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An ADP/ATP-Specific Mitochondrial Carrier Protein in the Microsporidian *Antonospora locustae*

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The mitochondrion is one of the defining characteristics of eukaryotic cells, and to date, no eukaryotic lineage has been shown to have lost mitochondria entirely. In certain anaerobic or microaerophilic lineages, however, the mitochondrion has become severely reduced that it lacks a genome and no longer synthesizes ATP. One example of such a reduced organelle, called the mitosome, is found in microsporidian parasites. Only a handful of potential mitosomal proteins were found to be encoded in the complete genome of the microsporidian *Encephalitozoon cuniculi*, and significantly no proteins of the mitochondrial carrier family were identified. These carriers facilitate the transport of solutes across the inner mitochondrial membrane, are a means of communication between the mitochondrion and cytosol, and are abundant in organisms with aerobic mitochondria. Here, we report the characterization of a mitochondrial carrier protein in the microsporidian *Antonospora locustae* and demonstrate that the protein is heterologously targeted to mitochondria in *Saccharomyces cerevisiae*. The protein is phylogenetically allied to the NAD^+ transporter of *S. cerevisiae*, but we show that it has high specificity for ATP and ADP when expressed in *Escherichia coli*. An ADP/ATP carrier may provide ATP for essential ATP-dependent mitosomal processes such as Hsp70-dependent protein import and export of iron–sulfur clusters to the cytosol.

Keywords: microsporidia; mitosome; mitochondrion; ATP transporter

Introduction

For many years, it was hypothesized that certain anaerobic and microaerophilic eukaryotes were in fact amitochondriate. In particular, the lineages containing such parasites as *Giardia intestinalis*, *Trichomonas vaginalis*, *Entamoeba histolytica*, and the phylum Microsporidia were proposed to primitively lack mitochondria because they diverged before the mitochondrial endosymbiosis took place.[1] The lack of clearly discernable mitochondria in some of these lineages alternatively led to the suggestion that mitochondria were lost due to their parasitic, anaerobic, or microaerophilic lifestyle.[2–7] More recently, however, thorough immunoelectron micrographic examinations of several such 'amitochondriates' have invariably revealed the presence of cryptic and highly reduced organelles descended from mitochondria.[8–11] These structures are typically known as mitosomes or hydrogenosomes, depending on their measured or inferred biochemical properties (in some cases, hydrogenosomes are not cryptic in the sense that they were not detected, but their relationship to mitochondria was not clear). Mitosomes and hydrogenosomes are derived from mitochondria but are highly reduced both biochemically and physically. With interesting exceptions, they typically lack biochemical hallmarks of mitochondria such as electron transport chain and Krebs cycle, morphologically distinguishing features such as cristae, and a genome.

In the microsporidian parasites, a metabolic profile of the mitosome has been pieced together from the complete genome of the vertebrate parasite *Encephalitozoon cuniculi*,[12] but this profile contains

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Abbreviations used: MCF, mitochondrial carrier family; AAC, ADP/ATP carrier; CAT, carboxyatractyloside; BKA, bongkrekic acid; PLP, pyridoxal 5′-phosphate.

many incomplete pathways and partial reactions that are not well understood.[13] One such gap is that, in the absence of an electron transport chain, there is no obvious mechanism for ATP synthesis within the organelle. This is in contrast to the hydrogenosomes of trichomonads and some chytrid fungi, where ATP synthesis occurs via substrate-level phosphorylation rather than oxidative phosphorylation,[14] and is significant because ATP is essential for some of the pathways that do seem to be present.

Indeed, many reactions within mitochondria require import and export of metabolites across the inner mitochondrial membrane, which is typically achieved by a family of mitochondrial carrier proteins with diverse substrates: the mitochondrial carrier family, or MCF.[15] These have a characteristic tripartite protein structure of three repeats each passing in and out of the mitochondrion, forming a pore across the inner mitochondrial membrane. Genes for MCF proteins are abundant in genomes of aerobic eukaryotes; for example, the yeast genome encodes 35 such proteins.[16] These proteins are also typically encoded in the genomes of eukaryotes with hydrogenosomes or mitosomes, where they are not so numerous but generally include an ADP/ATP transporter. A single MCF protein has been reported from the genome of *E. histolytica*,[17] and although this gene does not encode a classic ADP/ATP carrier (AAC), it has been shown to take up ATP in exchange for ADP.[18] Five genes for uncharacterized MCF proteins have been identified in the *T. vaginalis* genome.[19] Like *E. histolytica*, ADP/ATP counterexchange across the mitochondrial membrane is achieved in *T. vaginalis* and *Trichomonas gallinae* by another class of MCF that is structurally distinct from typical AAC proteins.[20,21] In contrast, the hydrogenosomal ADP/ATP transporters in the anaerobic chytrid *Neocallimastix* share structural and functional similarities to mitochondrial AACs.[22,23] Overall, different lineages with reduced mitochondria have recruited different MCF members to maintain ADP/ATP counterexchange.

