Trichomonas hydrogenosomes contain the NADH dehydrogenase module of mitochondrial complex I

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Hydrogenosomes are double-membraned ATP-producing and hydrogen-producing organelles of diverse anaerobic eukaryotes[1]. In some versions of endosymbiotic theory they are suggested to be homologues of mitochondria[2–4], but alternative views suggest they arose from an anaerobic bacterium that was distinct from the mitochondrial endosymbiont[5,6]. Here we show that the 51-kDa and 24-kDa subunits of the NADH dehydrogenase module in complex I, the first step in the mitochondrial respiratory chain[7], are active in hydrogenosomes of *Trichomonas vaginalis*. Like mitochondrial NADH dehydrogenase, the purified *Trichomonas* enzyme can reduce a variety of electron carriers including ubiquinone, but unlike the mitochondrial enzyme it can also reduce ferredoxin, the electron carrier used[1] for hydrogen production. The presence of NADH dehydrogenase solves the long-standing conundrum of how hydrogenosomes regenerate NAD^+ after malate oxidation. Phylogenetic analyses show that the *Trichomonas* 51-kDa homologue shares common ancestry with the mitochondrial enzyme. Recruitment of complex I subunits into a H_2-producing pathway provides evidence that mitochondria and hydrogenosomes are aerobic and anaerobic homologues of the same endosymbiotically derived organelle.

The evolutionary origins of *Trichomonas* hydrogenosomes remain debated because, unlike mitochondria or plastids, they lack an associated genome[8]. Because the phylogenetic position of *Trichomonas* is unresolved, its relationship to mitochondrion-bearing eukaryotic lineages is also unclear[4]. Characters suggesting that *Trichomonas* hydrogenosomes and mitochondria share a common origin include the surrounding double membrane, a common mode of division, similar overall physiology, common protein import pathways and conserved mechanisms of iron–sulphur-cluster assembly[9–12]. However, *Trichomonas* hydrogenosomes also contain enzymes that are typically found in anaerobic bacteria[1], including pyruvate:ferredoxin oxidoreductase and the iron [Fe]-hydrogenase that makes hydrogen. The presence of these enzymes, which are atypical for mitochondria, has recently[6] been used to resurrect an old hypothesis[5] for the origins of hydrogenosomes through symbioses involving anaerobic bacteria distinct from the mitochondrial endosymbiont.

We identified homologues in *Trichomonas vaginalis* of the mitochondrial 51-kDa (NuoF, named Tvh-47) and 24-kDa (NuoE, named Tvh-22) subunits of the catalytic flavoprotein component of mitochondrial complex I (Fig. 3). Complex I, also called NADH:ubiquinone oxidoreductase, is the first part of the mitochondrial respiratory chain[7], so its presence in *Trichomonas*—which lacks cytochromes[1]—was unexpected. Conceptual translation of both genes revealed that they preserve key functional residues[7] (Fig. 3) and both contain putative leader sequences resembling those on proteins known to localize to the *Trichomonas* hydrogenosome[13]. Localization experiments on cell fractions with the use of a specific antibody raised against the recombinant *Trichomonas*

