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Acetate:Succinate CoA-transferase in the Hydrogenosomes of *Trichomonas vaginalis*

IDENTIFICATION AND CHARACTERIZATION[*] [S]

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Acetate:succinate CoA-transferases (ASCT) are acetate-producing enzymes in hydrogenosomes, anaerobically functioning mitochondria and in the aerobically functioning mitochondria of trypanosomatids. Although acetate is produced in the hydrogenosomes of a number of anaerobic microbial eukaryotes such as *Trichomonas vaginalis*, no acetate producing enzyme has ever been identified in these organelles. Acetate production is the last unidentified enzymatic reaction of hydrogenosomal carbohydrate metabolism. We identified a gene encoding an enzyme for acetate production in the genome of the hydrogenosome-containing protozoan parasite *T. vaginalis*. This gene shows high similarity to *Saccharomyces cerevisiae* acetyl-CoA hydrolase and *Clostridium kluyveri* succinyl-CoA:CoA-transferase. Here we demonstrate that this protein is expressed and is present in the hydrogenosomes where it functions as the *T. vaginalis* acetate:succinate CoA-transferase (*Tv*ASCT). Heterologous expression of *Tv*ASCT in CHO cells resulted in the expression of an active ASCT. Furthermore, homologous overexpression of the *Tv*ASCT gene in *T. vaginalis* resulted in an equivalent increase in ASCT activity. It was shown that the CoA transferase activity is succinate-dependent. These results demonstrate that this acetyl-CoA hydrolase/transferase homolog functions as the hydrogenosomal ASCT of *T. vaginalis*. This is the first hydrogenosomal acetate-producing enzyme to be identified. Interestingly, *Tv*ASCT does not share any similarity with the mitochondrial ASCT from *Trypanosoma brucei*, the only other eukaryotic succinate-dependent acetyl-CoA-transferase identified so far. The trichomonad enzyme clearly belongs to a distinct class of acetate:succinate CoA-transferases. Apparently, two completely different enzymes for succinate-dependent acetate production have evolved independently in ATP-generating organelles.

Trichomonas vaginalis is the most prevalent sexually transmitted parasite with annually over 100 million cases worldwide. This parasite is an aerotolerant anaerobe that lacks mitochondria, the characteristic energy producing organelle of most eukaryotic organisms. However, trichomonads do contain other double membrane-bound organelles (1, 2) called hydrogenosomes (3), which are evolutionarily related to mitochondria (4–9). These hydrogenosomes convert pyruvate into acetate and carbon dioxide, resulting in hydrogen formation and ATP production (10–12).

Upon entry into the hydrogenosome, pyruvate is oxidatively decarboxylated by pyruvate:ferredoxin oxidoreductase (PFO,[3] EC 1.2.7.1), yielding carbon dioxide and acetyl-CoA. The electrons released in the oxidation of pyruvate by PFO are transferred to ferredoxin and subsequently to protons, producing hydrogen gas. The acetyl-CoA formed by PFO is converted into acetate by an acetate:succinate CoA-transferase (ASCT), which transfers the CoA-group from acetyl-CoA to succinate. The succinyl-CoA derived from this reaction is then converted back into succinate by succinyl-CoA synthetase (SCS, EC 6.2.1.4), thereby producing ATP (12). ATP production in hydrogenosomes occurs exclusively via substrate level phosphorylation, using this ASCT/SCS cycle. ASCT is the only enzyme involved in *T. vaginalis* hydrogenosomal carbohydrate metabolism that has never been identified. In fact, acetate is not only produced in the hydrogenosomes of the parabasalid *T. vaginalis*, but also in the hydrogenosomes of certain ciliates (*e.g. Nyctotherus ovalis*), amoeboflagellates (*e.g. Psalteriomonas lanterna*) and in chytridiomycete fungi (*e.g. Neocallimastix*) (3, 12–14). However, the enzyme responsible for acetate formation has never been identified in any hydrogenosomal organism.

In addition to hydrogenosomes, ASCT activity is also known to be present in the anaerobically functioning mitochondria of metazoa that produce acetate, such as those of the parasitic helminths *Fasciola hepatica* and *Ascaris suum* (15, 16). Moreover, ASCT activity has been identified in the strictly aerobically functioning mitochondria of procyclic forms of the trypanosomatid *Trypanosoma brucei* (17). Therefore, the ASCT/SCS cycle is the only catabolic pathway present in both

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[3] The abbreviations used are: PFO, pyruvate:ferredoxin oxidoreductase; ASCT, acetate:succinate CoA-transferase; *Tv*ASCT, *T. vaginalis* acetate:succinate CoA-transferase; UTR, untranslated region.

mitochondria and hydrogenosomes. Identification of genes encoding mitochondrial or hydrogenosomal ASCT could therefore provide valuable information to unravel the evolutionary relation between these organelles.

The formation of acetate from acetyl-CoA as an end product of energy metabolism is observed in many prokaryotes and in a few eukaryotic species, all predominantly living in anaerobic or hypoxic environments (18, 19). Besides the formation of acetate by ASCT activity (Fig. 1, *reaction 1*), three alternative pathways for acetate production from acetyl-CoA have been identified: (i) acetate can be produced from acetyl-CoA via a two-step mechanism in which acetyl-phosphate occurs as an intermediate, involving the enzymes phosphate acetyltransferase (EC 2.3.1.8) and acetate kinase (EC 2.7.2.1), as occurs in many eubacteria (Fig. 1, *reaction 2*) (20); (ii) acetate can be produced from acetyl-CoA via an acetyl-CoA synthetase (EC 6.2.1.13, also known as acetate thiokinase) a process that concomitantly produces ATP from ADP in a reaction that is known to occur in several archaea and in the amitochondriate protists *Giardia intestinalis* and *Entamoeba histolytica*, (Fig. 1, *reaction 3*) (21, 22); (iii) furthermore acetate can be produced via hydrolysis of acetyl-CoA catalyzed by an acetyl-CoA hydrolase (EC 3.1.2.1), as is present in *Saccharomyces cerevisiae* (23, 24) (Fig. 1, *reaction 4*).

