Schistosoma mansoni sporocysts contain rhodoquinone and produce succinate by fumarate reduction

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SUMMARY

Although schistosomes were thought to be one of the few parasitic helminths that do not produce succinate via fumarate reduction, it was recently demonstrated that sporocysts of *Schistosoma mansoni* produce, under certain conditions, succinate in addition to lactate. This succinate production was only observed when the respiratory chain activity of the sporocysts was inhibited, which suggested that succinate is produced by fumarate reduction. In this report the presence of essential components for fumarate reduction was investigated in various stages of *S. mansoni* and it was shown that, in contrast to adults, sporocysts contained a substantial amount of rhodoquinone which is essential for efficient fumarate reduction in eukaryotes. This rhodoquinone was not made by modification of ubiquinone obtained from the host, but was synthesized *de novo*. Furthermore, it was shown that complex II of the electron-transport chain in schistosomes has the kinetic properties of a dedicated fumarate reductase instead of those of a succinate dehydrogenase. The presence of such an enzyme, together with the substantial amounts of rhodoquinone, shows that in *S. mansoni* sporocysts succinate is produced via fumarate reduction. Therefore, the energy metabolism of schistosomes does not differ in principle from most other parasitic helminths, which are known to rely heavily on fumarate reduction.

Key words: anaerobic energy metabolism, phosphoenolpyruvate carboxykinase, ubiquinone, fumarate reductase, helminth, parasite.

INTRODUCTION

The life-cycle of *Schistosoma mansoni* comprises distinct stages, that differ in their energy metabolism. The free-living stages of *S. mansoni*, miracidia and cercariae, have an aerobic energy metabolism in which endogenous glycogen is mainly catabolized to CO_2 via the Krebs cycle (Van Oordt, Tielens & Van den Bergh, 1989; Tielens *et al.* 1991*a*; Tielens, 1994). Adult schistosomes have besides a significant capacity to produce energy via aerobic pathways, a fermentative metabolism, as they excrete, in addition to CO_2, large amounts of lactate (Bueding, 1950; Thompson *et al.* 1984; Van Oordt *et al.* 1985). Adult schistosomes and filarial nematodes, which also excrete mainly lactate, are often called 'homolactic fermenters' (Bueding, 1950; Köhler, 1991). Most other parasitic helminths, however, have a completely different energy metabolism, degrading glucose to fermentation products like succinate, propionate and acetate (Smyth & Halton, 1983; Köhler & Voigt, 1988; Saz, 1990; Tielens, 1994).

S. mansoni sporocysts, the parasitic stage inside the intermediate host, have an energy metabolism different from that of the adults inside the final host. Sporocysts are facultative anaerobes and adjust their energy metabolism to the variable conditions inside the snail. In the presence of oxygen they derive most of their energy from the aerobic degradation of glucose to CO_2, but also produce lactate, whereas under the anaerobic conditions which occasionally occur in the snail, they switch to the production of lactate and succinate (Tielens *et al.* 1992). This succinate is produced via a pathway that involves phosphoenolpyruvate carboxykinase (PEPCK), as $^{14}CO_2$ was incorporated in the excreted succinate and succinate production was reduced by 3-mercaptopicolinic acid, a specific inhibitor of PEPCK (Tielens *et al.* 1992). Therefore, oxaloacetate produced from phosphoenolpyruvate by PEPCK is probably used for succinate production. It is uncertain, however, by which process this oxaloacetate is subsequently metabolized to succinate. Most adult parasitic helminths, like *Ascaris suum*, *Haemonchus contortus* and *Fasciola hepatica*, form succinate by the reduction of fumarate. This fumarate is produced from malate, which has been produced from phosphoenolpyruvate via PEPCK and a subsequent reduction of the formed oxaloacetate (Tielens, 1994; Komuniecki & Harris, 1995). However, other eukaryotes, like *Leishmania* promastigotes (Cazzulo, 1992; Blum, 1993), also excrete succinate as an end-product of glucose catabolism and, recently, it was demonstrated that this succinate is for a large part produced by Krebs cycle activity (Van Hellemond,

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Van der Meer & Tielens, 1997). Therefore, further analysis of the process by which *S. mansoni* sporocysts produce succinate was needed, also to clarify the observed differences in energy metabolism between sporocysts and adult schistosomes.