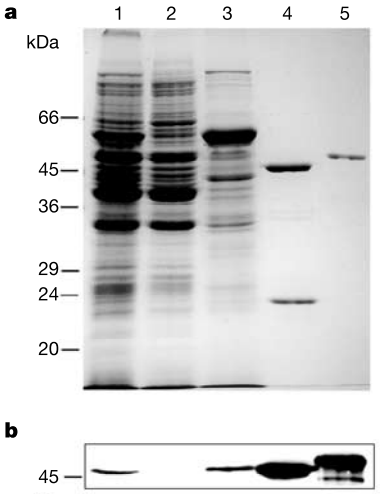

Figure 1 Localization of *Trichomonas* NADH dehydrogenase subunits. **a**, Coomassie-stained SDS–polyacrylamide-gel electrophoresis analysis of *Trichomonas* subcellular fractions: total homogenate (lane 1), cytosol (lane 2) and hydrogenosomes (lane 3). Lane 4, subunits of NADH dehydrogenase (Tvh-47 and Tvh-22 proteins (apparent molecular mass 47 and 23 kDa, respectively)) purified to homogeneity from *T. vaginalis* hydrogenosomes. Lane 5, recombinant Tvh-47 subunit (apparent molecular mass 54 kDa). **b**, Parallel analysis of the proteins by western blotting and the anti-Tvh-47 polyclonal antibody.

51-kDa homologue revealed that the protein is localized to the hydrogenosomal fraction (Fig. 1); this is confirmed by homologous transfection experiments demonstrating that Tvh-47 co-localizes with malic enzyme, a biochemical marker[14] for the *Trichomonas* hydrogenosome (Fig. 2). Mass spectrometry of the active NADH dehydrogenase purified from *Trichomonas* hydrogenosomes confirmed the identity of the Tvh-47 and Tvh-22 proteins (Figs 1 and 3).

Uncovering the evolutionary origins of these *Trichomonas* pro-

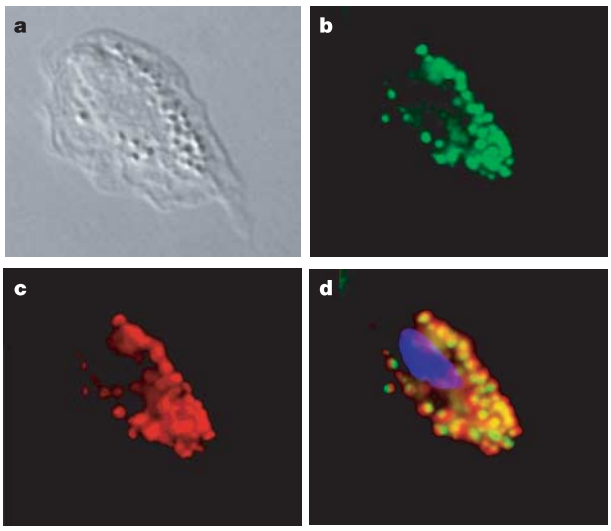

Figure 2 Localization of Tvh-47 within *Trichomonas* hydrogenosomes. **a**, Differential interference contrast image of a single transfected *Trichomonas vaginalis* cell. **b**, The same cell expressing an episomal plasmid containing Tvh-47 tagged with a carboxy-terminal haemagglutinin tag, probed with a mouse anti-haemagglutinin monoclonal antibody and Alexa Fluor-488 (green) donkey anti-mouse Ig. **c**, Localization of the hydrogenosomal marker protein malic enzyme, using rabbit anti-(malic enzyme) polyclonal antibody and Alexa Fluor-546 (red) donkey anti-rabbit Ig. **d**, Merge of **b** and **c** showing co-localization of the two proteins. The nucleus was stained blue with 4′,6-diamidino-2-phenylindole.