The first gene encoding a eukaryotic ASCT was identified in *T. brucei* (25). This gene showed high sequence similarity to mammalian succinyl-CoA:3-oxoacid CoA-transferase (SCOT, EC 2.8.3.5). However, no sequence homolog of this ASCT gene from *T. brucei* could be detected in the *T. vaginalis* genome data base at TIGR. On the other hand, we could identify in the *T. vaginalis* data base another candidate for acetate production by BLAST searches using the sequence of acetyl-CoA hydrolase from *S. cerevisiae* (ACH1) (van Weelden, PhD thesis, Utrecht University, 2005). Earlier studies showed that some of the gene products of the acetyl-CoA hydrolase/transferase family actually function as CoA transferases (26, 27). In this study we investigated the expression, localization, and enzymatic function of this acetyl-CoA hydrolase/transferase homolog in *T. vaginalis*. We describe the first hydrogenosomal ASCT enzyme and demonstrate that it has a completely independent origin compared with the mitochondrial ASCT from *T. brucei.*

EXPERIMENTAL PROCEDURES

Purification of Native TvASCT—T. vaginalis NIH-C1 strain (ATCC 30001) was cultured as described (28) and trichomonads from 120 ml of culture were collected by centrifugation at 1300 × *g* for 3 min and washed in 150 mM NaCl solution before being homogenized by ultrasonication (Soniprep 150) in 1.4 ml of milliQ water. Subsequently 100 μl of 1% (v/v) Triton X-100 was added, and the homogenate was centrifuged for 30 min at 10,000 × *g* at 4 °C. The resulting supernatant was used for purification of *Tv*ASCT by column chromatography.

MonoQ anion exchange chromatography was performed with a monoQ HR5/5 FPLC column (Amersham Biosciences Fine Chemicals, Uppsala, Sweden). The column was equilibrated with 15% (v/v) glycerol in 20 mM Hepes pH 8.3, at a flow rate of 0.5 ml/min, after which 1 ml of supernatant was loaded. UV absorption was recorded at 254 nm and fractions of 2 ml were collected during the entire run. The column was eluted for 10 min with 15% (v/v) glycerol in 20 mM Hepes pH 8.3, followed by a linear gradient increasing from 0 mM NaCl to 200 mM NaCl in 64 min, and from 200 mM NaCl to 1 M NaCl in 24 min.

ASCT Activity Assay—The activity of ASCT was determined by a radioactive assay as described before (17). In brief, the assay mix contained 1 mM [1-^{14}C]acetyl-CoA (0.2 MBq, Amersham Biosciences), 50 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 50 mM succinate (pH 7.4), and 0.05% (v/v) Triton X-100. The assay was started by addition of the lysate, incubated at 20 °C for 30 min, and terminated by the addition of ice-cold trichloroacetic acid (6% w/v, final concentration). Reaction products were then separated by anion-exchange chromatography and quantified by liquid scintillation counting.

Gene Identification and Recombinant Expression—BLAST searches (29) for acetyl-CoA hydrolase related sequences in the available *T. vaginalis* TIGR genome data base were performed using acetyl-CoA hydrolase from *S. cerevisiae* (ACH1 YBL015W) as a template. Based on the obtained sequence (corresponding to contig 921979, annotated in GenBank™ as XM_001330141) two specific oligonucleotides containing BamHI and PstI sites (underlined) at the initiating methionine and 90-bp downstream of the stop codon, respectively, were designed: 5′-TATAGGATCCATGTTGTCATCATCTTCACGTGCTATC-3′ and 5′-TATACTGCAGAAATTATACCACATTCCCACCCTC-3′. PCR amplification was performed and the predicted 1600-bp DNA fragment was purified from the gel and ligated into the eukaryotic expression vector pCDNA3.1+ and the bacterial expression vector pQE30. The sequences of the resulting constructs were verified by sequencing. *Tv*ASCT (in pCDNA 3.1+) was transfected into CHO K-1 cells using Lipofectamine Reagent (Invitrogen, Karlsruhe, Germany), following the standard, manufacturer's protocol. Cells were harvested 24 h after transfection.

Antibody Preparation—Recombinant *Tv*ASCT (in pQE30) was expressed by M15 (pREP4) *Escherichia coli* and purified from inclusion bodies by subsequent lysis with lysosyme (1 mg/ml), sonication (Soniprep 150), and centrifugation at 10,000 × *g* for 30 min at 4 °C. The pellet was washed two times with 1% (v/v) Triton X-100 in phosphate-buffered saline (PBS) and 1 time with PBS only, after which the pellet was dissolved in 8 M urea, 50 mM glycine, 100 mM Tris-HCl, pH 8.0. Solubilized recombinant *Tv*ASCT was loaded on an SDS-PAGE gel (9%) and 400 mg of the 57-kDa recombinant protein was excised. The excised protein was confirmed to be *Tv*ASCT by nano-electrospray tandem mass spectrometry on Q-TOF2 (see below), after which antisera against *Tv*ASCT were raised in rabbits by Eurogentec (Liège, Belgium).

