A Broad Distribution of the Alternative Oxidase in Microsporidian Parasites

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Abstract

Microsporidia are a group of obligate intracellular parasitic eukaryotes that were considered to be amitochondriate until the recent discovery of highly reduced mitochondrial organelles called mitosomes. Analysis of the complete genome of Encephalitozoon cuniculi revealed a highly reduced set of proteins in the organelle, mostly related to the assembly of iron-sulphur clusters. Oxidative phosphorylation and the Krebs cycle proteins were absent, in keeping with the notion that the microsporidia and their mitosomes are anaerobic, as is the case for other mitosome bearing eukaryotes, such as Giardia. Here we provide evidence opening the possibility that mitosomes in a number of microsporidian lineages are not completely anaerobic. Specifically, we have identified and characterized a gene encoding the alternative oxidase (AOX), a typically mitochondrial terminal oxidase in eukaryotes, in the genomes of several distantly related microsporidian species, even though this gene is absent from the complete genome of E. cuniculi. In order to confirm that these genes encode functional proteins, AOX genes from both A. locustae and T. hominis were over-expressed in E. coli and AOX activity measured spectrophotometrically using ubiquinol-1 (UQ-1) as substrate. Both A. locustae and T. hominis AOX proteins reduced UQ-1 in a cyanide and antimycin-resistant manner that was sensitive to ascofuranone, a potent inhibitor of the trypanosomal AOX. The physiological role of AOX microsporidia may be to reoxidise reducing equivalents produced by glycolysis, in a manner comparable to that observed in trypanosomes.

Introduction

Microsporidia are a large and diverse group of eukaryotic intracellular parasites that infect a wide variety of animal lineages, including humans [1]. Although once thought to be early branching eukaryotes, they are now widely accepted to be very atypical parasitic fungi [2,3,4,5]. They are highly adapted to the infection process, and many typical eukaryotic features have been simplified, reduced, or lost completely. Microsporidian genomes are reduced and organised as the peroxisome, mitochondria and Golgi apparatus are absent or altered from their canonical forms [6,7,8].

In particular, microsporidian mitochondria have been severely reduced into biochemically and physically streamlined “mitosomes” [8]. Mitosomes lack their own genome, and there is no evidence from the nuclear genome of any microsporidian for genes encoding any of the respiratory chain complexes or an F₃-ATP synthase complex. In the absence of the ability to synthesize ATP through oxidative phosphorylation, microsporidia appear to import ATP directly from their host cell via ATP translocases located in the cell membrane [9,10], using a transporter which may have been acquired by lateral gene transfer from bacterial energy parasites such as Chlamydia and Rickettsia [11]. Identification of which mitochondrial-derived genes have been retained in the complete genome of Encephalitozoon cuniculi, together with immunolocalization studies in E. cuniculi and Trachipleistophora hominis, suggest that the major functional role for the mitosome is not in energy generation, but instead the assembly of iron-sulphur clusters for export to the cytoplasm [6,12,13].

Biochemical and genomic evidence generally point to glycolysis as the major route of energy generation in most microsporidia [6,9]. In order for ongoing glycolytic activity to be sustainable, however, some mechanism to reoxidise reducing equivalents produced by this pathway is also required. Of the few proteins associated with the microsporidian mitosomes that are not involved in iron-sulfur cluster assembly, one is glyceraldehyde-3-phosphate dehydrogenase. This enzyme is the mitochondrial component of the glyceraldehyde-3-phosphate shuttle, a pathway used in
some eukaryotes to move reducing equivalents into mitochondria [14]. Both cytosolic and mitochondrial components of this shuttle are encoded in the genomes of several microsporidia that have been well studied [6,15], and it has been suggested that this could provide a mechanism sustaining glycolysis in the cytosol by reoxidising glycerol-3-phosphate [9]. However, the E. cuniculi mitochondrial glycerol-3-phosphate dehydrogenase does not appear to be located in the mitochondrion any longer [12], and even if a working shuttle was present, there is no obvious mechanism for reoxidation of the co-reduced FAD produced by this shuttle in the genome of E. cuniculi [6]. In the bloodstream form of Trypanosoma brucei parasites, the mitochondrial glycerol-3-phosphate dehydrogenase is coupled to an alternative oxidase (AOX) that together achieve this process [16], and a similar system has been postulated to be present in the apicomplexan parasite Cryptosporidium parvum [17].

