a potential chemotherapeutic target.

In contrast to the bloodstream forms, pyruvate is not the end product of glucose metabolism in the procyclic form [44–46]. Instead, it is further metabolized to acetate, succinate or L-alanine. Finally, succinate is fed into the TCA cycle, and ATP is produced partially by substrate-level phosphorylation and oxidative phosphorylation through mitochondrial ATP synthase coupled with the cytochrome-mediated electron transport chain (Figure 4b). The primary energy source in the procyclic form is amino acids, particularly proline and threonine, and the rate of glycolysis is reduced [44,45].

Several recent reports dramatically alter the concept of energy metabolism in procyclic cells [46]. It has been shown that, although the procyclic form utilizes oxidative phosphorylation for ATP production, substrate-level phosphorylation is the primary pathway and is essential for the survival of the procyclic form. Moreover, in addition to the cytochrome-mediated electron transport chain, the procyclic form maintains a low level of TAO activity (Figure 3b) (Figure 4b). Inhibition of either one of these two pathways of electron transport by specific inhibitors is not lethal; however, inhibition of both pathways at the same time completely inhibits the growth within two days, suggesting that mitochondrial electron transport is necessary for the survival of the parasite.

Recently, it has been shown that increasing TAO protein expression by genetic manipulation in the procyclic form causes the parasite to preferentially utilize TAO for its respiration [47] (Figure 5). Under such conditions the oxygen consumption through the cytochrome pathway is reduced, possibly because of decreased amounts of proteins in the cytochrome pathway. This is unique for *T. brucei*, because similar increases in AOX protein levels in plants and fungi do not alter the cellular respiration pattern [48]. Thus, electron partitioning between the cytochrome-dependent and TAO-dependent pathways is more flexible in trypanosomes than the electron sharing between the similar two terminal oxidases found in other systems. Furthermore, in the insect form of the parasite, the access to two terminal oxidases for electron transport appears to be beneficial to cope with environmental factors such as nutrient and oxygen availability, which fluctuate in the insect gut and the salivary glands. Thus, it appears that TAO is crucial for both the bloodstream and procyclic forms of *T. brucei*.

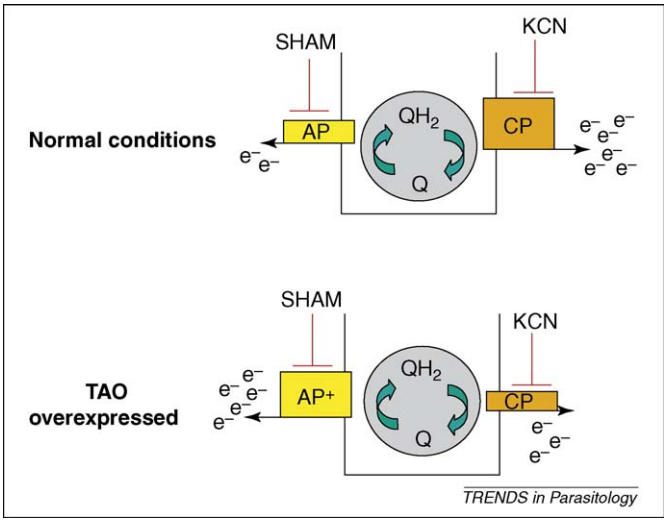

Figure 5. Electron partitioning between the cytochrome-dependent (CP) and alternative TAO-dependent (AP) pathways in the *T. brucei* procyclic form. The circle represents the ubiquinone pool. In the procyclic form of *T. brucei*, there is no threshold level of ubiquinol for TAO to be in operation. Under normal conditions, electrons flow primarily through CP and AP capacity is reduced. When TAO is overexpressed, electrons flow primarily through AP and CP capacity is reduced. SHAM and KCN are specific inhibitors for AP and CP, respectively.