Western Blot Analysis—Samples for Western blot analysis were subjected to SDS-PAGE on a 7 or 10% gel and blotted onto nitrocellulose membranes. Blots were blocked with PBS-Tween (0.05%, v/v) containing 5% (w/v) dried milk powder and incubated with 1:10,000 diluted anti-*Tv*ASCT rabbit serum in PBS-Tween (0.05%, v/v) containing 5% (w/v) dried milk powder. Subsequently, blots were washed three times with PBS-Tween (0.05%, v/v), followed by incubation with 1:10,000 GAR/IgG(H+L)/PO immunoconjugate (Nordic Immunology, Tilburg, The Netherlands or Sigma-Aldrich, München, Germany) in PBS-Tween (0.05%, v/v) containing 5% (w/v) dried milk pow-

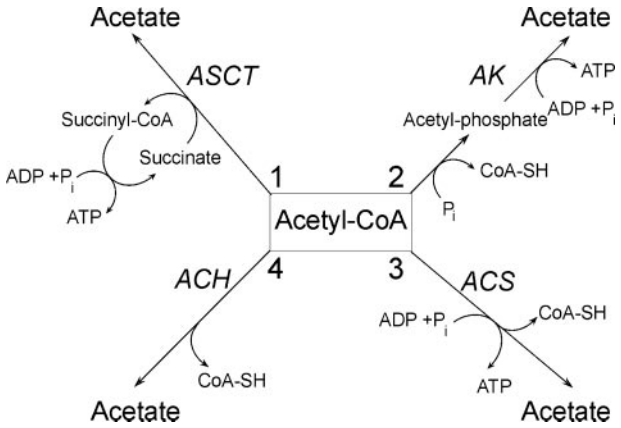

FIGURE 1. **Enzymatic conversion of acetyl-CoA into acetate.** In prokaryotes and eukaryotes four distinct possibilities have been described for the conversion of acetyl-CoA into acetate. In reaction 1, acetate production via ASCT is depicted. Reaction 2 shows the two-step pathway using phosphate acyltransferase and acetate kinase. Reaction 3 depicts acetate production via acetyl-CoA synthetase, and reaction 4 schematically represents the hydrolysis of acetyl-CoA, as performed by acetyl-CoA hydrolase. Abbreviations: *ASCT*, acetate:succinate CoA-transferase; *AK*, acetate kinase; *ACH*, acetyl-CoA hydrolase; *ACS*, acetyl-CoA synthetase.

der. After washing three times more with PBS-Tween (0.05%, v/v), signals were visualized using Supersignal chemiluminescent substrate (Pierce). Signals were detected and quantified using a Molecular Imager Chemidoc XRS System with Quantity One software (version 4.6.3) (Bio-Rad) or Lumi-Film Chemiluminescent Detection films (Roche Applied Science). Hydrogenosome isolation, two-dimensional electrophoresis, protein in-gel digestion with trypsin, mass spectrometry, and protein identification were carried out as described (30).

Neighbor-Net Analysis—Protein sequences were retrieved from GenBank™ and aligned using ClustalW (31). Protein LogDet distances (32) were calculated with the LDDist program (33). All gapped positions in the alignment were removed. The network of sequence similarity was constructed with Neighbor-Net (34) and visualized with Splitstree (35). The dataset is available upon request.

Selectable Transformation of T. vaginalis Strain T1—The *Tv*ASCT gene was amplified with the specific primers described above but containing NdeI and BamHI sites and introduced into plasmid pTagvag2 (36) between a *Tv*α-SCS 5′-UTR and a *Tv*malic enzyme 3′-UTR to yield pTagvagASCT. *T. vaginalis* T1 cells were electroporated with pTagvagASCT and selected in TYM medium supplemented with G418 (Sigma-Aldrich).

Northern Blot Analysis—Total RNA was purified from *T. vaginalis* cells with the TRIzol reagent (Invitrogen, Karlsruhe, Germany) following the manufacturer's instructions. RNA was transferred to HybondN nylon membrane (GE Healthcare, München, Germany) and hybridized with the radioactively labeled coding sequences of *Tv*ASCT or *Tv*Actin. Signals were detected on SR imaging plates with a FLA3000 Fluorescence scanner version 1.8E and quantified with the Image Gauge software (version 3.0) (Fuji Photo film Co., Düsseldorf, Germany).

RESULTS

Detection of ASCT Candidate Gene—Four different enzymes are known to catalyze the conversion of acetyl-CoA into acetate (Fig. 1). The *T. vaginalis* genome was analyzed for the presence of homologs of the acetate:succinate CoA transferase of *T. brucei*, acetate kinases and acetyl-CoA synthetases of several organisms, and acetyl-CoA hydrolase of *S. cerevisiae*. For the first three acetate producing enzymes no significant sequence homologs could be detected in the data base, only for the latter one, the acetyl-CoA hydrolase, significant hits were found. Four high-scoring segment pairs (TVAG_395550, TVAG_113870, TVAG_164890, TVAG_393850) were identified with an open reading frame of 1569 bp encoding a polypeptide of 523 amino acids. The calculated molecular mass of this protein is 57 kDa with a theoretical pI of 7.99 (ProtParam tool). Computer analysis of the predicted protein revealed 55% sequence identity to acetyl-CoA hydrolase from *S. cerevisiae*.

Data base searches with the derived amino acid sequence of this acetyl-CoA hydrolase homolog from *T. vaginalis* produced no significant matches to typical eukaryotic homodimeric CoA-transferases from eukaryotes such as succinyl-CoA:3-oxoacid CoA-transferase from human or acetate:succinate CoA-transferase from *T. brucei*. However, significant similarity was observed with a family of proteins of both eukaryotic and prokaryotic species. These proteins are annotated as acetyl-CoA hydrolase/acetyl-CoA transferase/succinyl-CoA:CoA-transferase (Fig. 2). Here we define the *T. vaginalis* homolog of this family of proteins as *T. vaginalis* acetate:succinate CoA-transferase (*Tv*ASCT), as our further experiments showed that this *T. vaginalis* gene codes for an acetate:succinate CoA transferase.