AOX is a cyanide-insensitive terminal oxidase that is typically located on the inner surface of the inner mitochondrial membrane. It branches from the main respiratory chain at the level of the ubiquinone pool, results in the net reduction of oxygen to water, and is non-protonmotive [18,19,20]. It has been found in some prokaryotic lineages, including alpha-proteobacteria [21], and has a wide but discontinuous distribution across eukaryotes: it is widely distributed in plants, and has also been found in a handful of invertebrate animals [22,23,24,25]. In parasitic protists, the distribution of AOX is also uneven: it is known from the genomes of several microsporidia [6]. In the bloodstream form of Trypanosoma cruzi parasites, the mitochondrial glycerol-3-phosphate dehydrogenase is coupled to an alternative oxidase (AOX) that together achieve this process [16], and a similar system has been postulated to be present in the apicomplexan parasite Cryptosporidium parvum [17].

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206, which is most unusual since it is highly conserved and has been proposed to play either a structural or catalytic role [18]. In *A. locustae* the tryptophan has been replaced by serine whilst in *T. hominis* it has been replaced by alanine. Similar to other parasite AOXs however, none of the cysteines postulated to play a role in the regulation of AOX activity in plants [40], are present in either *A. locustae* or *T. hominis*.

Mitoprot I predicted both microsporidian AOX sequences to encode amino-terminal mitochondrial transit peptides, and the *T. hominis* AOX protein was also predicted by Predotar and TargetP 1.1 to have a mitochondrial targeting peptide. In order to test the degree of conservation and functionality of potential targeting signals, full-length proteins were expressed in *S. cerevisiae* cells fused to a green fluorescent reporter protein. Expression in yeast shows that GFP overlays mitotracker fluorescence, indicating successful heterologous targeting for both proteins (Figure 2).

**Phylogenetic analysis**

The phylogenetic relationship among alternative oxidases is in general poorly resolved. There are several well-supported clades, including the microsporidia, the ascomycete fungi, and the basidiomycete fungi, but the fungi do not form a single well-supported clade (Figure 3A), similar to results recovered in earlier AOX phylogenies [27]. The strong support uniting AOX from *A.

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**Figure 1. Alignment of *A. locustae*, *T. hominis*, *S. guttatum* and *T. brucei* AOX sequences.** The four-helix bundles are underlined with a solid line. The putative quinone binding site is underlined with a broken line. Conserved amino acid sites are marked with a star and semi conserved sites are marked with dots.

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Methods. It is important to note that, although the specific activities reported in Table 1 have been corrected for non-reducing gels, as is the case within the thermogenic plant *Sauronatum guttatum*, although in the case of *A. locustae* the monomer is not very prominent. (Figure 4). In *E. coli* membrane fractions containing either *A. locustae* or *T. hominis* recombinant AOX (rAOX), ubiquinol-1 oxidase activity indicates that the activities of both proteins are as expected for AOX (Table 1). In both cases, 1 μM antimycin A, 2 μM myxothiazol and 1 mM potassium cyanide were included in the assay system to ensure inhibition of the cytochrome bo and bd complexes of *E. coli*, and the specific activities reported in Table 1 have been corrected for auto-oxidation of ubiquinol-1 in the absence of membranes (see methods). It is important to note that, although *A. locustae* rAOX was more active than *T. hominis* rAOX, both proteins were equally sensitive to 10 nM ascorfuranone (Table 1), the very specific and potent inhibitor of the trypanosomal alternative oxidase [37]. Furthermore, it is apparent from Table 1 that the specific activities of these microsporidia are considerably higher than those reported for rAOX from *C. parvum* [17] but comparable to those observed with overexpression studies of *T. brucei* rAOX in *E. coli* membranes [42].

**Discussion.**

The genome of *E. cuniculi* has served as a model for microsporidian metabolism since it was completed [6], however, it has never been clear how this model organism dealt with the reducing potential built up through ongoing glycolysis, since it lacks a terminal oxidase. Here we show that this model does not reflect microsporidia as a whole, because alternative oxidase has a broad distribution amongst microsporidian parasites. This distribution remains discontinuous, however, because we can say with some confidence that AOX is not present in either the *E. cuniculi* or *N. cerenae* genomes, which have been sequenced to near completion [6,32]. It also appears to be absent from the genome of *E. bieneusi*, although this genome is not completely sampled [31].