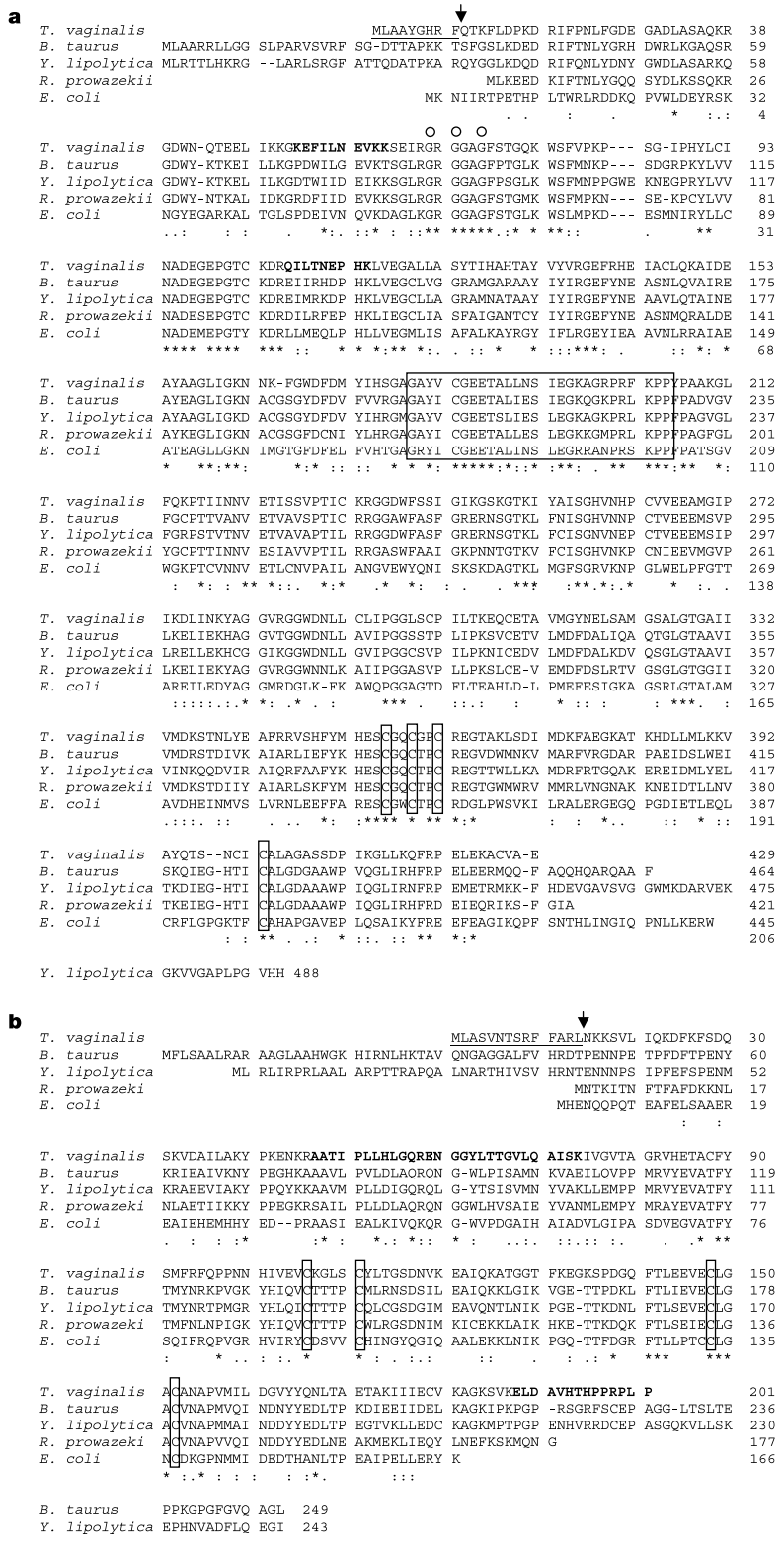

Figure 3 Sequences of NuoF and NuoE. **a**, Sequence alignment of *Trichomonas vaginalis* 47-kDa subunit of complex I (NuoF) with mitochondrial (*Bos taurus*, GenBank A39362; *Yarrowia lipolytica*, AAF65194) and eubacterial (*Rickettsia prowazekii*, NP_22050; *Escherichia coli*, NP_311195) homologues. Glycines of the NADH-binding motif $GXGGXGX_3G$ are indicated by open circles. The FMN-binding site (GA/MGA/RYV/ICGEERA/SLL/IE/NSL/IEG) and conserved cysteines motif coordinating the [4Fe–4S] cluster ($CX_2CX_2CX_{38-40}C$) are boxed. **b**, Sequence alignment of *T. vaginalis* 22-kDa subunit of complex I (NuoE) with mitochondrial (*Bos taurus*, B30113; *Yarrowia lipolytica*, CAB65523) and eubacterial (*Rickettsia prowazekii*, NP_220737; *Escherichia coli*, NP_311196) homologues. Invariable cysteines coordinating the [2Fe–2S] cluster ($CX_4CX_{35-36}CX_3C$) are boxed. N-terminal pre-sequences of *T. vaginalis* subunits are underlined. An arrow indicates cleavage sites predicted by PSORT II (http://psort.nibb.c.jp/). Sequences obtained by matrix-assisted laser desorption ionization Q-TOF are in bold. Asterisks indicate fully conserved residues, colons strongly conserved residues, and full stops weakly conserved residues.