Anti-TvASCT Antibody Generation—For Ab generation *Tv*ASCT was expressed in *E. coli*, which resulted in minor amounts of soluble *Tv*ASCT and predominantly in insoluble *Tv*ASCT in inclusion bodies. Neither the complete lysate containing all soluble proteins, nor soluble *Tv*ASCT purified on nickel-nitrilotriacetic acid resin (Qiagen) showed any detectable ASCT activity. This is in contrast to recombinant expression of the *Clostridium kluyveri* succinyl-CoA:CoA transferase (*cat1*), in *E. coli*, which was active, even though measured enzyme activities were very low (27). *Tv*ASCT purified from inclusion bodies was subsequently used for antibody generation, which resulted in rabbit sera containing polyclonal antibodies against *Tv*ASCT. These antibodies specifically recognized the purified recombinant His tag *Tv*ASCT, the recombinant His tag *Tv*ASCT in a total lysate of transfected *E. coli*, and also the native *T. vaginalis* ASCT in a total lysate (Fig. 3). On the other hand, these antibodies did not recognize any other protein present in a total lysate of *E. coli* transfected with an empty vector. To further confirm the specificity of the anti-*Tv*ASCT antibodies, proteins of the complete hydrogenosomal fraction of *T. vaginalis* were separated on a two-dimensional gel, blotted and probed with the *Tv*ASCT antiserum. This resulted in specific detection of only *Tv*ASCT (Supplementary data) because the spots detected were confirmed to be *Tv*ASCT by ESI-Q-TOF-MS/MS analysis.

Expression and Subcellular Localization of TvASCT—Two specific oligonucleotides were designed against the open reading frame in contig 921979. This ORF corresponds to the *T. vaginalis* gene XM_001330141 in GenBank™. A 1569-bp fragment could be amplified by PCR using *T. vaginalis* cDNA.

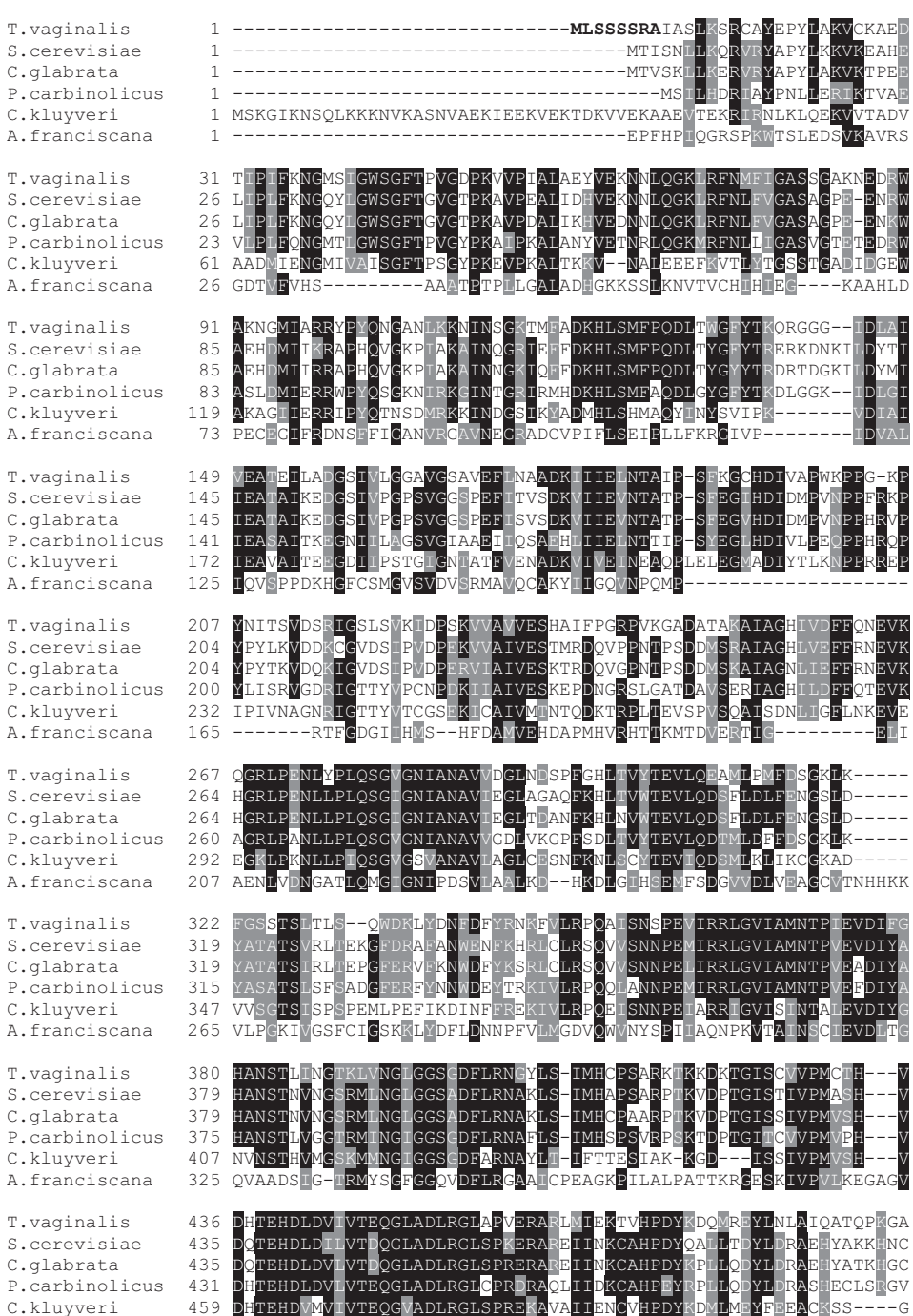

FIGURE 2. **Alignment of acetyl-CoA hydrolases/transferases.** Alignment of the gene product of contig 921979 from *Tv*ASCT, with the gene products from *S. cerevisiae ACH1*, P32316; *Candida glabrata* Q6FPF3; *Pelobacter carbinolicus* YP357793; *Clostridium kluyveri Cat1*, P38946, and *Artemia franciscana* AAP68833. Identical residues among 4 of 6 aligned proteins are shown in *black*, whereas conserved residues among 4 of 6 aligned proteins are *shaded*. The putative N-terminal targeting sequence of *Tv*ASCT is printed in *bold*.