Unique functions of TAO

There are several bodies of evidence suggesting that the surface coat protein expression is somehow linked to the pattern of carbohydrate metabolism in the procyclic form. The two types of the major surface protein procyclin that are expressed in the procyclic form are GPEET and EP. These two types differ mainly in their internal pentapeptide (GPEET) or dipeptide (EP) repeat motifs [49]. The abundance of EP and GPEET procyclins is regulated during the development of *T. brucei* in the fly gut. It has been found recently that the inhibition of TAO activity by SHAM repressed, whereas the overexpression of TAO increased, the expression of the GPEET procyclin [47,49]. These data suggest that electron flow through TAO is involved in the expression of the surface protein. GPEET expression is also regulated by different environmental factors and other metabolic activities. The presence of glycerol in the medium increased the expression of GPEET by increasing the stability of its mRNA through a glycerol-responsive element located at the 3′ untranslated region [49,50]. A decrease in environmental oxygen concentration, and inhibition of the mitochondrial acetate:succinate CoA transferase (ASCT) cycle, downregulates GPEET expression [49,50]. How these mitochondrial activities regulate the expression of GPEET is unclear. However, perhaps either inhibition of TAO by SHAM or inhibition of the ASCT cycle increases production of cellular reactive oxygen species (ROS) [51], which might be the second messenger for the regulation of specific gene expression. TAO also possibly has roles in the regulation of the programmed cell death (PCD)-like phenomena that occur in the bloodstream forms under stress such as cold temperature or high cell density [52].

TAO as a potential chemotherapeutic target for African trypanosomiasis

Among the four drugs that are used to treat African trypanosomiasis, eflornithine is the only one that has a definite target site in *T. brucei* [53]. The others, suramin, pentamidine and melarsoprol, were discovered more than 50 years ago, are toxic, target multiple sites, are painful to administer and are susceptible to the development of resistance [53]. In spite of its efficacy, a major drawback for eflornithine is that it is only effective against *T. gambiense*, which causes the chronic form of the disease. It is not effective for the acute form of the disease caused by *T. rhodesiense*. Furthermore, this drug is also not ideal for immunocompromised patients because it is not trypanocidal but trypanostatic; thus, an active host immune system is necessary to clear the growth-arrested parasites [54]. New drugs that are well tolerated and have defined biochemical targets are therefore urgently needed to treat this disease.

Because TAO is unique and essential to the bloodstream form of trypanosomes and not found in mammals, it represents a potential chemotherapeutic target. However, several previous approaches showed that the inhibition of TAO by SHAM or different benzohydroxamide derivatives does not kill the parasite, but the addition of excess glycerol with SHAM abolishes the parasite very quickly both *in vitro* and *in vivo* [55]. The reason for the requirement for glycerol along with SHAM is that this combination inhibits both the aerobic and anaerobic pathways for ATP production.

Although initially the SHAM plus glycerol combination was demonstrated to kill trypanosomes within several minutes, Helfert *et al.* [43] showed that the bloodstream form could not survive under anaerobic conditions for longer time periods. SHAM at a concentration of 40 μM kills the bloodstream form of the parasite within 24 h under *in vitro* conditions [43]. Yabu *et al.* [56,57] also showed that the repeated administration of ascofuranone, a very potent inhibitor of TAO, could effectively cure infection in mice without the addition of glycerol. These new findings alter the previous concept and suggest that the inhibition of TAO alone is trypanocidal. All these findings serve to increase the potential of TAO as a target for chemotherapeutic intervention in African trypanosomiasis.

Concluding remarks

There is no doubt that TAO is an important molecule in African trypanosomes. It has a significant role in cellular energy metabolism. It is also involved in the cellular redox balance, cell signaling that leads to regulation of gene expression and in the control of cell death. A further understanding of its mechanism of action is urgently required. Obviously, more work is needed to establish conclusively that TAO is essential *in vivo* using an animal model system with more appropriate genetic tools. Work is now in progress to purify and crystallize active recombinant protein for designing structure-based specific inhibitors. A high-throughput screening for small-molecule inhibitors of TAO is also a suitable approach for finding novel trypanocidal drugs.