teins pushes phylogenetics to its limits because, like many parasite proteins, the *Trichomonas* proteins are divergent (Fig. 3). Tvh-22 was too poorly conserved and short to yield much insight into its origins, so we concentrated on the longer Tvh-47. Initial phylogenetic analyses of Tvh-47 and 51-kDa proteins, using a gamma-correction for site-rate variation and the empirical WAG matrix[15], placed the *Trichomonas* sequence outside the mitochondrial and alpha-proteobacterial sequences. However, we also recovered alternative trees that placed the *Trichomonas* Tvh-47 protein within the mitochondrial clade that could not be rejected with the AU test[16]. Notably, the *Trichomonas* branch was much longer than the other branches in the tree and the Tvh-47 protein had a different amino acid composition from the other sequences, phenomena that are known to cause severe problems for phylogenetic analyses[17,18]. To mitigate these phenomena we recoded each amino acid according to the six groups of chemically related amino acids that commonly replace one another[15,19,20] and reanalysed the data. This recoding is related to transversion analysis and RY coding[21] of DNA sequences, and like these methods had the effect of shortening long branches and homogenizing the amino acid composition between sequences. It also allowed us to estimate a general time-reversible matrix specific to our data set. In these analyses, the best tree placed the *Trichomonas* Tvh-47 sequence within the mitochondrial clade (Fig. 4, Supplementary Table 1 and Supplementary Fig. 1). Thus, at the very least, our analyses cannot reject the hypothesis of a common origin for the *Trichomonas* and mitochondrial 51-kDa proteins, and the more sophisticated analysis prefers it.

Mitochondrial complex I catalyses the transfer of electrons from NADH to the lipid-soluble electron carrier ubiquinone in the respiratory chain[7]. The NADH dehydrogenase activity is associated with the 51-kDa and 24-kDa subunits of complex I. *Trichomonas* Tvh-47 and Tvh-22, which co-purify as an active heterodimeric enzyme from hydrogenosomes (Fig. 1, Supplementary Table 2), reduced, in an NADH-dependent manner, several electron carriers (Supplementary Table 3) including ubiquinone and *Trichomonas* 2Fe–2S ferredoxin. Kinetic data suggest that ferredoxin is the natural electron acceptor in the hydrogenosome (Supplementary Table 4). The *Trichomonas* enzyme is insensitive to rotenone, which blocks electron transfer in mitochondria by binding to complex I subunits that form the ubiquinone binding site; such units are missing from the *Trichomonas* enzyme.