The genome of the microsporidian *E. cuniculi* is unusual, since not a single MCF protein was identified, raising the question of how its mitosomes import ATP or whether they synthesize it internally.[24] We recently identified a small fragment of an MCF gene in an expressed sequence tag survey of the insect-infecting microsporidian *Antonospora locustae*.[25] This gene shared the highest sequence similarity to an NAD^+ carrier from *Saccharomyces cerevisiae*, previously annotated as a pyruvate carrier.[26] Because of the lack of MCF genes in the *E. cuniculi* genome and the importance of ATP in mitosome function, here we have characterized the expression and substrate specificity of this protein. We show that this protein encodes mitochondrial targeting information that is recognized by the yeast mitochondrial import machinery and that it preferentially transports ATP and ADP but not NAD^+. Overall, these observations suggest that at least some microsporidian mitosomes retain ADP/ATP counterexchange mechanisms but have adopted a distinct class of MCF protein to facilitate this process.

Results

Sequence features

The fully sequenced *A. locustae* MCF gene was 900 bp in length, corresponding to a protein of 299 amino acids. The protein displays the tripartite structure typical of MCF proteins, predicted by TMpred[27] to comprise a total of six transmembrane domains with the N-terminus to the outside (Fig. 1). Phylogenetic analysis showed the *A. locustae* MCF protein to fall within a large clade of adenine nucleotide carrier proteins with modest support (61–65% bootstrap support). Specifically, however, it falls within a strongly supported clade (82–92% bootstrap support) composed of FAD/NAD/folate transporters, most closely allied with an uncharacterized protein from *Dictyostelium discoideum*, though with weak support (Fig. 2).

Adenine nucleotide transporters share the conserved amino acid motif G-[IVLM] at one of the substrate contact points.[28] This motif is also conserved in the *A. locustae* protein (Fig. 1). However, the RRRMMM motif characteristic of typical AACs

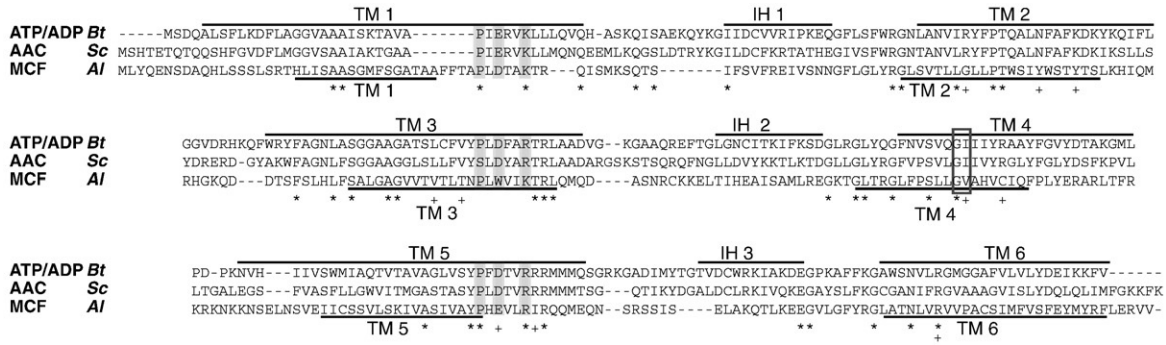

Fig. 1. Alignment of the *A. locustae* (*Al*) MCF protein to homologues of the bovine AAC protein and the *S. cerevisiae* (*Sc*) AAC1. Known transmembrane domains and internal mitochondrial helices are labeled on the *Bos taurus* (*Bt*) protein. Predicted transmembrane domains in the *A. locustae* protein are also labeled. Conserved amino acids are labeled by *. Sites known to interact with CAT in *B. taurus* protein are indicated by +. Gray-shaded amino acids show sites of the PX(D/E)XX(K/R) motif, which are not fully conserved across all proteins. TM, transmembrane; IH, internal helix.

is absent, as are residues involved in the interactions with carboxyatractyloside (CAT), known to effectively inhibit activity in classic ADP/ATP translocases.[29] The three helices of the mitochondrial carriers are thought to be held together in a salt bridge aided by a P-X-[DE]-X-X-[RK] motif,[30] which is also conserved in the first and third helices of the *A. locustae* protein but only partially conserved in the second helix (Fig. 1).

