Proc. Natl. Acad. Sci. USA
Vol. 95, pp. 3036–3041, March 1998
Evolution

Trypanosomatidae produce acetate via a mitochondrial acetate:succinate CoA transferase

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Edited by P. Borst, Netherlands Cancer Institute, Amsterdam, The Netherlands, and approved December 29, 1997 (received for review July 1, 1997)

ABSTRACT **Hydrogenosome-containing anaerobic protists, such as the trichomonads, produce large amounts of acetate by an acetate:succinate CoA transferase (ASCT)/succinyl CoA synthetase cycle. The notion that mitochondria and hydrogenosomes may have originated from the same α-proteobacterial endosymbiont has led us to look for the presence of a similar metabolic pathway in trypanosomatids because these are the earliest-branching mitochondriate eukaryotes and because they also are known to produce acetate. The mechanism of acetate production in these organisms, however, has remained unknown. Four different members of the trypanosomatid family: promastigotes of *Leishmania mexicana mexicana*, *L. infantum* and *Phytomonas* sp., and procyclics of *Trypanosoma brucei* were analyzed as well as the parasitic helminth *Fasciola hepatica*. They all use a mitochondrial ASCT for the production of acetate from acetyl CoA. The succinyl CoA that is produced during acetate formation by ASCT is recycled presumably to succinate by a mitochondrial succinyl CoA synthetase, concomitantly producing ATP from ADP. The ASCT of *L. mexicana mexicana* promastigotes was further characterized after partial purification of the enzyme. It has a high affinity for acetyl CoA (K_m 0.26 mM) and a low affinity for succinate (K_m 6.9 mM), which shows that significant acetate production can occur only when high mitochondrial succinate concentrations prevail. This study identifies a metabolic pathway common to mitochondria and hydrogenosomes, which strongly supports a common origin for these two organelles.**

Most, but not all, eukaryotes possess mitochondria for the degradation of pyruvate, the end product of glycolysis. In some eukaryotes without mitochondria, pyruvate is further degraded inside hydrogenosomes, cellular organelles that are bounded by a double membrane, like mitochondria (1–5). Hydrogenosomes originally were discovered in trichomonads (6) and subsequently were detected also in a variety of unrelated organisms, like some ciliates and fungi (7). The evolutionary origin of hydrogenosomes is still under debate. Functional and morphological considerations led to the suggestion that hydrogenosomes and mitochondria are related organelles (8).

Trypanosomatids are the earliest branching eukaryotes that have retained mitochondria. They belong to the order Kinetoplastida and contain a number of characteristic organelles, such as glycosomes, the site of the first enzymes of the Embden–Meyerhof pathway of glycolysis, and a kinetoplast, a specialized region of the single mitochondrion containing a highly concatenated form of the mtDNA (9). Trypanosomatids have an energy metabolism in which mainly partially oxidized end products, like pyruvate, succinate, and acetate, are excreted. All of the analyzed trypanosomatids produce acetate to a certain extent during their life cycle (10–13), and the amount of acetate produced may differ widely depending on the species, stage, and environmental conditions. In the promastigote forms of *Leishmania* spp. and procyclic forms of *T. brucei*, acetate is a main end product of glucose metabolism. It is assumed that this acetate is produced from acetyl CoA (10–12). However, the process by which trypanosomatids produce acetate is unknown.

Acetate production from acetyl CoA can occur via three distinct pathways as detected in different organisms. Many eubacteria produce acetate from acetyl CoA via a two-step reaction (reaction 1a and 1b; EC 2.3.1.8 and EC 2.7.2.1, respectively) in which acetyl phosphate occurs as an intermediate (14). In archea (15), *Selenomonas ruminantium* (16), and the amitochondriate protists *Entamoeba histolytica* (17) and *Giardia lamblia* (18), acetate is produced from acetyl CoA via an acetyl CoA synthetase (also known as acetate thiokinase, EC 6.2.1.13). In this process, the formation of acetate from acetyl CoA concomitantly produces ATP from ADP (reaction 2). The hydrogenosome-containing trichomonads (19, 20) and the anaerobic fungus *Neocallimastix* (21) as well as the parasitic helminth *F. hepatica* (22–24) are believed to produce acetate from acetyl CoA by a pathway comprising two reactions that separate the formation of acetate by acetate:succinate CoA transferase (ASCT) (EC 2.3.1.99) and the conservation of the energy of the thioester bond by succinyl CoA synthetase (EC 6.2.1.4) (reactions 3a and 3b). However, the enzyme that transfers CoA from acetate to succinate (reaction 3a), so far, has not been characterized yet.