The presence in *Trichomonas* hydrogenosomes of the NADH

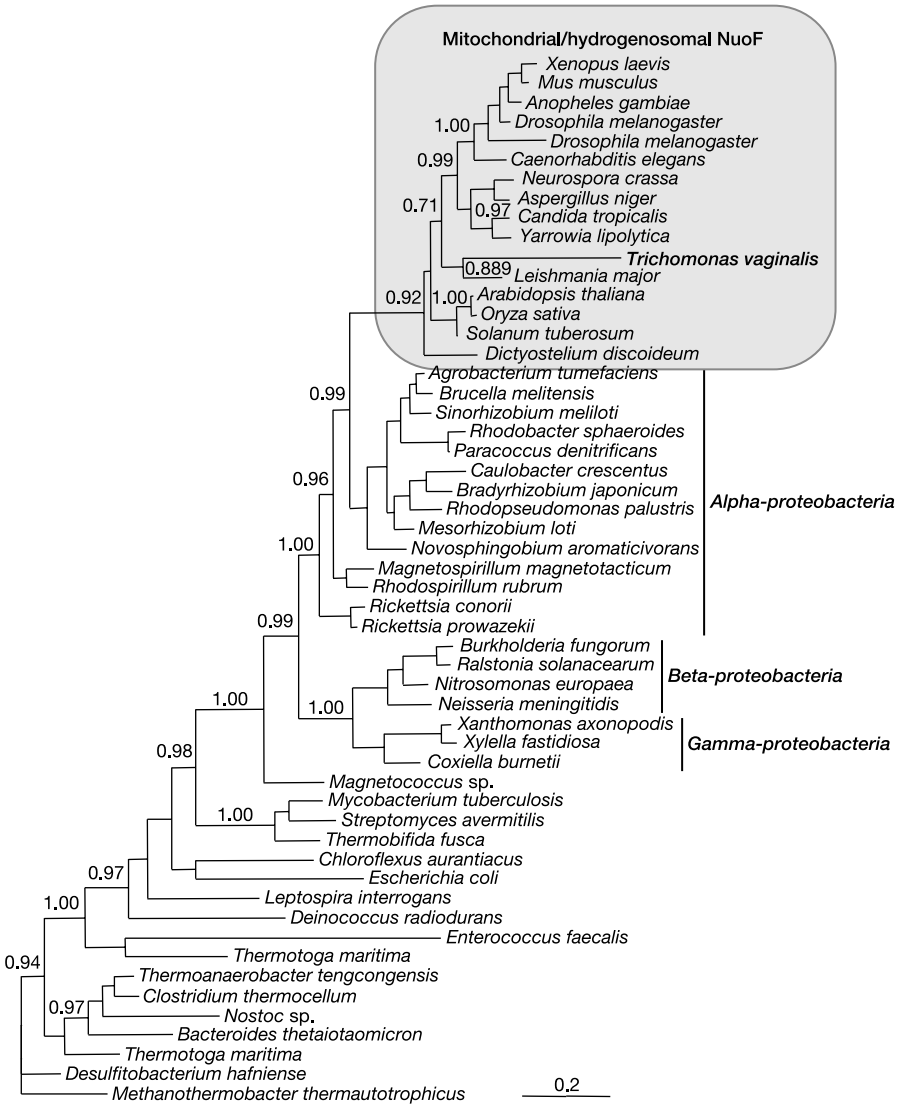

Figure 4 Phylogenetic analyses of NuoF homologues. Bayesian consensus tree of a general time-reversible substitution matrix estimated[18] from amino acid sequences recoded into the six Dayhoff groups: ASTGP, DNEQ, RKH, MVIL, FYW and C[19]. Among-site rate heterogeneity was modelled by using a four-category gamma correction with a fraction of invariant sites (pinvar). All parameters, including the composition and substitution rate matrix, were free, and the analysis used the Metropolis-coupled MCMC strategy from MrBayes[29]. Posterior probabilities for selected branches are shown at nodes. Scale bar indicates number of changes per site.