Localization of the microsporidian MCF in yeast cells

MCF proteins are generally targeted to the mitochondrion, but a few members of the family are localized to other cellular compartments and may function in peroxisomes (and glyoxosomes) or plastids.[31–33] Since microsporidia lack these organelles, the *A. locustae* protein is most likely localized to the mitosome. Unfortunately, *A. locustae* also lacks a tractable system for immunolocalization and its mitosome has not been directly identified to date, although the genome contains many of the same mitosome-derived proteins as *E. cuniculi* and the organelle almost certainly does exist. Nevertheless, to investigate its likely location in the cell, we expressed *A. locustae* MCF protein in yeast cells to look for evidence of conserved mitochondrial targeting signals that are recognized by the yeast mitochondrial targeting machinery. Whereas

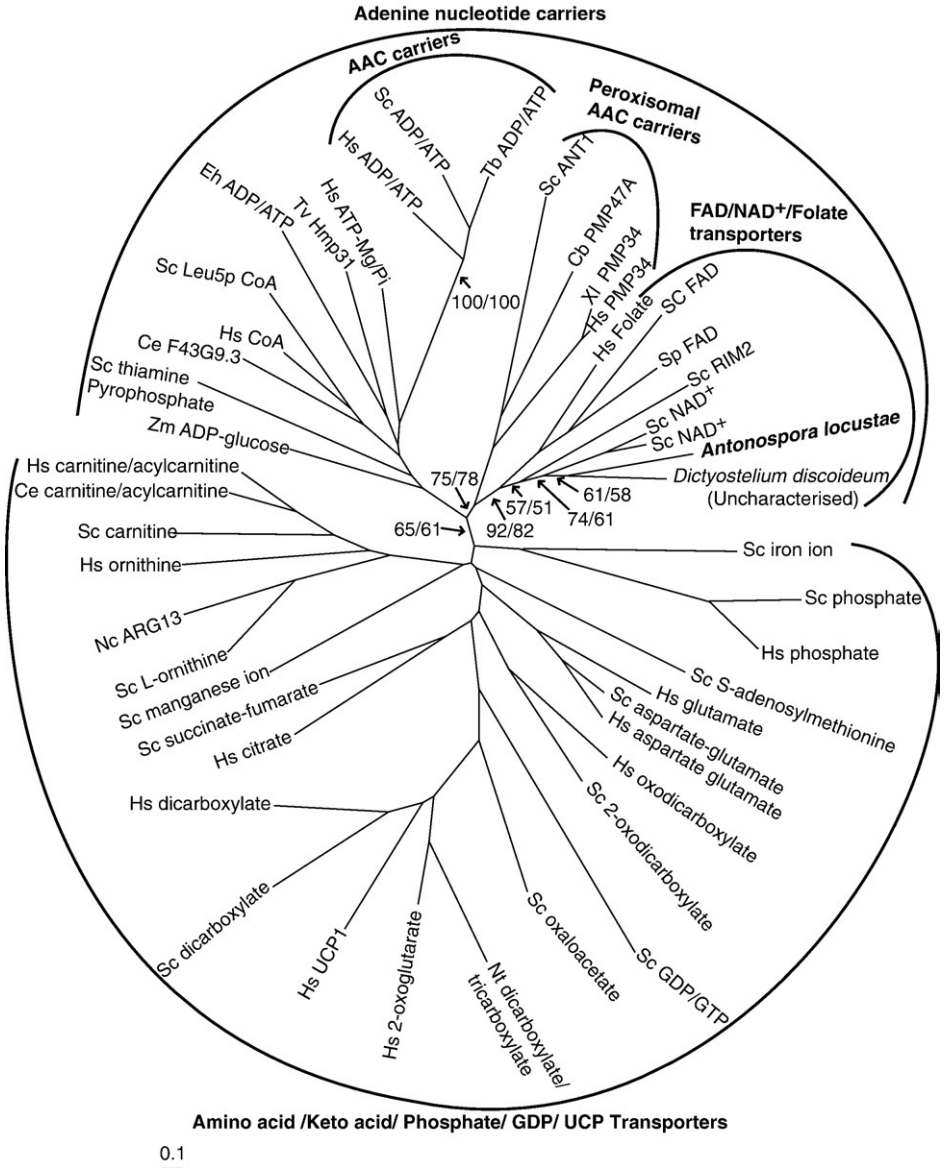

Fig. 2. Phylogenetic analysis of the *A. locustae* MCF protein, showing its relationship to other mitochondrial carrier protein sequences. The tree shown is a ProML tree with PhyML and ProML bootstrap values (left and right, respectively) shown at nodes of interest with over 50% support. Nc, *Neurospora crassa*; Sc, *S. cerevisiae*; Sp, *Schizosaccharomyces pombe*; Nt, *Nicotiana tabacum*; Xl, *Xenopus laevis*; Cb, *Candida boidinii*; Zm, *Zea mays*; Tb, *Trypanosoma brucei*; Eh, *E. histolytica*; Tv, *T. vaginalis*; Ce, *Caenorhabditis elegans*.

most mitochondrial proteins are targeted via an N-terminal leader, some proteins, including AACs from yeast or animals, can be targeted via internal targeting signals.[34] There is no obvious N-terminal targeting signal within the *A. locustae* MCF sequence, nor is it predicted to be targeted to a mitochondrion using MitoProt,[35] a program that detects N-terminal targeting signals. Nevertheless, it is successfully recognized by the yeast cell mitochondrial import machinery (Fig. 3), indicating the presence of conserved, possible internal, mitochondrial targeting signals.

Substrate specificity

Of all the functionally annotated *S. cerevisiae* MCF proteins, the amino acid sequence of the *A. locustae* protein shares highest sequence similarity to the NAD^+ carrier, which is also described to accept nicotinic acid adenine dinucleotide, (d)AMP, deoxyguanosine monophosphate, and, to a lesser extent, uridine monophosphate, trimethyl phosphate, (d)ADP, and deoxyguanosine diphosphate as substrates.