1a. acetyl CoA + P_i ⇄ acetyl phosphate + CoA SH
1b. acetyl phosphate + ADP ⇄ acetate + ATP
2. acetyl CoA + ADP + P_i ⇄ acetate + ATP + CoA SH
3a. acetyl CoA + succinate ⇄ acetate + succinyl CoA
3b. succinyl CoA + ADP + P_i ⇄ succinate + ATP + CoA SH

In this study, we analyzed the pathway of acetate production in trypanosomatids. We showed that formation of acetate occurs via an ASCT both in promastigotes of *Leishmania* spp. and *Phytomonas* spp. as well as in procyclics of *T. brucei*. This report demonstrates the existence of a mitochondrial ASCT in *L. mexicana mexicana* promastigotes and describes for the first time the catalytic properties of an ASCT. The identification of an ASCT/succinyl CoA synthetase cycle in the mitochondria of trypanosomatids is additional evidence in support of a common origin of mitochondria and hydrogenosomes.

MATERIALS AND METHODS

Chemicals and Biomaterials. Succinyl CoA, acetyl CoA, and Dowex 2X8 (200-to 400-mesh) were obtained from Sigma.

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This paper was submitted directly (Track II) to the *Proceedings* office.
Abbreviations: ASCT, acetate:succinate CoA transferase; EC, enzyme classification.
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[1-^{14}C]Acetate (2.15 GBq/mmol) and [1-^{14}C]acetyl CoA (2.04 GBq/mmol) were obtained from Amersham, and [1,4-^{14}C]succinic acid (2.18 GBq/mmol) was obtained from Dupont/NEN. All of the other chemicals were of analytical grade. *L. infantum* promastigotes (MCAM/ES/88/1SS441DOBA) (25) and procyclics of *T. brucei* stock 427 (26) were a kind gift of E. Pinelli (Department of Immunology, Faculty of Veterinary Medicine, Utrecht University, The Netherlands) and A. W. C. A. Cornelissen (Department of Parasitology and Tropical Veterinary Medicine, Faculty of Veterinary Medicine, Utrecht University, The Netherlands), respectively. Promastigotes of *L. mexicana mexicana* (NHOM/B2/84/BEL46) (27), *L. infantum* and *Phytomonas* sp. (28), and procyclics of *T. brucei* were all cultured at 28°C in SDM-79 medium (29) under a gas phase of air/CO_2 (95/5%). *F. hepatica* adults were isolated from infected sheep livers obtained at a slaughterhouse.

Homogenization and Subcellular Localization. Homogenates of hamster hearts, *F. hepatica* adults, and of cells of late log phase cultures of trypanosomatids (13) were prepared in 20 mM Hepes (pH 7.4) with an Ultra Turrax homogenizer (IKA, Staufen). Where indicated, endogenous ions and cofactors were removed by filtration of the homogenate using a Sephadex G50 (Pharmacia) gel filtration column (25 × 0.5 cm).

Subcellular fractionation by using sucrose gradient centrifugation was carried out in a vertical Beckman VTi 50 rotor as described (30, 31). The homogenates used for this subcellular fractionation were prepared by grinding of the cells using silicon carbide as described (30). Presentation of results is shown as in Beaufay and Amar-Costesec (32). Studies on the enzyme release from digitonin-treated cells were performed as described (33). Marker enzyme assays were performed as described (34).

ASCT Activity Assay. ASCT activity was determined by a radioactive enzyme assay containing 50 mM succinate (pH 7.4), 1 mM [1-^{14}C]acetyl CoA (0.2 MBq), 50 mM Tris·HCl (pH 7.4), 10 mM $MgCl_2$, and 0.05% (vol/vol) Triton X-100, unless otherwise indicated. The reaction was performed at 20°C and terminated after 20 min by the addition of ice-cold trichloroacetic acid (10% final concentration, wt/vol), and subsequently, the assay mixtures were cooled on ice. Acetate and acetyl CoA were separated by anion exchange chromatography, by using an acetate form of a Dowex 2X8 anion exchange column (5 × 30 mm), as described by Sladik *et al.* (35). The assay mixture was centrifuged (2 min at 10,000 × *g*) and 200 μl of the resulting supernatant was loaded on top of the column. The column was eluted with 2 ml portions of distilled water (three times), 0.2 M NaCl (four times), and 1 M NaOH (two times). Fractions (2 ml) were collected for liquid scintillation counting. All values were corrected for blank incubations.