Acknowledgements

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References

1 Moore, A.L. and Siedow, J.N. (1991) The regulation and nature of the cyanide-resistant alternative oxidase of plant mitochondria. *Biochim. Biophys. Acta* 1059, 121–140
2 Lukes, J. *et al.* (2005) Unexplained complexity of the mitochondrial genome and transcriptome in kinetoplastid flagellates. *Curr. Genet.* 48, 277–299
3 Vanlerberghe, G.C. and McIntosh, L. (1997) Alternative oxidase: from gene to function. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 48, 703–734
4 Grant, P.T. and Sargent, J.R. (1960) Properties of L-α-glycerophosphate oxidase and its role in the respiration of *Trypanosoma rhodesiense*. *Biochem. J.* 76, 229–237
5 Clarkson, A.B. *et al.* (1989) Respiration of the bloodstream forms of the parasite *Trypanosoma brucei brucei* is dependent on a plant-like alternative oxidase. *J. Biol. Chem.* 264, 17770–17776
6 Fairlamb, A.H. (2003) Chemotherapy of human African trypanosomiasis: Current and future prospects. *Trends Parasitol.* 19, 488–494
7 Hill, G.C. (1976) Electron transport systems in kinetoplastids. *Biochim. Biophys. Acta* 456, 149–193
8 Bayne, R.A. *et al.* (1969) Isolation of bodies containing the cyanide insensitive glycerophosphate oxidase of *Trypanosoma equiperdum*. *Comp. Biochem. Physiol.* 30, 1049–1054

9 Opperdoes, F.R. *et al.* (1977) Localization of glycerol-3-phosphate oxidase in the mitochondrion and particulate NAD^+-linked glycerol-3-phosphate dehydrogenase in the microbodies in the bloodstream form of *Trypanosoma brucei*. *Eur. J. Biochem.* 76, 29–39

10 Fairlamb, A.H. and Bowman, I.B.R. (1977) The isolation and characterization of particulate sn-glycerol-3-phosphate oxidase from *Trypanosoma brucei*. *Int. J. Biochem.* 6, 659–668

11 Evans, D.C. and Brown, R.C. (1973) The inhibitory effect of a aromatic hydroxamic acids on the cyanide insensitive terminal oxidase of *Trypanosoma brucei*. *Trans. R. Soc. Trop. Med. Hyg.* 67, 258–268

12 Minagawa, N. *et al.* (1996) An antibiotic, ascofuranone, specifically inhibits respiration and *in vitro* growth of long slender bloodstream forms of *Trypanosoma brucei brucei*. *Mol. Biochem. Parasitol.* 81, 127–136

13 Affourtit, C. *et al.* (2002) Exploring the molecular nature of alternative oxidase regulation and catalysis. *FEBS Lett.* 510, 121–126

14 Moore, A.L. *et al.* (2002) Function of the alternative oxidase: is it still a scavenger? *Trends Plant Sci.* 7, 478–481

15 Chaudhuri, M. *et al.* (1995) Identification and partial purification of a stage specific 33 kDa mitochondrial protein as the alternative oxidase of *Trypanosoma brucei brucei* bloodstream trypanosomes. *J. Eukaryot. Microbiol.* 42, 467–472

16 Chaudhuri, M. *et al.* (1998) Biochemical and molecular properties of the *Trypanosoma brucei* alternative oxidase. *Mol. Biochem. Parasitol.* 95, 53–68

17 Bass, H.S. *et al.* (1990) Solubilization and partial purification of glycerol-3-phosphate oxidase from mitochondria of *Trypanosoma brucei*. *Exp. Parasitol.* 70, 486–489

18 Chaudhuri, M. and Hill, G.C. (1996) Cloning, sequencing and functional activity of the *Trypanosoma brucei brucei* alternative oxidase. *Mol. Biochem. Parasitol.* 83, 125–129

19 Nihei, C. *et al.* (2002) Trypanosome alternative oxidase as a target of chemotherapy. *Biochim. Biophys. Acta* 1587, 234–239