[36] Phylogenetic analyses placed the *A. locustae* protein in the same subgroup as the NAD^+ carrier within a larger clade of NAD^+, FAD^+, and folate transporting proteins (Fig. 2). However, MCF proteins are highly divergent from species to species and from substrate to substrate, and phylogeny is not necessarily a good indicator of substrate specificity in this family, especially in mitosome- and hydrogenosome-containing lineages.[18,21] We therefore carried out a biochemical characterization of the *A. locustae* MCF protein to more directly determine its preferred substrate. Heterologous assays for substrate specificity in *Escherichia coli* have been used to characterize several proteins from this family, including mitochondrial, hydrogenosomal, and also plastidic nucleotide carriers belonging to the MCF.[20,23,37] Using *E. coli* BL21 (DE3) pLysS cells, we found no detectable protein expression and no accumulation of radioactively labeled ATP, ADP, or NAD^+ into induced cells when compared to the control (data not shown). Therefore, we used the *E. coli* strain Rosetta2 (DE3) pLysS, which is described to improve expression of full-length proteins because it contains additional plasmid-encoded tRNA genes whose products decode normally rare codons. Although the *A. locustae* mitosomal MCF is most similar to NAD^+ carriers, induced Rosetta2 cells were not able to import NAD^+ (data not shown) but instead imported ATP and ADP at significant levels. ATP (Fig. 4a) and ADP (Fig. 4b) uptake was time linear within the first 1 min and declined progressively until 30 min. Noninduced Rosetta2 cells imported only trace amounts of labeled nucleotides over the same time frame (Fig. 4a and b).

To assess whether other nucleotides are also transported by the *A. locustae* MCF protein, substrate specificity of the recombinant protein was analyzed by competition. Import of radioactively labeled ATP was performed in the presence of 20-fold excess of structurally related, nonlabeled nucleotides and derivates (Fig. 5a). Only ATP and ADP had significant inhibitory effects on [α^{32}P]ATP uptake (100%). The calculated residual activity of about 25% in the presence of either ATP or ADP was only slightly in excess of background observed in noninduced control cells (about 20%). Excess of AMP also resulted in about a 40% reduction of ATP import, but none of the other potential substrates produced more than a slight reduction of nucleotide transport (Fig. 5a), indicating a high specificity.