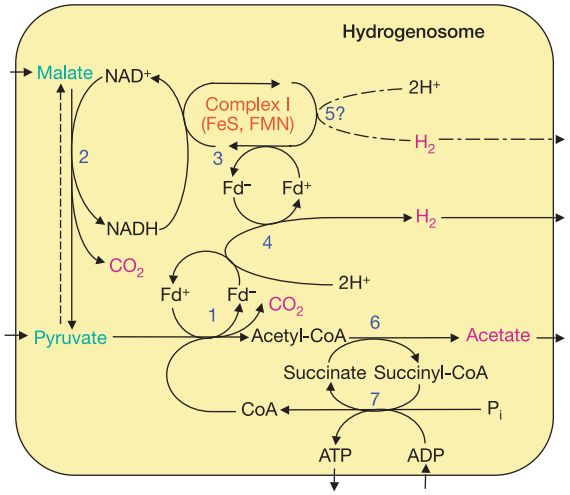

Figure 5 A schematic diagram illustrating the possible roles of NADH dehydrogenase from complex I in hydrogenosomal metabolism of *Trichomonas vaginalis*. Enzymes are indicated as follows: 1, pyruvate:ferredoxin oxidoreductase; 2, NAD-dependent malate dehydrogenase (decarboxylating); 3, NADH:ferredoxin oxidoreductase activity of the 51-kDa (Tvh-47) and 24-kDa (Tvh-22) catalytic flavoprotein component of complex I; 4, ferredoxin-dependent Fe-hydrogenase; 5, hypothetical NAD-dependent 65-kDa Fe-hydrogenase; 6, acetate:succinate CoA-transferase; 7, succinate thiokinase.

dehydrogenase components from mitochondrial complex I fills important gaps in the redox balance of hydrogenosome metabolism[22,23]. The enzyme can replenish the NAD^+ pool by reoxidizing NADH produced by the hydrogenosomal malic enzyme (Fig. 5). It can also catalyse the previously elusive NADH:ferredoxin oxidoreductase activity, to provide an alternative source of reducing power to that provided by pyruvate:ferredoxin oxidoreductase[1]. Its suspected interactions (Fig. 5) with the *Trichomonas* 65-kDa Fe-hydrogenase, which contains similar domains to the 75-kDa subunit of mitochondrial complex I (ref. 7) and to bacterial NAD-reducing hydrogenase[24], have yet to be demonstrated experimentally.

Our findings and previously published data[9–12] indicate that mitochondria and *Trichomonas* hydrogenosomes are homologues *sensu* Owen[25], meaning 'the same organ [here organelle] in different animals under every variety of form and function'. This hypothesis explains the shared similarities between the two organelles most simply and therefore is the one that must be rejected before alternative models are deemed necessary. Furthermore, the hypothesis[6] that the *Trichomonas* hydrogenosome could be the product of a second invasion of eukaryotic cells by an anaerobic bacterium does not account for the observation, unless further invasions are posited, that hydrogenosomes also occur in fungi[4] and diverse ciliate lineages[26]. The 'atypical anaerobic metabolic enzymes', whose presence was used to underpin this hypothesis[6], are also not unique to hydrogenosomes. A protein comprising a homologous pyruvate:ferredoxin oxidoreductase fused with NADPH-cytochrome P450 reductase occurs in *Euglena* mitochondria[27], and a homologous iron [Fe]-hydrogenase is found in the plastids of green algae and the cytosol of *Entamoeba* and *Giardia*[4]. By contrast, the presence of complex I components of mitochondrial ancestry in hydrogenosomes underscores their common endosymbiotic origin and uncovers a potential new route to hydrogen production in these anaerobic versions of mitochondria. □

Methods

Organisms, cultivation and cell fractionation

T. vaginalis strains T1 and G3 were maintained in Trypticase–yeast extract–maltose (TYM) medium with 10% heat-inactivated horse serum at 37 °C. Cytosolic and hydrogenosomal fractions were prepared as described[10]. Hydrogenosomes were isolated by isopycnic centrifugation on a 45% Percoll gradient[10].

Identification of a NuoF-like gene in *T. vaginalis*

A clone identified in a *T. vaginalis* G3 EST library encoding a nearly complete 51-kDa subunit/NuoF homologue was used to isolate a full-length sequence (Tvh-47) from a *T. vaginalis* T1 λ ZAPII genomic library[28].