20 Suzuki, T. *et al.* (2004) Molecular cloning and characterization of *Trypanosoma vivax* alternative oxidase (AOX) gene, a target of the trypanocide ascofuranone. *Parasitol. Int.* 53, 235–245

21 Suzuki, T. *et al.* (2005) Alternative oxidase (AOX) genes of African trypanosomes: phylogeny and evolution of AOX and plastid terminal oxidase families. *J. Eukaryot. Microbiol.* 52, 374–381

22 Hertz-Fowler, C. *et al.* (2004) Gene DB: a resource for prokaryotic and eukaryotic organisms. *Nucleic Acids Res.* 32, D339–D343

23 Stenmark, P. and Nordlund, P. (2003) A prokaryotic alternative oxidase present in the bacterium *Novosphingobium aromaticivorans*. *FEBS Lett.* 552, 189–192

24 Berthold, D.A. and Stenmark, P. (2003) Membrane-bound diiron carboxylate proteins. *Annu. Rev. Plant Biol.* 54, 497–517

25 McDonald, A. and Vanlerberghe, G.C. (2004) Branched mitochondrial electron transport in the animalia: presence of alternative oxidase in several animal phyla. *IUBMB Life* 56, 333–341

26 Hakkaart, G.A. (2006) Allotopic expression of a mitochondrial alternative oxidase confers cyanide resistance to human cell respiration. *EMBO Rep.* 7, 341–345

27 Siedow, J.N. *et al.* (1994) The active site of cyanide-resistant oxidase from plant mitochondria contains a coupled binuclear iron center. *FEBS Lett.* 362, 10–14

28 Andersson, M.E. and Nordlund, P. (1999) A revised model of the active site of alternative oxidase. *FEBS Lett.* 449, 17–22

29 Ajayi, W.U. *et al.* (2002) Site-directed mutagenesis reveals the essentiality of the conserved residues in the putative diiron active site of the trypanosome alternative oxidase. *J. Biol. Chem.* 277, 8187–8193

30 Albury, M.S. *et al.* (1998) A highly conserved glutamate residue (Glu-270) is essential for plant alternative oxidase activity. *J. Biol. Chem.* 273, 30301–30305

31 Berthold, D.A. (1998) Isolation of mutants of the *Arabidopsis thaliana* alternative oxidase (ubiquinol:oxygen oxidoreductase) resistant to salicylhydroxamic acid. *Biochim. Biophys. Acta* 1364, 73–83

32 Sakajo, S. *et al.* (1993) Characterization of the alternative oxidase protein in the yeast *Hansenula anomala*. *FEBS Lett.* 318, 310–312

33 Berthold, D.A. *et al.* (2002) EPR studies of the mitochondrial alternative oxidase: evidence for a diiron carboxylate center. *J. Biol. Chem.* 277, 43608–43614

34 Chaudhuri, M. *et al.* (2002) Trypanosome alternative oxidase is regulated post-transcriptionally at the level of RNA stability. *J. Eukaryot. Microbiol.* 49, 263–269

35 Bienen, E.J. *et al.* (1991) Mitochondrial development in *Trypanosoma brucei brucei* transitional bloodstream forms. *Mol. Biochem. Parasitol.* 45, 185–192

36 Bienen, E.J. *et al.* (1991) *Trypanosoma* (Nannomonas) *congolense*: changes in respiratory metabolism during the life cycle. *Exp. Parasitol.* 73, 403–412

37 Chaudhuri, M. *et al.* (2005) Trypanosome alternative oxidase exists as a monomer in *Trypanosoma brucei* mitochondria. *Parasitol. Res.* 96, 178–183

38 Umbach, A.L. and Siedow, J.N. (1993) Covalent and noncovalent dimmers of the cyanide-resistant alternative oxidase protein in higher plant mitochondria and their relationship to enzyme activity. *Plant Physiol.* 103, 845–854

39 Rhoads, D.M. *et al.* (1998) Regulation of the cyanide-resistant alternative oxidase of plant mitochondria. Identification of the cysteine residue involved in α-keto acid stimulation and intersubunit disulfide bond formation. *J. Biol. Chem.* 273, 30750–30756