In a consecutive anion-chromatographic analysis of the separated end product, it was checked and confirmed that this reaction product was indeed acetate. Using a Bio-Rad AG 1-X8, 100- to 200-mesh column (60 × 1.1 cm), from which acetate elutes at a very specific retention volume (36), it was demonstrated that the end product of the enzyme assays was acetate in all organisms investigated. Furthermore, by using the AG 1-X8 column, a mixture of the reaction product and an equivalent amount of labeled acetate, as standard, eluted as a single-labeled peak at the retention volume of acetate, which contained the expected double amount of label.

To identify all substrates and products of the ASCT reaction, we used the following conditions: 50 mM [1-^{14}C]acetate (0.2 MBq) in the presence of several succinyl CoA concentrations (0.2–8 mM) or 50 mM [1,4-^{14}C] succinate (0.2 MBq) in the presence or absence of 1 mM acetyl CoA. The reaction products were separated as described above after which the CoA-containing fraction was hydrolyzed by 1 M NaOH. Subsequently, the samples were analyzed on a Bio-Rad AG 1-X8, 100- to 200-mesh column (60 × 1.1 cm) (36). The hydrolyzed CoA fractions contained labeled acetate and succinate, showing that acetyl CoA and succinyl CoA were formed when acetate or succinate were used as respective substrates. Protein was determined by a Lowry method as described by Bensadoun and Weinstein (37) using BSA as a standard. Kinetic analyses were performed with EZ-FIT (38).

Partial Purification of ASCT. Homogenates of *L. mexicana mexicana* promastigotes were solubilized further by the addition of 1% (vol/vol, final concentration) Triton X-100. Subsequently, the homogenate was treated with DNase I (Boehringer Mannheim) for 15 min at 4°C and centrifuged for 10 min at 14,000 × *g*. The supernatant was applied on a fast protein liquid chromatography Superose 6 gel filtration column (Pharmacia) that was eluted at 0.4 ml/min with buffer A, containing 20 mM Hepes and 1 mM EDTA (pH 7.4). The fractions containing ASCT activity were pooled and loaded on a fast protein liquid chromatography Mono Q-anion exchange column (Pharmacia) that was equilibrated with buffer A. ASCT was eluted from this column using a linear gradient from 0 to 150 mM NaCl in buffer A in 10 min at 1 ml/min. The fractions containing ASCT activity were pooled and used for kinetic characterization of the enzyme.

RESULTS

Kinetics of ASCT. We investigated the process by which acetate is produced from acetyl CoA in homogenates of *L. infantum* promastigotes. We observed that when the assay mixture contained Mg^{2+}, ADP, and P_i but no succinate, only a minimal amount of acetate was produced from acetyl CoA by homogenates of *L. infantum* promastigotes (not shown), which indicates that in *L. infantum* promastigotes, acetate production from acetyl CoA did not occur via simple hydrolysis of acetyl CoA nor by the action of an acetyl CoA synthetase. Therefore, we looked for the presence of an ASCT. As shown in Fig. 1, acetate was produced from acetyl CoA when succinate was present in the assay mixture. The amount of acetate produced was linear with time as well as with the amount of protein present in the incubation (Fig. 1). These results demonstrate that acetate was produced enzymatically from acetyl CoA by a succinate-dependent process.

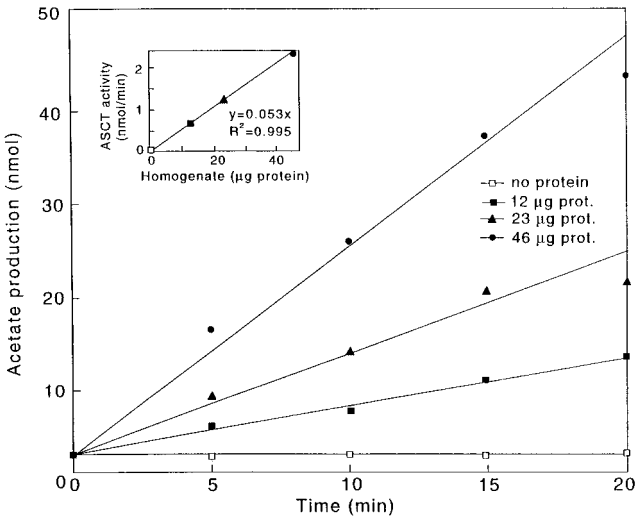

FIG. 1. ASCT activity in homogenates of *L. infantum* promastigotes. ASCT activity was determined in homogenates of *L. infantum* promastigotes, containing 12, 23, or 46 μg of protein, as described in *Materials and Methods*. The assay mixture contained 10 mM succinate, 2 mM ADP, and 10 mM P_i. (*Inset*) slopes of the graphs of acetate production vs. time are plotted against the amount of homogenate. One example of two independent experiments is shown.