The fragment was isolated and sequence analysis confirmed the amplification of the *Tv*ASCT gene. This result clearly demonstrates that the gene is expressed at the mRNA level.

ASCT activity in *T. vaginalis* has been localized to hydrogenosomes (12), and therefore, the *Tv*ASCT protein sequence is expected to contain a mitochondrial-like targeting sequence, as the import system into hydrogenosomes is comparable to that of mitochondria (37–39). The eight N-terminal amino acids of *Tv*ASCT (Fig. 2) and of its three paralogs in the *T. vaginalis* genome (Supplemental data), strongly fit the consensus of hydrogenosomal targeting sequences (40). The programs TargetP (41), WoLF Psort (42), and MitoPred (43) predicted *Tv*ASCT to contain a mitochondrial targeting sequence. Therefore *Tv*ASCT expression at the protein level was investigated in purified hydrogenosomes.

Hydrogenosomal proteins of *T. vaginalis* were separated by isoelectric focusing in the first dimension and by SDS-PAGE in the second dimension. Two 57-kDa protein spots were excised from the Coomassie-stained gels, digested with trypsin and analyzed by ESI-Q-TOF-MS/MS (Supplemental data). Sequenced peptides were compared with the *T. vaginalis* genome data base (TIGR) and all originated from the same ORF in contig 921979 (Table 1). A Western blot of cellular fractions probed with anti-*Tv*ASCT antibody showed strong accumulation of *Tv*ASCT in the hydrogenosomal fraction (Fig. 4). These observations clearly demonstrate that the *Tv*ASCT protein is expressed and located inside hydrogenosomes. The *Tv*ASCT gene encodes a protein with ASCT activity.

Expression of TvASCT in CHO Cells—CHO K1 cells were transfected with *Tv*ASCT, and the cells were harvested after 24 h. The cells transfected with *Tv*ASCT showed a 15-fold increase in ASCT activity, whereas cells containing the empty vector showed no increased ASCT activity (Fig. 5). The amount of ASCT protein in transfected CHO cells was, however, very low and the average ASCT activity was 7 nmol/min per mg protein, which is two orders of magnitude lower than the ASCT activity in *T. vaginalis* cells (Fig. 6). The observed conversion of a minute

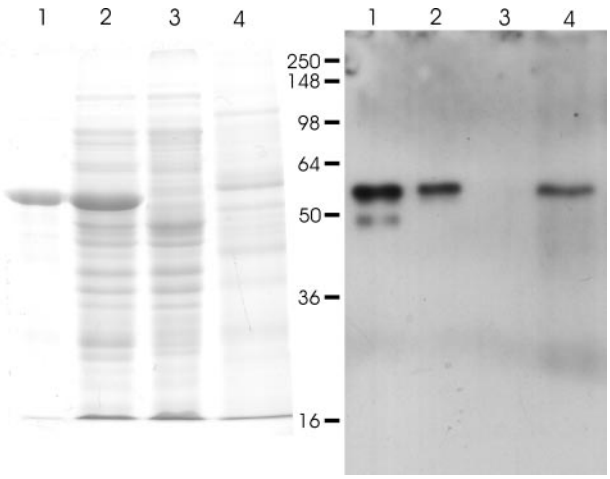

FIGURE 3. **Specificity of antiserum against *Tv*ASCT.** Coomassie-stained SDS-PAGE gel and a Western blot probed with the antiserum raised against recombinant *Tv*ASCT. The following protein samples were loaded; purified recombinant His-tagged *Tv*ASCT (*lane 1*), a total lysate of *E. coli* M15(REP4) transfected with pQE30-*Tv*ASCT (*lane 2*), or an empty pQE30 vector (*lane 3*), and a total lysate of *T. vaginalis* (*lane 4*). For the Coomassie-stained gel, 5 mg of protein was loaded in *lane 1* and 50 mg of protein in *lanes 2–4*. For the Western blot, 10 μg of protein was loaded in *lanes 1–3*, whereas 10 mg of protein was loaded in *lane 4*.

TABLE 1

Peptide sequences of protein spots S54 and S55

Mass (Da)	Sequence[a]	*Tv*ASCT[b]
1114	CAYEPYLAK	16–24
1033	AEDTIPIFK	28–36
1750	SIGWSGFTPVGDPK	40–53
1331	VVPIALAEYVEK	54–65
1459	IIIELNTAIPSFK	179–191
1557	TSLTLSQWDK	326–335
1252	LYDNFDFYR	336–344
1670	AISNSPE	353–359
1304	LVNGLGGSGDFLR	391–403
1489	EYLNLAIQATQPK	481–493

[a] Peptide sequences identified from the described 57-kDa protein spots by mass spectrometry after two-dimensional-gel separation of isolated hydrogenosomal proteins from *T. vaginalis*.

[b] The position of the identified peptides in the amino acid sequence of the TvASCT protein as encoded on contig 921979.

amount of acetyl-CoA by untransfected CHO cells might be caused by side activity of the mammalian succinyl-CoA:3-oxoacid CoA-transferase (SCOT, EC 2.8.3.5) (Ref. 17).