Our negative PCR results from *S. eae* and *A. (Brachiola) algerae* are less conclusive (these have previously been shown to have a high AT content that may prevent the successful amplification of the AOX gene by degenerate PCR [43]), but it suggests the gene may also be absent in several other lineages. Whilst *G. plecoglossi*, *T. hominis* and *S. lophii* are quite closely related and within the Marinosporidia clade, *Antonospora locustae* falls within the distantly related Aquasporidia clade as defined by molecular and ecological analysis [41] (Figure 3C). As we know that the alternative oxidase is present in at least two major clades, and in many fungi, the most parsimonious explanation for its distribution in microsporidia is that it was present in their last common ancestor, but has been lost in *E. cuniculi* and probably other lineages during their more recent evolutionary history.

Analysis of the AOX sequences from *A. locustae* and *T. hominis* reveals that both possess the iron-and substrate-binding motifs found in other AOXs. In *S. guttatum*, Tyr-253 has been shown to be involved in substrate binding, and Tyr-275 to be critical for catalytic activity [19,44], and both of these are also conserved in microsporidia. The absence of Trp-206 in *A. locustae* and *T. hominis* AOX sequences is somewhat surprising, as it is conserved across all other known mitochondrial AOX sequences. Since *A. locustae* and *T. hominis* AOX sequences are demonstrably functional (Table 1), Trp-206 cannot play a universally critical role in electron transport, but it may have a role in other mitochondrial AOXs as helping to anchor the protein to the leaflet of the inner mitochondrial membrane in a manner seen with other monotopic membrane proteins [19,20,45].

The demonstration that *A. locustae* and *T. hominis* rAOX have a high quinol oxidase activity that is sensitive to ascorfuranone at nanomolar concentrations not only solves a significant puzzle in microsporidian metabolism, but also offers a new avenue of treatment for some microsporidian species and further “in tissue culture” trials can establish the efficiency of the drug across the life cycle of the microsporidian. There is currently considerable interest in this antibiotic, originally isolated from the phytopathogenic fungus *Ascochyta visiae*, for its potential promise in the treatment of trypanosomiasis and cryptosporidiosis. The fact that it also appears to potently inhibit the microsporidian AOX may give the drug a more widespread use than previously considered. Of course several of the microsporidia that parasitise humans lack the AOX (e.g. *E. cuniculi* and *E. bieneusi*), but for other human parasites (e.g. *T. hominis*) the AOX is clearly a potential target, and may also be in other unexplored lineages (e.g., *Vittaforma cornea*).

With respect to the potential function of AOX in microsporidia a possible role may be similar to that proposed in the bloodstream form of some trypanosomes. In the bloodstream form of *Trypanosoma brucei*, where glucose is abundant and there is no conventional respiratory chain [16], ATP synthesis is switched from oxidative phosphorylation to substrate level phosphorylation.
Figure 3. Phylogenetic analyses of microsporidian AOX sequences. A. Global MrBayes AOX phylogeny, posterior probabilities and PhyML bootstraps from an analysis of 500 bootstrapped datasets are shown above and below respectively, key and well supported clades (>70% Bootstrap). B. Short alignment PhyML phylogeny including the translated amplified sequences from all four microsporidia. Bootstrap support from 100 datasets is shown next to microsporidian nodes. C. Microsporidian distribution of the alternative oxidase gene plotted onto a phylogeny of microsporidian SSU rDNA sequences. Scale bars in all trees indicate substitutions per site.

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Glycolysis is contained within a glycosome, a membrane-bound organelle containing glycolytic enzymes. In this system, reducing equivalents generated by glycolysis in the form of glycerol-3-phosphate are shuttled out of the glycosome and re-oxidised by a glycerol-3-phosphate dehydrogenase (G3PDH) located on the outer surface of the inner membrane. G3PDH itself reduces the mitochondrial ubiquinone pool that in turn is then re-oxidised by the alternative oxidase. In this way, glycerol-3-phosphate within the glycosome is continuously being re-oxidised to supply further substrate for the net oxidation of NADH [16]. Thus in an indirect manner mitochondrial alternative oxidase activity maintains the NADH/NAD balance within the glycosomes. In addition to the alternative oxidase, however, trypanosomes also possess a glycerol kinase that under anaerobic conditions helps to maintain the glycosome NADH/NAD balance by converting glycerol-3-phosphate to glycerol [16].