ATP transport activity and inhibition of the *A. locustae* MCF protein

To determine the kinetic properties of the *A. locustae* MCF protein, we analyzed ATP and ADP uptake in the presence of rising substrate concentrations. The recombinant carrier exhibited apparent affinities of about 31.2(±4.4) μM for ATP and of about 35.1(±4.6) μM for ADP and transported ATP with a V_{max} of 598.8(±61.1) pmol mg protein^{-1} h^{-1} and ADP with a V_{max} of 579.0(±69.2) pmol mg protein^{-1} h^{-1}. Accordingly, the recombinant carrier transports both ATP and ADP with nearly identical apparent affinities and maximal velocities.

Because the *A. locustae* MCF sequence lacks motifs associated with common ATP-transporter inhibitors, we determined the efficacy of several common inhibitors on ATP uptake into *E. coli* cells. Lysozyme was added prior to transport measurement to improve penetration across the outer membrane and access of the inhibitors to the carrier protein.[20] Treatment of *E. coli* cells with lysozyme had no significant influence on the ATP transport or on the competitive influence of 20-fold excess of ATP or ADP (Fig. 5a and b). Bongkrekic acid (BKA) or CAT are highly specific inhibitors of mitochondrial AACs.[38,39] However, structurally different MCF

Fig. 3. Targeting of the *A. locustae* MCF protein into yeast mitochondria. Confocal microscopy of yeast cells expressing an *A. locustae* MCF–GFP fusion protein and costained with MitoTracker Red. Filters selective for GFP (left) and MitoTracker Red (center) were used. The merged GFP and mitotracker fluorescence images are shown on the right.

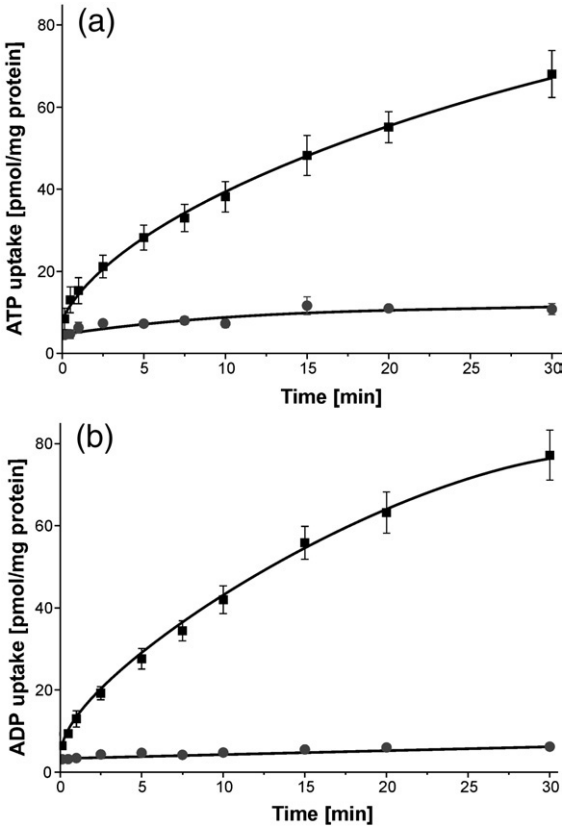

Fig. 4. Time dependency of α^{32}P-labeled ATP and ADP uptake into *E. coli* cells. IPTG-induced (squares) or noninduced (circles) Rosetta2 cells harboring the expression construct encoding the *A. locustae* mitosomal MCF gene were incubated in phosphate buffer medium containing 50 μM labeled ATP (a) or ADP (b) for the indicated time periods.

proteins that also accept adenine nucleotides as substrates are not comparably inhibited by BKA or CAT.[20,33,36,37] At the concentration tested, neither BKA nor CAT had a detectable effect on the *A. locustae* MCF activity (Fig. 5b). In addition, only slight inhibition was observed with mersalyl, which is known to be a powerful inhibitor of the peroxisomal adenine nucleotide carrier[33] and of the mitochondrial NAD carrier[36] from *S. cerevisiae*. In contrast, pyridoxal 5′-phosphate (PLP), which effectively inhibits the plastidic adenine nucleotide uniporter from potato,[37] did reduce ATP uptake, and simultaneous application of PLP and 4,4′-diisothiocyanatostilbene-2,2′-disulfonate, both potential inhibitors of plastidic phosphate translocators,[40] nearly completely inhibited ATP uptake (Fig. 5b).

