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Short communication

Hydrogenosome-localization of arginine deiminase in *Trichomonas vaginalis*

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ABSTRACT

The arginine dihydrolase (ADH) pathway has an analogous function to the urea cycle in mitochondria-containing cells, by removing nitrogen from amino acids and generating ATP. Subcellular localization of the ADH pathway enzymes in *Trichomonas vaginalis* revealed that arginine deiminase (ADI) localizes to the hydrogenosome, a mitochondrion-like organelle of anaerobic protists. However the other enzymes of the ADH pathway, ornithine carbamyltransferase and carbamate kinase localize to the cytosol. Three gene sequences of *T. vaginalis* ADI (ADI 1–3) were identified in the *T. vaginalis* genome, all having putative mitochondrial targeting sequences. The ADI sequences were cloned and used to probe *T. vaginalis* using a carboxyterminal di-hemogglutinin epitope tag which demonstrated co-localization with malic enzyme confirming the hydrogenosome localization of this enzyme.

The arginine dihydrolase (ADH) pathway catalyzes the conversion of arginine to ornithine and ammonia via the enzymes arginine deiminase (ADI), catabolic ornithine carbamyltransferase (OCT), and carbamate kinase. Cumulatively the pathway removes nitrogen from arginine with the generation of ATP, and therefore performs an analogous function to the urea cycle of vertebrates. The ADH pathway is present in some protists such as *Trichomonas vaginalis* [17] and *Giardia intestinalis* [15], as well as some Gram positive bacteria, e.g. *Streptococcus* sp. [8], Gram negative bacteria, e.g. *Pseudomonas* sp. [1], and some Mollicutes, e.g. *Mycoplasma hominis*, *M. genitalium* [10], where it has been proposed to function as an alternative ATP generating mechanism. The enzymes of the pathway have been characterized in several organisms notably *Mycoplasma* sp., *G. intestinalis*, *T. vaginalis* and *Tritrichomonas foetus* [12,17,18]. Based upon subcellular studies with *T. vaginalis* all enzymes of the pathway were localized in the cytosol with the exception of arginine deiminase (ADI), which was mainly associated with sedimentable cell components, and its co-localization with the plasma membrane was suggested [18]. The availability of the complete genome sequence for *T. vaginalis* [6] enables studies for the localization of ADI in *T. vaginalis* to be confirmed. BLAST search of the *T. vaginalis* genome database (TrichoDB, http://trichdb.org/trichdb/) using *G. intestinalis* ADI (accession number XP_001705755) as query revealed the presence of three copies of the ADI gene (TvADI-1, TVAG_467820; TvADI-2, TVAG_344520 and TvADI-3, TVAG_183850) coding for proteins with calculated molecular weight 46–47,000. Using ClustalX [14] and BioEdit software the *T. vaginalis* sequences were aligned with *Mycoplasma arginini* for which a crystal structure has been determined [2], which revealed the presence of conserved residues involved in the substrate binding and/or the enzyme active site (supplementary Fig. s1). The positional equivalent of the catalytic triad Cys397, His268, Glu213 determined in *M. arginine* ADI [2] is conserved in the TvADI-2 (Cys408, His283, Glu230) and TvADI-3 (Cys405, His281, Glu228), while the Cys405 is replaced by Ser405 in TvADI-1 (Fig. s1). All three putative TvADI sequences contained mitochondria-like N-terminal targeting pre-sequences with predicted cleavage site for processing peptidase [3] (Fig. s1) and high probability of mitochondrial localization estimated by PSORT II (http://psort.hgc.jp/) (56–65%) and TargetP (mTP values 0.500–0.710). These predictions suggested the localization of ADI with *T. vaginalis* hydrogenosomes, an anaerobic form of mitochondria in these parasites. To investigate cellular localization three genes coding for *T. vaginalis* ADI were amplified by PCR from

Abbreviations: ADH, arginine dihydrolase; ADI, arginine deiminase; OCT, ornithine carbamyltransferase.

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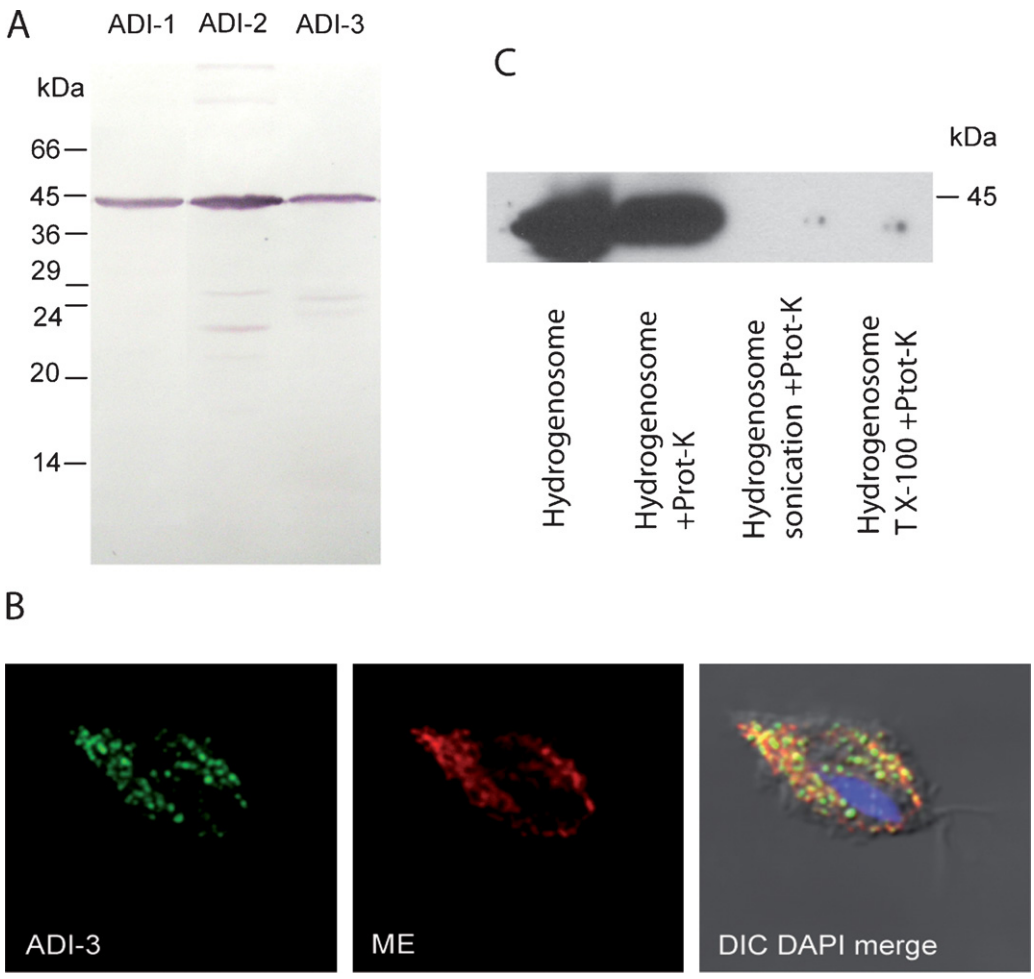