It is plausible that most microsporidia rely on a similar system and that AOX fulfils the role of the terminal oxidase, as shown in Figure 5. Whether the microsporidian AOX functions in the mitosome or cytosol is not completely certain, but its very presence in the cell and its carrying out the functions we have demonstrated in vitro significantly change our view of microsporidian metabolism and drug sensitivity in either event. Overall, the presence of an N-terminal leader with characteristics of a transit peptide, together with the likely mitochondrial origin of the protein, all suggest a mitosomal location is most plausible. This also fits well with previously unusual observations on the glycerol-3-phosphate shuttle. Localization studies on mitochondrial glycerol-3-phosphate dehydrogenase in *E. cuniculi* show no evidence that the enzyme is confined to mitochondria or specifically localized there, unlike ferredoxin, frataxin, ISCU and ISCS [12,13], and in *E. bieneusi* the gene appears to be absent altogether [31]. This suggests that the glycerol shuttle has been displaced in these microsporidia, which is functionally consistent with the absence of the alternative oxidase protein in both species.

### Methods

**Characterisation of AOX genes in microsporidia**

The *A. locustae* alternative oxidase sequence was retrieved from the GMOD MBL *A. locustae* database and used to design degenerate primers to amplify a fragment of the alternative oxidase gene from *T. hominis*, *G. plecoglossi* and *S. lophii* (Forward 5'-GAAACWGTWGCWGCWGTNCCNGG-3', Reverse 5'-ATW-GCTTCTTCTTCNAKRTANCCNAC-3'). Degenerate PCR was carried out on DNA from *E. cuniculi* to exclude the possibility of contamination with other species.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Specific Activity μmol QH2 oxidised min⁻¹ mg⁻¹</th>
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<tbody>
<tr>
<td><em>A. locustae</em> rAOX in <em>E. coli</em> membranes</td>
<td>2.2</td>
</tr>
<tr>
<td>+10 nM ascofuranone</td>
<td>0.2</td>
</tr>
<tr>
<td><em>T. hominis</em> rAOX in <em>E. coli</em> membranes</td>
<td>1.6</td>
</tr>
<tr>
<td>+10 nM ascofuranone</td>
<td>0.1</td>
</tr>
<tr>
<td><em>C. parvum</em> rAOX in <em>E. coli</em> membranes [17]</td>
<td>0.03</td>
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**Figure 5. Hypothetical scheme of function of the alternative oxidase in the microsporidian cell.** Microsporidian cells are known to contain glycolytic enzymes, though no obvious mechanism exists for reoxxdising NADH to NAD⁺. The glycerol-3-phosphate shuttle is encoded in many microsporidian genomes. If this shuttle is coupled to an alternative oxidase protein in the mitosome, it could potentially represent a mechanism for regenerating NAD⁺. doi:10.1371/journal.ppat.1000761.g005
that the AOX gene is present in the genome within the subtelomeric regions that have not been fully assembled [6]. This gave negative results. Negative degenerate PCR results were found for Brachiola algerae and Edhazardia aedis. The full-length gene was amplified from T. hominis DNA and RNA obtained from purified spores from cultures maintained in rabbit kidney cells at Rutgers, State University of New Jersey. The 5’ prime end of the gene was amplified using RLM-RACE using primers designed from within that fragment amplified by degenerate PCR. The first round of PCR yielded a product truncated at the 5’ end. Primers were then designed from within that fragment to obtain the presumed full-length gene. A splinkerette strategy was used to obtain 3’ end of the gene [38]. Amplified PCR products were cloned using the TOPO TA cloning system (Invitrogen) and sequenced using Big Dye 3.2 (ABI). Mitochondrial transit peptides were predicted using Mitoprot I [46], Predotar [47], and TargetP 1.1 [48]. (New sequences are deposited in the GenBank Database under the accession numbers GU221909-GU221911).

**Heterologous expression in yeast**

DNA fragments corresponding to *A. locustae* and *T. hominis* AOX open reading frames were amplified by PCR using primers that generated in-frame restriction sites. PCR products were cloned upstream of green fluorescent protein (GFP-S65T) under the control of the MET25 promoter [49] for analysis by confocal or fluorescence microscopy. Constructs were then transformed into the diploid yeast strain JK9-3da/a ([ura3-52]/[ura3-52] uaa5-32/ uaa5-32 rme1/rme1 tp1,tp1 his4/his4 GAL+/GAL+ HMLa/HMLa), and plated on uracil and thiostrepton ([Gal-/Gal+]), Trp+ and His+ plates for growth and assay analysis. Yeast cells were visualized using the Zeiss meta confocal microscope.