Table 1. CoA transfer by the acetate:succinate CoA-transferase

Labeled substrate	CoA donor	Labeled product formation, nmol·min^{-1}·mg^{-1} protein	
50 mM (0.2 MBq)	10 mM	[^{14}C]Succinyl CoA	[^{14}C]Acetyl CoA
Succinate	Acetyl CoA	81.9 ± 3.5	–
Succinate	none	0.5 ± 1.0	–
Acetate	Succinyl CoA	–	20.8 ± 1.0
Acetate	none	–	0.1 ± 0.2

The acetate:succinate CoA transferase activity in *L. mexicana mexicana* promastigotes was measured in both directions as described in *Materials and Methods*. Shown are the results of three experiments ± SD.

Succinate dependency of the conversion of acetyl CoA to acetate could be explained also by an allosteric activation of an acetyl CoA synthetase-like enzyme rather than by the postulated ASCT. Therefore, we have verified that such an acetyl CoA synthetase was absent from an *L. mexicana mexicana* homogenate by removing by gel filtration all of the endogenous cofactors and ions such as ADP, ATP, P_i, and CoA SH. The addition of ADP and P_i did not enhance the succinate-dependent production of acetate from acetyl CoA (not shown), which demonstrated that a succinate-dependent acetyl CoA synthetase was not present.

Subsequently, the direct transfer of CoA from acetate to succinate by the ASCT in homogenates of *L. mexicana mexicana* was demonstrated by the production of ^{14}C-labeled succinyl CoA from [1,4-^{14}C]succinate, which was enhanced significantly when acetyl CoA was added (Table 1). In addition, the reaction could be reversed because incubation of homogenates with [1-^{14}C]acetate resulted in greatly increased formation of ^{14}C-labeled acetyl CoA when succinyl CoA was present (Table 1). Furthermore, this reaction showed Michaelis–Menten kinetics with respect to succinyl CoA (apparent K_m 0.80 ± 0.10 mM), demonstrating that succinyl CoA functions as a substrate in this reverse reaction. For the characterization of the kinetic properties of ASCT in the forward reaction, we used a partially purified enzyme preparation (see *Materials and Methods*) that had a sixfold-increased ASCT activity (Table 2). The conversion of acetyl CoA to acetate showed Michaelis–Menten kinetics with respect to both substrates (Fig. 2). The affinity for acetyl CoA was higher than for succinate because the apparent K_m was 0.26 ± 0.03 mM and 6.9 ± 0.7 mM, respectively (Fig. 2).

These results clearly demonstrate that *L. mexicana mexicana* promastigotes produced acetate from acetyl CoA by a single reaction catalyzed by an ASCT because: (*i*) ASCT activity could be purified partially by a two-column step procedure; (*ii*) the conversion of acetyl CoA to acetate obeyed Michaelis–Menten kinetics with respect to both acetyl CoA and succinate, and by using labeled acetyl CoA or succinate, the products were shown to be acetate and succinyl CoA, respectively; and (*iii*) in the backward reaction, the conversion of acetate to acetyl CoA was shown to be succinyl CoA-dependent and again followed Michaelis–Menten kinetics with respect to both substrates, and by using labeled acetate, the product was shown to be acetyl CoA.

In addition to promastigotes of *L. mexicana mexicana* also homogenates of the other trypanosomatids, such as procyclics of *T. brucei* and promastigotes of *L. infantum* and *Phytomonas* sp., produced acetate from acetyl CoA (Fig. 3). Also in homogenates of *F. hepatica* adults, a parasitic helminth that is assumed to produce acetate via ASCT *in vivo* (22–24), acetate was produced from acetyl CoA (Fig. 3). However, hamster heart, which does not metabolize acetyl CoA to acetate *in vivo*, was not capable of producing acetate from acetyl CoA *in vitro* (Fig. 3). The observed production of acetate from acetyl CoA in the trypanosomatids and *F. hepatica* was dependent highly on succinate (Fig. 3), which demonstrates that also in all of these organisms, only a very small amount of acetate was formed from acetyl CoA via hydrolysis of acetyl CoA; the main formation was catalyzed by an ASCT because these reactions also exhibited Michaelis–Menten kinetics comparable with the results obtained for *L. mexicana mexicana* promastigotes (not shown). The apparent K_m for succinate of the ASCT of *T. brucei*, *Phytomonas*, and *F. hepatica* was 2.3 mM, 3.5 mM, and 4.9 mM, respectively. Hence, these results show that both trypanosomatids and parasitic helminths contained an ASCT for the production of acetate from acetyl CoA.