40 Horvath, A. *et al.* (2000) Translation of the edited mRNA for Cytochrome *b* in trypanosome mitochondria. *Science* 287, 1639–1640

41 Umbach, A.L. and Siedow, J.N. (2000) The cyanide resistant alternative oxidases from fungi *Pichia stipitis* and *Neurospora crassa* are monomeric and lack regulatory features of the plant enzyme. *Arch. Biochem. Biophys.* 378, 234–245

42 Opperdoes, F.R. (1987) Compartmentation of carbohydrate metabolism in trypanosomes. *Annu. Rev. Microbiol.* 41, 127–151

43 Helfert, S. *et al.* (2001) Roles of triosephosphate isomerase and aerobic metabolism in *Trypanosoma brucei*. *Biochem. J.* 357, 117–125

44 Bochud-Allemann, N. and Schneider, A. (2002) Mitochondrial substrate level phosphorylation is essential for growth of procyclic *Trypanosoma brucei*. *J. Biol. Chem.* 277, 32849–32854

45 Coustou, V. *et al.* (2003) ATP generation in the *Trypanosoma brucei* procyclic form. *J. Biol. Chem.* 278, 49625–49635

46 Besteiro, S. *et al.* (2005) Energy generation in insect stages of *Trypanosoma brucei*: metabolism in flux. *Trends Parasitol.* 21, 185–191

47 Walker, R. *et al.* (2005) The effect of over-expression of the alternative oxidase in the procyclic forms of *Trypanosoma brucei*. *Mol. Biochem. Parasitol.* 139, 153–162

48 Vanlerberghe, G.C. *et al.* (1994) Molecular genetic alteration of plant respiration. *Plant Physiol.* 106, 1503–1510

49 Vassella, E. *et al.* (2004) Expression of a major surface protein of *Trypanosoma brucei* insect forms is controlled by the activity of mitochondrial enzymes. *Mol. Biol. Cell* 15, 3986–3993

50 Vassella, E. *et al.* (2000) A major surface glycoprotein of *Trypanosoma brucei* is expressed transiently during development and can be regulated post-transcriptionally by glycerol or hypoxia. *Genes Dev.* 14, 615–626

51 Fang, J. and Beattie, D.S. (2003) Alternative oxidase present in procyclic *Trypanosoma brucei* may act to lower the mitochondrial production of superoxide. *Arch. Biochem. Biophys.* 414, 294–302

52 Tsuda, A. *et al.* (2005) Expression of alternative oxidase inhibits programmed cell death-like phenomenon in bloodstream form of *Trypanosoma brucei rhodesiense*. *Parasitol. Int.* 54, 243–251

53 Docampo, R. and Moreno, S.N.J. (2003) Current chemotherapy of human African trypanosomiasis. *Parasitol. Res.* 90, S10–S13

54 Burri, C. and Brun, R. (2003) Eflornithine for the treatment of human African trypanosomiasis. *Parasitol. Res.* 90, S49–S52

55 Clarkson, A.B. and Brohn, F.H. (1976) Trypanosomiasis: an approach to chemotherapy by the inhibition of carbohydrate catabolism. *Science* 194, 204–206

56 Yabu, Y. *et al.* (2006) Chemotherapeutic efficacy of ascofuranone in *Trypanosoma vivax*-infected mice without glycerol. *Parasitol. Int.* 55, 39–43

57 Yabu, Y. *et al.* (2003) The efficacy of ascofuranone in a consecutive treatment on *Trypanosoma brucei brucei* in mice. *Parasitol. Int.* 52, 155–164

58 Olson, S.A. (1994) MacVector: an integrated sequence analysis program for Macintosh. *Methods Mol. Biol.* 25, 195–201

59 Rogers, J.S. and Swofford, D.L. (1998) A fast method for approximating maximum likelihoods of phylogenetic trees from nucleotide sequences. *Syst. Biol.* 47, 77–89