To understand the role of the *A. locustae* MCF protein in the physiological context of a cell, we determined whether it acts as a uniporter or in a counterexchange mode. *E. coli* cells expressing the recombinant carrier were incubated in the presence of [α^{32}P]ADP. After removal of external radioactivity, efflux of nucleotides in the presence of nonlabeled ATP, ADP, AMP, or phosphate buffer medium was analyzed by thin-layer chromatography (Fig. 6).

The release of total radioactivity (about 3100 counts) is comparable across the different samples,

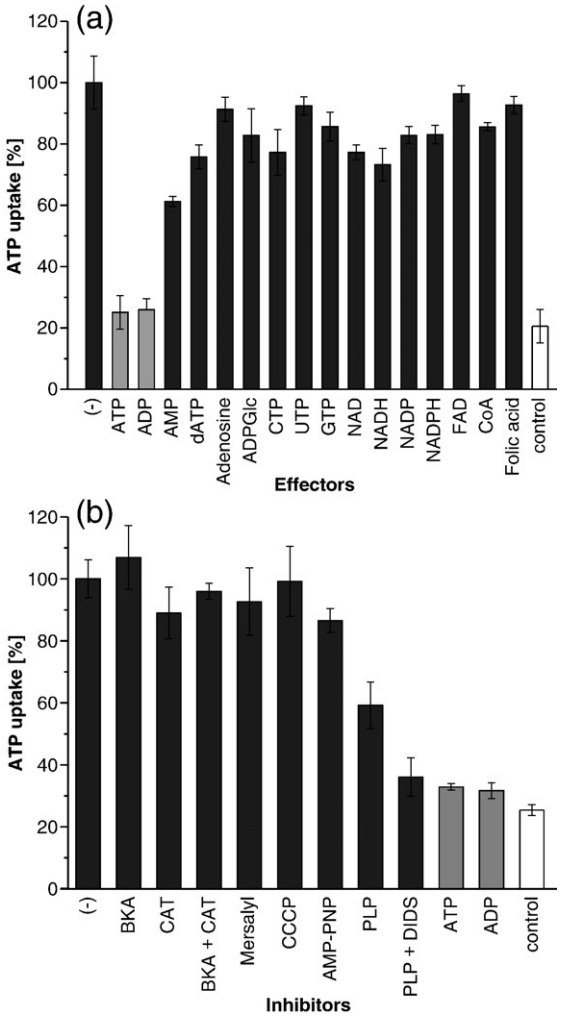

Fig. 5. Substrate specificity (a) and inhibition (b) of the *A. locustae* MCF protein. (a) The effects of ATP uptake by competition with several nucleotides or nucleotide derivates. Uptake of α^{32}P-labeled ATP by the recombinant mitosomal carrier from *A. locustae* was measured at a substrate concentration of 50 μM in three independent trials. Unlabeled effectors were applied in 20-fold excess. The rate of transport in the absence of effectors (−) was set to 100%. Rates of nucleotide transport in the presence of effectors are normalized to the nonaffected transport. The control (open bar) represents the nucleotide uptake into noninduced *E. coli* cells harboring the expression construct. (b) For inhibitor analysis, in three independent trials, *E. coli* cells were preincubated for 5 min with lysozyme (500 μg/ml) to allow penetration of the reagents across the outer membrane. Uptake was carried out for 12 min and stopped by rapid filtration. The final concentrations of the inhibitors were as follows: 10 μM CAT, 10 μM BKA, 0.2 mM mersalyl, 150 μM CCCP (carbonyl cyanide *m*-chlorophenylhydrazone), 1 mM AMP–PNP (adenylylimidodiphosphate), 2 mM PLP, 400 μM DIDS (4,4′-diisothiocyanatostilbene-2,2′-disulfonate), and 1 mM ATP or ADP.