Subcellular Localization of ASCT. Subcellular fractionation, by using sucrose gradient centrifugation, was carried out to localize the ASCT within the *Leishmania* cell (Fig. 4). The majority of the enzyme equilibrated in sucrose at a density of 1.18 g·cm^{-3}, which was similar to the mitochondrial components of the enzymes isocitrate dehydrogenase and malate dehydrogenase (30).

In addition, we also studied the localization of ASCT by analyzing the enzyme release from digitonin-treated *L. mexicana mexicana* promastigotes. This experiment showed that ASCT was localized in the mitochondria of these promastigotes because it was released from the cells at much higher digitonin concentrations than cytosolic and glycosomal marker enzymes and was released similar to the mitochondrial marker enzyme glutamate dehydrogenase (Fig. 5). The combination of these localization studies showed that the ASCT was present inside the mitochondrion of *L. mexicana mexicana* promastigotes.

Succinyl CoA Synthetase. The formation of acetate from acetyl CoA leads to the production of succinyl CoA, which is further metabolized because it is not excreted as such. Succinyl CoA is a known substrate for succinyl CoA synthetase, which

Table 2. Partial purification of acetate:succinate CoA transferase from *L. mexicana mexicana* promastigotes

Fraction	Volume, ml	Protein, mg	Specific activity ASCT, nmol/min·mg	Purification factor	Recovery, %
Homogenate	0.85	4.80	156.1	1	100
Supernatant	0.80	2.64	284.9	1.8	100
Superose 6	11.0	0.91	200.8	1.3	24
Mono Q	3.2	0.1	873.2	5.6	12

Acetate:succinate CoA transferase from *L. mexicana mexicana* promastigotes partially was purified as described in *Materials and Methods*. One example of two independent experiments is shown. ASCT, acetate:succinate CoA transferase.

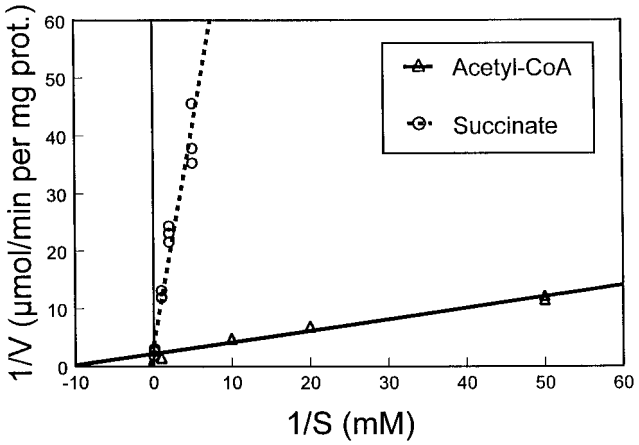

FIG. 2. Lineweaver–Burk plot of ASCT activity in a partially purified enzyme preparation from *L. mexicana mexicana* promastigotes. ASCT was purified partially as described in *Materials and Methods*. Shown are the results of a triplicate experiment for each substrate. The apparent K_m values for acetyl CoA and succinate were 0.26 ± 0.03 mM and 6.9 ± 0.7 mM, respectively, and the apparent V_{max} was 873 ± 32 nmol/min/mg protein.

is a citric acid cycle enzyme that catalyses the formation of succinate from succinyl CoA, producing concomitantly ATP from ADP (reaction 3b). Because the insect stages of trypanosomatids contain a fully developed mitochondrion with citric acid cycle activity (12), these trypanosomatids also contain succinyl CoA synthetase. Therefore, it is likely that in trypanosomatids, succinyl CoA synthetase is involved directly in recycling of succinyl CoA during acetate production.