Additional components of complex I on the *T. vaginalis* genome

Published sequences of eukaryotic complex I subunits were used in tBlastn searches to identify a homologue of the 24-kDa subunit of complex I (NuoE) on the genome of *T. vaginalis* strain G3 (TIGR: http://www.tigr.org/tdb/e2k1/tvg/).

Phylogenetic analyses

The *Trichomonas* NuoF and NuoE amino-acid sequences were aligned to a broad sample of homologous sequences from the NCBI. Bayesian phylogenetic analyses used MrBayes 3.0B4 (ref. 29) for amino acid alignments, and p4 (ref. 18) for alignments of recoded amino acids. In the latter, the amino acids were recoded into six categories corresponding to the PAM matrix (and most other matrices) as follows: (1) ASTGP, (2) DNEQ, (3) RKH, (4) MVIL, (5) FYW and (6) C. This allowed the use of a 6 × 6 general time-reversible rate matrix with free parameters rather than a fixed empirical matrix, composition and among-site rate variation parameters were also free.

Recombinant protein and antibody preparation

The complete Tvh-47 plus an amino-terminal His tag was expressed in *Escherichia coli* and purified under denaturing conditions on Ni(II)-nitrilotriacetate resin (Qiagen). A rabbit polyclonal antibody was raised against the recombinant protein as described[30].

T. vaginalis transformation

The plasmid TagVag-47 was constructed by amplifying the Tvh-47 ORF and ligating it into pCR2.1-TOPO plasmid (Invitrogen). The di-haemagglutinin ($(HA)_2$) epitope tag was amplified from Tviscs2–$(HA)_2$ (ref. 10) and cloned into pCR2.1-TOPO. A fragment containing Tvh-47 and $(HA)_2$ was then subcloned into pAP*luc*. The plasmid was digested by *Sac*II–*Nde*I and the 5′ untranslated region of AP65-1 was replaced by 340 base pairs of the 5′ untranslated region of *T. vaginalis* α-succinyl CoA synthetase. *T. vaginalis* cells were electroporated and selected in TYM medium supplemented with G418 (Sigma)[10].

Enzyme assays and purification of NADH dehydrogenase

For details of these procedures see Supplementary Information.

Recombinant ferredoxins

Recombinant *T. vaginalis* 2Fe–2S ferredoxin was produced as described[10]. Recombinant *Saccharomyces cerevisiae* mitochondrial 2Fe–2S ferredoxin, fused with a polyhistidine tag, was provided by A. Dancis (University of Pennsylvania).

Identification of peptides by mass spectrometry

Tryptic peptide fragments of purified NADH dehydrogenase subunits were sequenced by quadrupole time-of-flight mass spectrometry (Q-TOF) using an ULTIMA API nanospray mass spectrometer after separation by reverse-phase high-performance liquid chromatography (CapLC; Waters).

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Correspondence and requests for materials should be addressed to J.T. (tachezy@natur.cuni.cz) or T.M.E. (martin.embley@ncl.ac.uk). The sequences used in this paper have been deposited in the GenBank database under the accession numbers AY753550 and AY753551.

A candidate NAD$^+$ transporter in an intracellular bacterial symbiont related to Chlamydiae

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Bacteria living within eukaryotic cells can be essential for the survival or reproduction of the host[1,2] but in other cases are among the most successful pathogens[3,4]. Environmental Chlamydiae, including strain UWE25, thrive as obligate intracellular symbionts within protozoa; are recently discovered relatives of major bacterial pathogens of humans; and also infect human cells[4–7]. Genome analysis of UWE25 predicted that this symbiont is unable to synthesize the universal electron carrier nicotinamide adenine dinucleotide (NAD$^+$)[7]. Compensation of limited biosynthetic capacity in intracellular bacteria is usually achieved by import of primary metabolites[8–11]. Here, we report the identification of a candidate transporter protein from UWE25 that is highly specific for import of NAD$^+$ when synthesized heterologously in *Escherichia coli*. The discovery of this candidate NAD$^+$/ADP exchanger demonstrates that intact NAD$^+$ molecules can be transported through cytoplasmic membranes. This protein acts together with a newly discovered nucleotide transporter and an ATP/ADP translocase[12], and allows UWE25 to exploit its host cell by means of a sophisticated metabolic parasitism.