Overexpression of TvASCT in T. vaginalis—T. vaginalis T1 cells were transfected with *Tv*ASCT in pTagvag2 to yield strain *Tv*tagvagASCT and were selected for transient transformation with G418. Expression of *Tv*ASCT was quantified on mRNA, and protein levels in *Tv*tagvagASCT as well as wt *T.vaginalis* and *Tv*tagvagFrataxin cells (pTagvagFrataxin was kindly provided by Jan Tachezy, Prague) as controls and total cell lysates were assayed for ASCT activity. *Tv*ASCT transcript and protein levels in *Tv*tagvagASCT cells were increased to 409 ± 136% and 518 ± 171% of the wild type, respectively, while they remained largely unchanged in Frataxin-overexpressing controls (101 ± 10% and 95 ± 21%, respectively) (Fig. 6). ASCT activity in *T*vtagvagASCT was raised to 426 ± 54% of the wild type activity and again remained unchanged in *Tv*tagvagFrataxin (115 ± 10%) (Fig. 6).

Co-elution of ASCT Activity and TvASCT Protein in Different Protein Purifications—Three principally different column chromatography methods for protein purification were performed using complete *T. vaginalis* lysates. Fractions were collected and tested for both ASCT activity using an enzyme assay, and for the presence of the *Tv*ASCT protein by Western blot. In all experiments, using chromatography based on anion exchange, isoelectrofocusing and size exclusion, the elution pattern of ASCT activity corresponded to that of the *Tv*ASCT protein (Fig. 7 and Supplemental data). In all three purification methods ASCT was highly purified as it eluted in fractions containing only a small percentage of the total amount of protein. Combination of the anion exchange and electrofocusing columns could not be used for further purification of the ASCT enzyme since this always resulted in a complete loss of ASCT

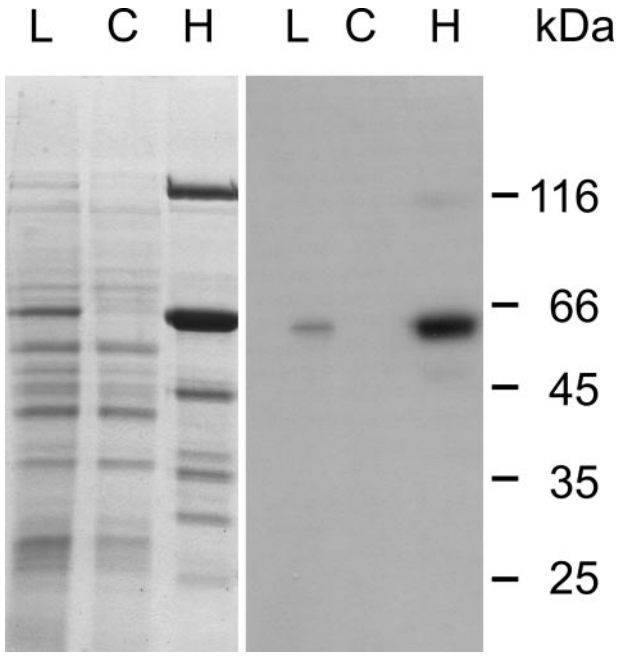

FIGURE 4. **ASCT of *T. vaginalis* is localized in hydrogenosomes.** Western blot of cellular fractions of *T. vaginalis* probed with anti-*Tv*ASCT rabbit serum shows localization of ASCT in the hydrogenosomal fraction. *L*, cell-free lysate; *C*, cytosolic fraction; *H*, hydrogenosomal fraction. Each lane contains 25 μg of protein separated on a 10% SDS-PAGE gel. *Left panel*, Coomassie-stained SDS-PAGE; *right panel*, Western blot.

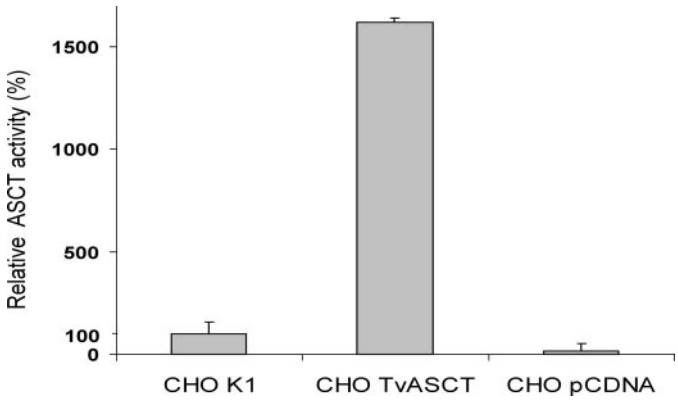

FIGURE 5. **Recombinant expression of *Tv*ASCT in CHO cells.** Wild-type CHO cells (CHO K1), CHO cells transfected with *Tv*ASCT (CHO *Tv*ASCT), and CHO cells transfected with empty pCDNA 3.1+ vector (CHO pCDNA) were analyzed for ASCT activity. ASCT activity in wild-type CHO cells was normalized to 100%. Shown are the means + S.D. of four measurements in two independent experiments. ASCT activity in CHO cells transfected with *Tv*ASCT was determined at 11.3 and 2.3 nmol/min·mg protein in the two different experiments.