When succinyl CoA synthetase functions in the direction of succinate production, it conserves the energy of the thioester bond by formation of GTP from GDP in higher eukaryotes, like mammals, or by formation of ATP from ADP in lower eukaryotes like trichomonads (19, 39) and trypanosomatids (40, 41). Thus, we tried to measure the formation of ATP or GTP during acetate production in homogenates of *L. mexicana mexicana*. However, it was not possible to detect any ATP or GTP in the assay mixture after incubation. This result is caused probably by the abundance of ATPases in the homogenate. Therefore, we measured the involvement of succinyl CoA synthetase in acetate production by an indirect method. The ASCT activity of homogenates was measured in the presence of 1 mM succinate, a concentration that is seven times lower

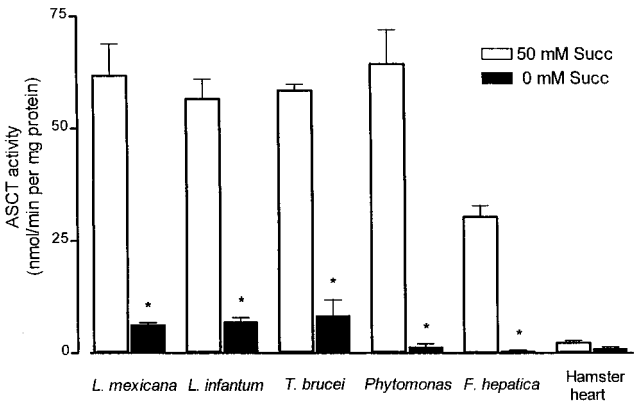

FIG. 3. The ASCT activity in trypanosomatids, *F. hepatica*, and hamster heart. ASCT activity was determined as described in *Materials and Methods*. Open bars represent the activity (acetate production) in the presence of 50 mM succinate, whereas closed bars represent the activity in the absence of succinate. Shown are the means ± SD of three independent experiments; *, significantly different from the ASCT activity in the presence of succinate. $P < 0.0005$.

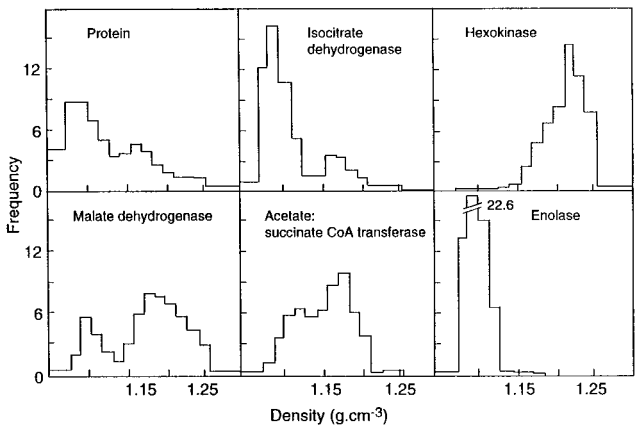

FIG. 4. Subcellular fractionation of an *L. mexicana mexicana* cell-free extract by sucrose gradient centrifugation. Marker enzymes with known subcellular localization are: enolase (cytosol), hexokinase (glycosome), isocitrate dehydrogenase (cytosol and mitochondrion), and malate dehydrogenase (mitochondrion and glycosome). The results are presented as normalized fractions with equal density increment.

than the apparent K_m for succinate in the ASCT catalyzed reaction. When ADP and P_i, the substrates for succinyl CoA synthetase of trypanosomatids, are added to the assay mixture, succinyl CoA synthetase should be able to recycle the produced succinyl CoA to succinate. On the other hand, when ADP and P_i would not be added to the assay mixture, succinate should be consumed during acetate formation and the succinyl CoA produced cannot be efficiently recycled because of the limited amount of endogenous ADP and P_i present in the homogenate. Therefore, the succinate concentration would decrease and the concentration of acetate and succinyl CoA in the assay mixture increases. Because the production of acetate via ASCT in the presence of 1 mM succinate would occur at a submaximal rate, the differences in substrate and product concentrations in the assay mixture caused by the presence or absence of an active succinyl CoA-recycling enzyme would result in a significantly different rate of acetate production. As shown in Fig. 6, the acetate production in the presence of ADP and P_i was increased significantly as compared with its production in the absence of ADP and P_i. This result indicates that