The genome sequence of UWE25 (ref. 7) contains five genes (*ntt1* to *ntt5*) with sequence homology to functionally characterized ATP/ADP translocases or nucleotide transporters (NTT) of other bacteria[13]. It is tempting to speculate that these genes encode transporters essential for survival of UWE25 by linking symbiont and host metabolism. However, the specific properties of these putative transporters can not be predicted from sequence analyses[14] but require heterologous expression in *E. coli*, leading to biochemical properties similar to the authentic carriers in their native membranes[14–18]. Heterologous expression of NTT1 of UWE25 and its biochemical characterization demonstrated that this transporter is used for energy parasitism by enabling uptake of host cell ATP in exchange with ADP[12].

In order to investigate whether NTT-type carriers of UWE25 also compensate for lack of nucleotide and NAD$^+$ biosynthesis, two further NTTs were functionally characterized in this study. NTT2 (516 amino acids) exhibits highest sequence identity (47%) to the ATP/ADP translocase CtNTT1 from *Chlamydia trachomatis*. NTT4 (431 amino acids) shows highest sequence identity (22%) to the plastidic ATP/ADP transporter AtNTT1 from *Arabidopsis thaliana*, exhibits nine predicted transmembrane domains, and lacks, in contrast to NTT2, several amino acid residues critical for nucleotide transport (Supplementary Fig. 1)[19].

Heterologously synthesized NTT2 transports ATP, GTP and UTP at high rates, but ADP import is much lower. This transport is specific because uninduced *E. coli* cells do not import labelled compounds (Supplementary Fig. 2a). This import closely resembles the characteristics of CtNTT2, a unidirectional nucleotide transporter from *C. trachomatis*[14]. The functional similarity of CtNTT2 and NTT2 is interesting, because the amino acid sequence of NTT2 exhibits the highest similarity to the ATP/ADP translocase CtNTT1 rather than CtNTT2 (Supplementary Fig. 1). Moreover, phylogenetic analysis indicates a closer relationship of UWE25 NTT2 to ATP/ADP translocases than to the chlamydial nucleoside-triphosphat uniporters (Supplementary Fig. 3). This observation illustrates that it is impossible to make reliable predictions on the transport characteristics of NTT-type proteins solely on the basis of amino acid sequence identities.

Heterologous expression of NTT4 demonstrated that this transporter does not accept nucleoside triphosphates, but instead catalyses ADP uptake. Uptake of radioactively labelled nucleotides by NTT4 depends upon induction of the corresponding gene (Supplementary Fig. 2b). Such a clear substrate preference for a nucleoside diphosphate, rather than nucleoside triphosphates, is unusual and prompted further transport analyses in the presence of putative inhibitors. Notably, the presence of unlabelled NAD$^+$ almost abolished [^{32}P]-ADP uptake, as did NADH and ADP, whereas NADP$^+$ and NADPH inhibited [^{32}P]-ADP uptake only slightly (Fig. 1a).

In order to reveal the properties of NTT4 in more detail, we analysed the transport of [adenylate-^{32}P]-labelled NAD$^+$. NTT4-expressing *E. coli* imported [^{32}P]-NAD$^+$ at a rate of about 80 nmol mg^{-1} of protein per hour, and the simultaneous presence of unlabelled NAD$^+$ (fivefold excess) inhibited uptake to less than 20% of the control rate (Fig. 1b). A high substrate specificity of NTT4 is further underlined by the fact that the substrate acceptance is determined in various domains of the NAD$^+$ molecule, as