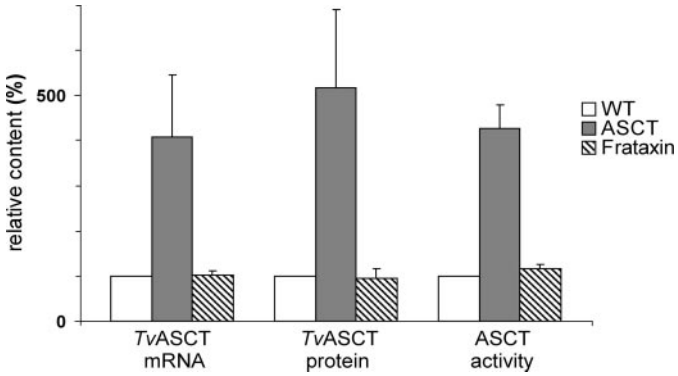

FIGURE 6. ***Tv*ASCT overexpression in *T. vaginalis*.** Wild-type *T. vaginalis* (*WT*), *T. vaginalis* transfected with *Tv*ASCT (*ASCT*), and *T. vaginalis* transfected with *Tv*Frataxin (Frataxin) were analyzed for *Tv*ASCT expression at the level of mRNA transcripts, protein, and enzyme activity by Northern blots, Western blots, and ASCT activity assays, respectively. *Tv*ASCT in wild-type *T. vaginalis* was normalized to 100%, which corresponded to an ASCT activity of 405 nmol/min·mg protein (±101). Shown are the means + S.D. of four measurements in two independent experiments.

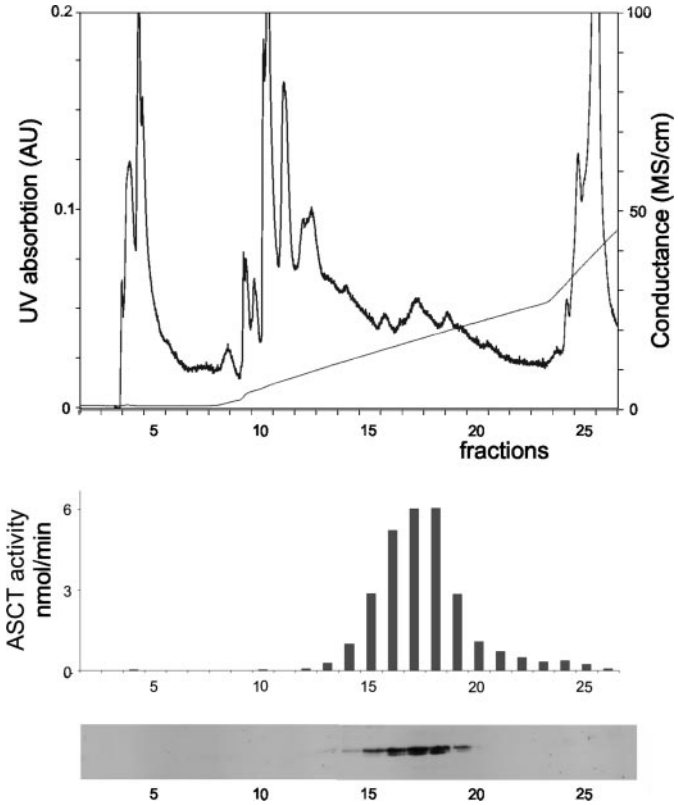

FIGURE 7. **Co-elution of ASCT activity and *Tv*ASCT protein in anion exchange chromatography.** *T. vaginalis* lysate was fractionated by anion exchange chromatography. The *top panel* shows the UV-absorption pattern during elution (left *y*-axis), whereas the *y*-axis on the *right* shows the conductance of the eluate. The number of collected fractions is indicated on the *x*-axis. The *middle panel* shows the ASCT activity present in each fraction, whereas the *bottom panel* depicts the detection of *Tv*ASCT protein on Western blot in each fraction.

activity that could not be regained in reconstitution experiments (not shown). In diluted lysates *Tv*ASCT activity always declined rapidly, although native *Tv*ASCT was stable for days at 4 °C in total cell lysate or during incubation for 15 min at 55 °C, while precipitation with 37.5% $(NH_4)_2SO_4$ resulted in only 10% activity loss (not shown).

Succinate Dependence and Apparent K_m for Succinate of TvASCT—Regarding the fact that *Tv*ASCT shows a high similarity to the protein family of acetyl-CoA hydrolases/transferases, the succinate dependence of *Tv*ASCT was re-examined. Using our specific and sensitive radioactive enzyme assay, in which substrates and end products were identified by anion exchange chromatography, we confirmed that *Tv*ASCT is succinate dependent, and therefore, is a true acetate:succinate CoA-transferase and not a hydrolase (Fig. 8, *panel A* and Ref. 12).

Furthermore, the affinity for succinate of the *Tv*ASCT enzyme as determined in a total *T. vaginalis* lysate, resulted in an apparent K_m of 0.36 mM (±0.03) (Fig. 8, *panel B*) and in an apparent K_m of 0.75 mM (±0.05) for *Tv*ASCT purified by anion exchange chromatography. Such partially purified enzyme preparations were also used to confirm that *Tv*ASCT activity is succinate-dependent (not shown). The apparent K_m values were calculated using direct linear plots in EZ-fit (44).

DISCUSSION

Recently, the draft genome sequence of *T. vaginalis* was published (40). The Supporting Online Material (SOM) of that manuscript (Table S22) shows a list of all hydrogenosomal proteins involved in carbohydrate metabolism and ASCT is the only enzyme lacking a complete annotation. Here we demonstrate that the homolog of the acetyl-CoA hydrolase/transferase protein family, present in the *T. vaginalis* genome data base, functions as the missing ASCT of *T. vaginalis*, for the following reasons. Firstly, heterologous expression of *Tv*ASCT in CHO cells resulted in the expression of an active ASCT enzyme albeit at a very low level when compared with *T. vaginalis* cells (Fig. 5). Second, homologous overexpression of *Tv*ASCT in *T. vaginalis* resulted in a more than 4-fold increase in *Tv*ASCT mRNA and protein levels and also ASCT activity (Fig. 6) Third, upon protein fractionation, using different chromatographic methods, the elution pattern of the activity of ASCT matched with the protein pattern of the acetyl-CoA hydrolase/transferase homolog (Fig. 7 and Supplemental data). Fourth, the *T. vaginalis* homologs of the acetyl-CoA hydrolase/transferase family contain a hydrogenosomal targeting signal (Fig. 2 and Supplemental data) and we have demonstrated the expression and hydrogenosomal localization of the gene product of at least one of these gene copies (Fig. 3). Finally, no homologs of any of the other known types of acetate producing enzymes (see Fig. 1) are present in the *T. vaginalis* genome, whereas multiple copies of homologs to the acetyl-CoA hydrolase/transferase protein family have been detected (Supplemental data).