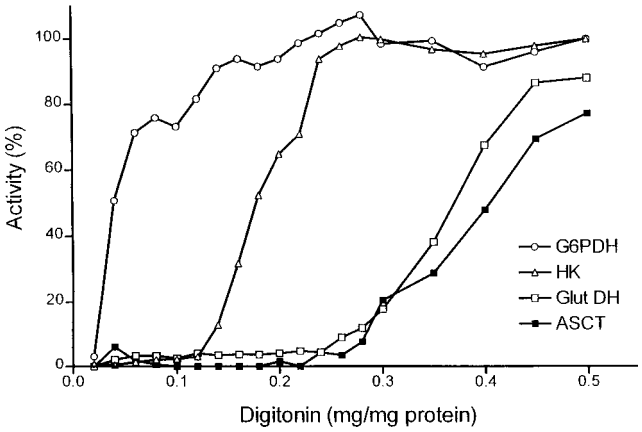

FIG. 5. Enzyme release from digitonin treated *L. mexicana mexicana* promastigotes. Enzyme release from digitonin-treated cells was performed as described in *Materials and Methods*. Marker enzymes with known subcellular localization are: glucose-6-phosphate dehydrogenase for the cytosol, hexokinase for the glycosome, and glutamate dehydrogenase for the mitochondrion. Glut DH, glutamate dehydrogenase; G6PDH, glucose-6-phosphate dehydrogenase; and HK, hexokinase.

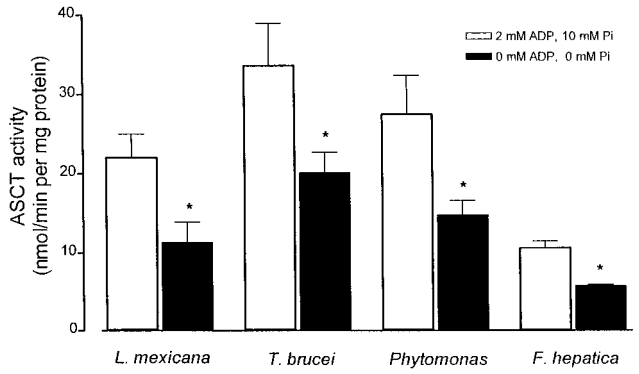

FIG. 6. The dependence of the ASCT activity on ADP and P_i in the presence of 1 mM succinate. ASCT activity was determined as described in *Materials and Methods* in the presence of 1 mM succinate. Open bars represent ASCT activity (acetate production) in the presence of ADP (2 mM) and P_i (10 mM), whereas closed bars represent enzyme activity in the absence of ADP and P_i. Shown are the means ± SD of three independent experiments; * indicates significantly different from the ASCT activity in the presence of ADP and P_i. $P < 0.02$.

during acetate production, the recycling of succinyl CoA by succinyl CoA synthetase must occur with the concomitant production of ATP from ADP and P_i. This recycling was demonstrated in all trypanosomatids tested as well as in *F. hepatica*.

DISCUSSION

Although the main function of both mitochondria and hydrogenosomes is the oxidation of pyruvate, these two organelles so far have not yet been shown to share any single common pathway. Nevertheless, recent analyses of several heat shock proteins or chaperonins (42–45) and the presence of mitochondrion-like targeting signals on hydrogenosomal enzymes (46–49), indicated that hydrogenosomes and mitochondria are related organelles (50). They probably originated from the same prokaryotic endosymbiont of the α group of proteobacteria (43) and hydrogenosomes might have evolved by an adaptation to anaerobic conditions.

Trichomonads and the anaerobic fungus *Neocallimastix* lack mitochondria, and both organisms contain hydrogenosomes (7). The relationship between the hydrogenosomes in *Neocallimastix* and those occurring in trichomonads and anaerobic ciliates is unclear yet. However, the recent observation that hydrogenosomes of *Neocallimastix* also are surrounded by a double membrane argues against a microbody origin for fungal hydrogenosomes and suggests a common origin for all hydrogenosomes (4).

Hydrogenosome containing organisms (19–21) produce acetate from acetyl CoA via the ASCT/succinyl CoA synthetase cycle. Because trypanosomatids are early branching eukaryotes with mitochondria that also produce acetate, it was reasoned that their mitochondria and the hydrogenosomes of anaerobic protists still might use the same pathway for acetate production.

In this paper, we demonstrated that all of the trypanosomatids studied (promastigotes of *L. mexicana mexicana*, *L. infantum* and *Phytomonas* sp. as well as procyclics of *T. brucei*) contained an ASCT that was involved directly in the conversion of acetyl CoA to acetate. The ASCT was characterized further after partial purification from *L. mexicana mexicana* promastigotes. This established the characterization of the ASCT reaction, although the presence of an ASCT has been implicated in the formation of acetate in a number of early evolving eukaryotes.