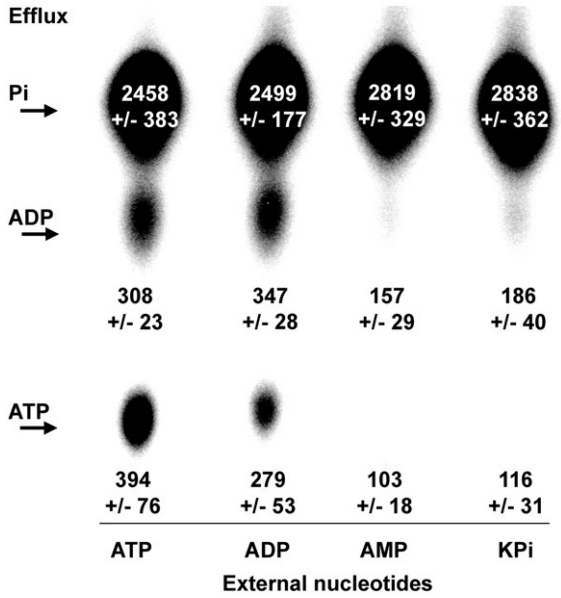

Fig. 6. Identification and quantification of exported radiolabeled nucleotides by thin-layer chromatography. *E. coli* cells expressing the recombinant mitosomal carrier from *A. locustae* were induced and incubated for 10 min in phosphate buffer containing 50 μM α^{32}P-labeled ADP. After removal of external radioactivity, efflux of nucleotides was analyzed in the presence of indicated adenine nucleotides at a concentration of 500 μM each or in the presence of phosphate buffer (KP_i). Back exchange was stopped after 5 min. Spots of ATP, ADP, and phosphate were excised and transferred to scintillation vessels, and radioactivity was quantified in a scintillation counter. Quantified radioactivity of three independent experiments is given at the corresponding spots. The thin layer shown represents a typical outcome.

indicating equal preloading of the *E. coli* cells with labeled ADP. The presence of external ATP or ADP caused efflux of radiolabeled ATP and ADP, whereas a concentration of 500 μM AMP or 50 mM phosphate buffer was not able to induce comparable efflux of both adenine nucleotides. Quantification of exported adenine nucleotides revealed that in the applied expression system, the homoexchange (ATP/ATP) and (ADP/ADP) is slightly preferred. In the presence of AMP or P_i, the release of phosphate by endogenous phosphate carriers is higher when compared to the samples incubated in the presence of counterexchange substrates of the mitosomal carrier. This is because import of nonlabeled adenine nucleotides in exchange with [α^{32}P]ATP and [α^{32}P]ADP reduces the amount of total internal radioactivity, which results in a lowered labeled phosphate-to-nonlabeled phosphate ratio.

Discussion

The fully sequenced genome of the microsporidian *E. cuniculi* was completely devoid of genes for MCF proteins, raising the question of how the highly reduced mitosome communicates with the rest of the cell. In particular, does the mitosome require ATP and if so how does it import it? We have now identified a single mitochondrial carrier protein in a distantly related microsporidian species, *A. locustae*. The *A. locustae* genome is not completely sequenced, and there is the possibility of other mitochondrial carrier proteins in this species. However, the function of the one found is noteworthy because our biochemical characterization reveals it to preferentially transport ATP and ADP with nearly similar affinities and maximal velocities and within the range of values for other characterized ADP/ATP transporters.[23]

This protein is a highly unusual carrier of ATP and ADP, since it is only distantly related to other AACs and lacks several sequence motifs characteristic of this type of carrier. One notable difference is the lack of a characteristic RRRMMM motif, which is common to most such carriers but lacking in a few such as the ADP/ATP translocases of *E. histolytica* and *Trichomonas* species.[18,21] Phylogenetic analyses indicate that the *A. locustae* protein is likely derived from some form of adenine nucleotide carrier, probably one that specifically transported folate, FAD^+, or NAD^+ and not a conventional AAC. Consistent with this, its activity is not inhibited by compounds such as BKA and CAT that are normally potent inhibitors of ADP/ATP transporters, likely because key residues that are known to interact with these toxins[29] are not conserved in the *A. locustae* protein. The protein therefore must have altered its function at some point in the evolution of microsporidia, and assuming no ATP synthesis takes place in the mitosome, to specifically transport ATP into the mitosome and ADP to the cytosol. This is reminiscent of the situation in *E. histolytica*,[18] and the recruitment of different members of the MCF to fulfill the process of ADP/ATP exchange may be a common theme in lineages where mitochondria are severely reduced or altered.