Fig. 1. Expression of ADI-1, ADI-2 and ADI-3 genes in *T. vaginalis*. (a) Each gene was expressed with C-terminal hemagglutinin tag in *T. vaginalis* and detected in cellular lysates by immunoblotting with anti-hemagglutinin monoclonal antibody. (b) Representative localization of ADI in *T. vaginalis* hydrogenosomes by confocal fluorescent microscopy. ADI-3, fluorescent microscopy of *T. vaginalis* expressing ADI-3. Mouse monoclonal anti hemagglutinin (ADI-HA) tag antibody was used to visualize ADI-3 (green); ME, fluorescent microscopy of rabbit polyclonal anti malic enzyme antibody (red); DIC DAPI merge, merged image of ADI-3-HA, ME image stained with DAPI (blue) to show the nucleus. (c) ADI localizes to the inside of hydrogenosomes. Treatment of intact hydrogenosomes with proteinase K (Prot-K) did not affect signal for ADI-1, while ADI-1 was not detected by immunoblotting when hydrogenosome membranes were disintegrated by sonication or Triton X-100 (T X-100) and subsequently treated with proteinase K. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

genomic DNA of *T. vaginalis* (strain T1) and sub-cloned to TagVag vector, which allows episomal expression of recombinant protein with carboxyterminal di-hemogglutinin epitope tag [4]. The molecular weight of the expressed proteins corresponded well with calculated values (Fig. 1a). *T. vaginalis* cells were transfected as described [4] and probed with a mouse monoclonal anti hemagglutinin antibody [4] (Fig. 1b). A hydrogenosome marker protein malic enzyme was used as a positive control and detected using a rabbit polyclonal anti malic enzyme antibody [4]. Images were obtained using immunofluorescence confocal microscopy which revealed that all three gene products localized to organelles organized along the axostyle and costa. A typical result of localization is shown for ADI-3 which co-localizes with the hydrogenosome protein marker, malic enzyme (Fig. 1b), ADI-1 and ADI-2 demonstrated similar results (not shown). To distinguish whether ADI is present inside the organelle or associated with the outer hydrogenosome membrane, hydrogenosomes were isolated from ADI-1 trasfected cells as described [5]. Intact, or disintegrated hydrogenosomes prepared by sonication or 0.5% Triton X-100, were treated with 50 μg/ml proteinase K for 20 min at 0 °C. The digestion was inhibited with 1 mM phenylmethylsulfonyl fluoride; the samples were acetone-precipitated and analysed by immunoblotting. While protease K treatment of intact hydrogenosomes did not affect ADI-1 signal, it was not detected when hydrogenosome membranes were disintegrated (Fig. 1c). This result strongly suggests that ADI-1 is present inside the hydrogenosome.

Subcellular localization of ADI activity was performed by differential centrifugation of whole cell extracts using 225 mM sucrose pH 8.0 in 15 mM Tris and 10 mM KCl. ADI activity was determined by measuring the formation of citrulline using 1 mM L-arginine in 40 mM HEPPS pH 8.0 [18]. In previous studies a pH of 6.0 was used which is sub-optimal for the *T. vaginalis* enzyme [18]. Under the assay conditions used in this study ADI was predominantly found in the hydrogenosome enriched fraction (Fig. 2a) based upon the distribution of malic enzyme, a marker for hydrogenosome-enriched fractions. The activity of malic enzyme was determined by measuring the absorbance change at 340 nm using 6 mM malate, 1 mM NAD and 0.66 mM $MnCl_2$ in pH 6.8 triethanolamine buffer [18] (Fig. 2b). Catabolic OCT was determined by measuring $^{14}CO_2$ release from L-[^{14}C-carbamyl] citrulline (17.2 mM L-[^{14}C-carbamyl] citrulline, 57.7 mCi/mmol; DuPont, N.E.N. Research Products) in 40 mM MES pH 6.0 [18]. As shown previously, catabolic OCT, which in aerobic eukaryotes localizes to the mitochondrion was found predominantly in the non-sediment able fraction (Fig. 2c) [18]. Integrity of the organelles was confirmed by performing all enzyme assays in isotonic buffered solutions (225 mM sucrose) to which