**Western blot analysis**

Separation of yeast mitochondrial proteins on non-reducing SDS-polyacrylamide gels, transfer to nitrocellulose membranes, and detection of AOX protein using monoclonal antibodies raised against the *S. guttatum* AOX [50] was performed as described previously [51].

**Functional assay**

The *A. locustae* and *T. hominis* gene sequences were amplified using Phusion High-Fidelity Taq (New England Biolabs) and cloned into the pet14b expression vector. Both constructs were used to transform E. coli strain C41, which is especially suited to the expression of transmembrane proteins. Bacterial membranes were prepared using 1.5 L Luria broth cultures, adapted from Berthold [52] and as described in detail by Crichton et al 2009 [53]. Flasks containing Luria Broth, 0.02% glucose, 0.002% FeSO4 and 50 μg ml−1 ampicillin were inoculated with 10 ml−1 starter culture, and incubated at 37°C for 4 hours. The temperature was reduced to 18°C, and the cultures were incubated for one hour prior to induction with 100 μM IPTG. After induction, the cultures were incubated for 18 hours at 18°C. Cells were then harvested using centrifugation at 11,000 g for 10 minutes. After initial centrifugation, cells were resuspended in 60 mM Tris-HCl (pH 7.5), 5 mM DTT, 300 mM NaCl and 0.1M PMSF and then sonicated for 8 minutes at 14 microns. After sonication, cell debris was removed by centrifugation at 12,000 × g for 15 minutes, and clear supernatant was further refined by a 2-hour ultracentrifugation step at 200,000 × g. Pellets from final spin were resuspended in 60 mM Tris-HCl (pH 7.5), 5 mM DTT, 300 mM NaCl and used for subcellular gel and assay analysis. Ubiquinol oxidase activity (AOX activity) was measured by recording the absorbance change of ubiquinol-1 at 278 nm ( Cary UV/vis -400 Scan spectrophotometer). Reactions were started by the addition of ubiquinol-1 (final concentration 150 μM, ε278 = 15,000 M−1cm−1) after 2 min preincubation at 25°C in the presence of rAlAOX and rThAOX in 50 mM Tris-HCl (pH 7.4). Endogenous ubiquinol activities were inhibited by inclusion of 1 μM antimycin A, 2 μM myxothiazol and 1 mM potassium cyanide in the assay medium.

**Phylogenetic analysis**

The *A. locustae* and *T. hominis* AOX amino acid sequences were aligned to 47 diverse protein sequences with representatives from animal, kinetoplastid, fungal, heterokont, plant and proteobacterial lineages. Sequences were aligned using ClustalW [54] and manually edited and masked. The alignment was analysed using Modelgenerator to select an appropriate model for amino acid change [55]. Phylogenetic trees were inferred using MrBayes 3 [56] with a Blosum62 matrix and with 2 runs each of 1000000 generations carried out on the freely available Biosoportal (www. bioportalauio.no). A burn-in of 400 trees was removed from each run and a consensus created from remaining trees. Five hundred bootstrapped data matrices were also analysed by maximum likelihood using PhyML 3.0 [57] with a JTT model of amino acid change and an estimated gamma parameter with four rate categories of amino-acid change. A second alignment restricted to the conserved area amplified by degenerate PCR from *S. lophii*, *G. plecoglossi* was also analysed. Trees were inferred and 100 bootstrap datasets analysed from this short alignment using PhyML, using the parameters described above. The SSU rRNA backbone phylogeny was also based on available SSU sequences from NCBI, which were aligned using ClustalW, manually edited and masked and analysed using PhyML 3.0 with a JC69 nucleotide substitution model with estimated gamma parameter and 4 categories of rate change.

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**Author Contributions**

Conceived and designed the experiments: BAPW ALM PJK. Performed the experiments: BAPW CE LB ALM. Analyzed the data: BAPW ALM. Contributed reagents/materials/analysis tools: YK KK ALM. Wrote the paper: BAPW ALM PJK.

**References**