We showed that ASCT is a mitochondrial enzyme in *L. mexicana mexicana* promastigotes, and therefore, acetate production by the enzyme leads to the concomitant production of succinyl CoA in the mitochondrial matrix. Our results suggest that succinyl CoA is recycled to succinate by the action of a succinyl CoA synthetase (reaction 3b), which has been localized in the mitochondria of trypanosomatids (30). Such a recycling of succinyl CoA by succinyl CoA synthetase is inferred from the fact that the ASCT activity in the presence of a low succinate concentration is stimulated by the addition of ADP, a substrate of the succinyl CoA synthetase reaction (Fig. 6). In this respect, it should be realized that the presence of succinyl CoA synthetase activity in itself is not questionable because these organisms have a functional citric acid cycle. These data suggest that the production of acetate from acetyl CoA *in vivo* does result in the production of ATP, which would be the explanation as to why pyruvate is converted to acetate and not excreted as such. Hence, we suggest that in trypanosomatids, the production of 1 mol acetate from glucose results in the formation of 2 mol ATP, rather than 1 mol, as was assumed until now.

The ASCT of *L. mexicana mexicana* promastigotes has a high affinity for acetyl CoA (K_m 0.26 mM), whereas it has a low affinity for succinate (K_m 6.9 mM). As a consequence, the intramitochondrial succinate concentration must be high (mM range) to support a significant acetate production by the ASCT. A high intracellular concentration of succinate is expected indeed to be present in these life cycle stages because next to acetate, succinate is one of the major end products of promastigotes (10–13).

The intramitochondrial succinate concentration is probably a key factor in switching mitochondrial metabolism from citric acid cycle activity to the production of acetate. When the citric acid cycle and the respiratory chain are unable to oxidize the supply of acetyl CoA, the concentration of NADH and citric acid cycle intermediates (including succinate) will increase. This will result in increased levels of both acetyl CoA and succinate, which then will lead to an increase in the formation of acetate by the ASCT. Acetate production thus will occur especially when the citric acid cycle cannot cope with the supply of acetyl CoA, and succinate and other citric acid cycle intermediates accumulate. This situation can occur for two reasons. First, the availability of oxygen may become limiting, which restricts the capacity of the respiratory chain and, therefore, citric acid cycle activity. Secondly, when nutrients are available abundantly more acetyl CoA may be formed than can be coped with and acetyl CoA and succinate levels will rise, thus leading to acetate formation. Such a regulation of carbohydrate metabolism in trypanosomatids would be in agreement with the kinetic properties of the ASCT, and drastic changes in the availability of oxygen and nutrients are known to occur during the life cycle of trypanosomatids, especially in the insect stages (51, 52). Thus, acetate formation then could be considered to be an overflow mechanism like lactate or ethanol formation in other organisms.

This study identifies the first metabolic pathway common to mitochondria and hydrogenosomes: an ASCT/succinyl CoA synthetase cycle. Interestingly, in organisms lacking compartmentation of metabolism, such as *Giardia* (18) and *Entamoeba* (17), this cycle is lacking and instead a single step (acetyl CoA synthetase) reaction occurs, suggesting that compartmentation and the presence of a cycle are linked. The presence of an ASCT/succinyl CoA synthetase cycle in the mitochondria of trypanosomatids can thus be considered as an additional piece of evidence in support of a common origin of mitochondria and hydrogenosomes.

We thank Prof. M. Müller (Rockefeller University, New York) for very valuable comments on the manuscript; Dr. S. Marché (International Institute of Cellular and Molecular Pathology, Brussels) for

providing fractions and marker-enzyme data of the localization study by using digitonin-treated cells; Dr. E. Pinelli (Department of Immunology, Faculty of Veterinary Medicine, Utrecht University, The Netherlands) and Prof. A.W.C.A. Cornelissen (Department of Parasitology and Tropical Veterinary Medicine, Faculty of Veterinary Medicine, Utrecht University, The Netherlands) for supplying insect stages of *L. infantum* and *T. brucei*, respectively; and F. K. Korver and H. Korver of the slaughterhouse in Baambrugge (The Netherlands) for supplying sheep livers infected with *F. hepatica*. This work was supported by the Life Science Foundation with financial aid from the Netherlands Organization for Scientific Research and by the Interuniversity Poles of Attraction Programme-Belgian State, Prime Minister's Office, Federal Office for Scientific, Technical and Cultural Affairs.

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