Taken together, the total of all our analyses clearly demonstrates that the gene product of this acetyl-CoA hydrolase/transferase homolog functions as the hydrogenosomal ASCT. Therefore, this gene can now be annotated as the *T. vaginalis* ASCT (*Tv*ASCT). *Tv*ASCT is thereby the first hydrogenosomal acetate-producing enzyme to be identified in any organism. The reason for the observed loss of activity during subsequent purification steps and the poor heterologous expression of *Tv*ASCT are unknown. In this respect it is unknown yet

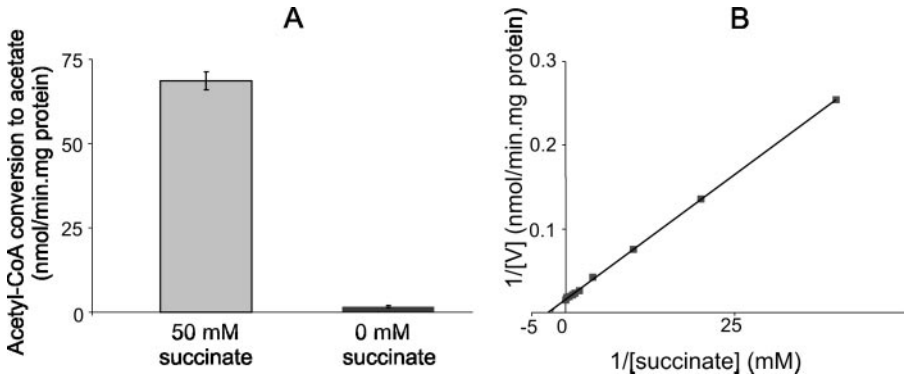

FIGURE 8. **Succinate dependence of *Tv*ASCT activity.** *Panel A* shows the conversion rate of acetyl-CoA to acetate in lysates of *T. vaginalis* in the absence or presence of succinate (50 mM). *Panel B* shows a Lineweaver-Burk plot of ASCT activity in *T. vaginalis* lysates in presence of distinct concentrations of succinate. The apparent K_m of *Tv*ASCT for succinate was 0.36 ± 0.03 mM, calculated from non-linear plots using the program EZ-fit (43).

whether or not *Tv*ASCT might need a cofactor or post-translational modification for maximal activity.

Even though *Tv*ASCT belongs to a protein family, which contains acetyl-CoA hydrolases as well as acetyl-CoA transferases, it is a true ASCT, because it converts acetyl-CoA to acetate in a succinate-dependent way (Fig. 8, *panel A*). This is consistent with the earlier reported characteristics of acetate production in *T. vaginalis* (12).

By measure of similarity, ASCT from *T. vaginalis* hydrogenosomes belongs to a family of proteins annotated as either acetyl-CoA hydrolases or acetyl- or succinyl-CoA transferases. However, it shares no similarity whatsoever with the functionally equivalent mitochondrial SCOT sequences from trypanosomes (25). A sequence similarity network of acetyl-CoA hydrolase/transferase family sequences (Supplemental data) displays two well defined major groups. Both groups contain prokaryotic as well as eukaryotic sequences. Some proteobacteria (*Rhodoferax ferrireducens*, *Geobacter sp.*or *Bordetella parapertussis*) and firmicutes (*Clostridium difficile*) are represented in both groups, suggesting an ancient prokaryotic gene duplication. The eukaryotic sequences are divided into two major clusters. All animal sequences form a monophyletic cluster in one of the two major groups of the network, while *T. vaginalis*, *D. discoideum*, and the fungi form a separate distinct cluster that is strongly associated with a set of δ-proteobacterial homologs in the other.

Functional annotation of most sequences in the network is based on sequence similarity rather than on any biochemical characterization and both major clusters contain sequences annotated as acetyl-CoA transferases, acetyl-CoA hydrolases, and acetyl-CoA transferase/hydrolase family members. Only four of these annotations are supported by biochemical data. The enzyme of the crustacean *Artemia franciscana* has 4-hydroxybutyrate transferase activity (45), while three enzymes of the other major cluster are succinyl-CoA transferase (*C. kluyveri*) (27), acetyl-CoA hydrolase (*S. cerevisiae*) (23), and ASCT (*T. vaginalis*) (this study), respectively. Thus clustering does not strictly correlate with function in this gene family.

Surprisingly, *Tv*ASCT does not share any similarity with the mitochondrial ASCT from *T. brucei*, the only other eukaryotic ASCT identified so far. Furthermore, trypanosomatid ASCT is kinetically different from *Tv*ASCT, as demonstrated by their different apparent K_m values for succinate; 2.8 ± 0.4 mM for *Leishmania mexicana* ASCT (17) and 0.75 ± 0.05 mM for *Tv*ASCT. Apparently, two completely different enzymes for succinate-dependent acetate production have evolved independently in ATP-generating organelles. The identification of *Tv*ASCT does not only illuminate the identity of the last, unidentified hydrogenosomal protein involved in carbohydrate metabolism, but it also means the identification of a completely different, second class of succinate-dependent acetyl-CoA transferases in eukaryotes.

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