Several ATP-dependent proteins or pathways are known from both *A. locustae* and *E. cuniculi* and are inferred from phylogeny or targeting studies to be mitosomal.[11,12,41] In particular, the ABC ATM1 transporters typically involved in exporting iron–sulfur clusters from the mitosome and the mitochondrial Hsp70 typically involved in importing proteins into the matrix are both normally ATP dependent. It is therefore most likely that ATP is required by the organelle, but there is no obvious pathway to generate it in any microsporidian and there is no transporter to import it in *E. cuniculi*. In light of this, our characterization of the *A. locustae* MCF protein as an ADP/ATP transporter fills a critical gap in the metabolism of microsporidian mitosomes as it is currently reconstructed. It remains curious, however, why this protein is present in *A. locustae* but absent from *E. cuniculi*, which presumably still must import essential metabolites and nucleotides across its mitosomal membranes. It is possible that this process is carried out by some alternative mechanism yet to be recognized, or it may be that the *E. cuniculi* organelle is functionally

reduced to a greater extent than in *A. locustae*, as has been observed in the mitosomal protein-import machinery.[41] If this is the case, it represents a particularly interesting case of differential mitochondrial degeneration in different lineages of microsporidia, because the absence of any mechanism to import ATP into the organelle would have widespread implications for its function in metabolism.

Materials and Methods

Amplification and sequencing of the full-length MCF gene

The 5′ end of the putative MCF sequence from *A. locustae* was amplified using primers designed to a small fragment of the gene identified in an expressed sequence tag project from *A. locustae*.[25] RNA was extracted from *A. locustae* spores and PolyA purified for 5′ RLM-RACE (Ambion/Applied Biosystems) as described previously.[25] Fragments were amplified with Hi-Fidelity *Taq* polymerase (Roche) and cloned using the TOPO TA system (Invitrogen). Positive colonies were sequenced using Big Dye 3.1 (Applied Biosystems). The 5′ fragment was aligned to the previously identified fragment to give the full-length gene. These sequence data are accessible from the GenBank database under accession number AY952290.

Phylogenetic analysis

The inferred protein sequence was aligned to a selection of 47 other mitochondrial carrier proteins to include functionally annotated isoforms from the yeast genome and selected members of carriers of different substrates from a variety of species. These were aligned using T-coffee[42] and manually edited using MacClade 4.07[42,43] to create an alignment of 235 amino acids. Phylogenetic trees were inferred by maximum likelihood using PhyML 2.4.4[44] and ProML 3.6.[44,45] Site-to-site rate variation was modeled on a gamma distribution using one invariable rate category and eight variable rate categories estimated using TREE-PUZZLE 5.2.[38] Bootstrap analysis of 100 data sets was carried out using both methods as described above, except that four variable rate categories were used in ProML analyses.

Heterologous expression in *S. cerevisiae*

The full-length *A. locustae* MCF gene was cloned into the yeast expression vector p416met25HDEL with an in-frame downstream GFP gene and expressed in JK9 yeast cells. Cells were costained with Mitotracker Red (Molecular Probes, Invitrogen) and were visualized by fluorescence microscopy using a Zeiss Axioplan microscope.

Heterologous expression of the mitosomal carrier in *E. coli*

The full-length *A. locustae* MCF sequence was cloned into the expression vector pET16b and expressed in the *E. coli* strains BL21 or Rosetta™2 (DE3) pLysS (Novagen, Heidelberg). *E. coli* cells harboring the expression vector containing the full-length sequence were cultivated in YT medium under selective conditions. Protein expression was induced at an optical density (OD_{600}) of 0.5 by addition of IPTG and conducted as previously described.[46]

Transport measurements on intact *E. coli* cells

In accordance with previously reported uptake studies, induced or noninduced (control) *E. coli* cells harboring the expression construct were added to 100 µl of 50 mM potassium phosphate buffer (KP_i), pH 7.0, containing α^{32}P-labeled ATP, ADP, or NAD^+ at indicated concentrations.[23,46,47] Uptake was performed at 30 °C for the indicated time spans and terminated by removal of external substrate.[47] Radioactivity in the cells was quantified in a scintillation counter (TriCarb 2500, Canberra-Packard, Dreieich).

Additionally, back-exchange studies were performed with *E. coli* cells expressing the recombinant mitosomal MCF to analyze the transport mode of this carrier. Induced *E. coli* cells were incubated for 10 min in 50 µM α^{32}P-labeled ADP, sedimented (3000*g*, 1 min, room temperature), and washed three times in phosphate buffer. Back exchange was carried out at 30 °C in phosphate buffer medium containing 10-fold excess of nonlabeled indicated adenine nucleotides.[46,48] The nature of the α^{32}P-labeled exported nucleotide was analyzed by thin-layer chromatography[47,49] and detected by phosphoimaging (Cyclon, PerkinElmer, Boston).

Acknowledgements

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