MATERIALS AND METHODS

Chemicals and biomaterials

Foetal calf serum was purchased from Gibco (Paisley, UK). Phenazine methosulfate, 2,6-dichlorophenolindophenol (DCIP) and ubiquinone standards containing 7, 9 or 10 isoprenoid units were obtained from Sigma (St Louis, USA). Bovine serum albumin was obtained from Boehringer (Mannheim, Germany). All other chemicals were of analytical grade.

S. mansoni adults were isolated from hamsters 45–50 days after infection. *S. mansoni* miracidia hatched from eggs isolated from livers of infected hamsters as described previously, and sporocysts were prepared by *in vitro* transformation of miracidia (Tielens *et al*. 1992). *S. mansoni* cercariae were shed from infected *Biomphalaria glabrata* snails for 3 h at 28 °C. Mussels (*Mytilus edulis*), oysters (*Crassostrea angulata*) and lugworms (*Arenicola marina*) were obtained at the Dutch coast. *F. hepatica* adults were isolated from infected sheep livers obtained at a slaughterhouse. Adults of *H. contortus* were a generous gift of Dr M. Eijsker (Department of Parasitology and Tropical Veterinary Medicine, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands).

Enzyme assays

Fumarate reduction activities were determined by a direct spectrophotometric assay with reduced benzyl viologen as electron donor as described by Ackrell *et al*. (1993). Succinate oxidation activities were measured by a spectrophotometric assay with phenazine methosulfate and DCIP as electron acceptors as described by Hägerhäll *et al*. (1992). The measured activity was corrected for non-specific reduction of DCIP by performing control assays with malonate instead of succinate. Enzyme assays were performed on mitochondrial fractions prepared from homogenates by differential centrifugation as described previously (Van Hellemond *et al*. 1995). However, the succinate oxidation and fumarate reduction activities of *C. angulata* abductor muscle, *A. marina* body wall muscle, *F. hepatica* metacercariae and *S. mansoni* sporocysts were determined in homogenates due to the limited amount of material available. All enzyme assays were performed at 25 °C, immediately after fractionation or homogenization of freshly collected material. Protein was determined by the Lowry method as reported by Bensadoun & Weinstein (1976), using bovine serum albumin as a standard.

Quinone analysis

Quinones were extracted following the procedure of Bligh & Dyer (1959) and separated and quantified (using ubiquinone-7 as internal standard) by HPLC with a reversed-phase RP-18 column as described before (Van Hellemond *et al*. 1995). Homogenates of approximately 160 adults, 10^6 miracidia, 10^6 cercariae and 10^6 sporocysts were used for quinone analysis. The retention in reverse-phase HPLC of rhodoquinone of all investigated stages was identical to rhodoquinone-10 of *F. hepatica* adults. Because of the limited availability and small biomass of sporocysts, miracidia and cercariae, only rhodoquinone-10 extracted from adults was further confirmed to be indeed a rhodoquinone: the absorbancy ratio (275 nm/283 nm) was less than 1 and identical to rhodoquinone of *F. hepatica*, and the u.v. spectrum was similar to that of benzoquinones and sensitive to reduction with dithionite (Parsons & Rudney, 1965).

RESULTS AND DISCUSSION

Fumarate reduction in S. mansoni *sporocysts*

Schistosomes were thought to be one of the few parasitic helminths that do not produce succinate via fumarate reduction, a very common process in parasites. Recently, however, sporocysts of *S. mansoni* were shown to produce succinate in addition to lactate during anaerobic conditions (Tielens *et al*. 1992). That report suggested that this succinate was produced via fumarate reduction, as CO_2 is incorporated in excreted succinate, and succinate production is stimulated by cyanide and inhibited by 3-mercaptopicolinic acid.

Reduction of fumarate is the reversal of the oxidation of succinate, which occurs in the Krebs cycle by succinate dehydrogenase, also known as complex II of the respiratory chain. Fumarate reduction and succinate oxidation are catalysed *in vivo* by homologous but distinct enzymes in *Escherichia coli* (Ackrell *et al*. 1992). Furthermore, eukaryotes that reduce fumarate during anoxia but oxidize succinate during aerobic conditions are also believed to contain 2 distinct enzymes for these reactions (Roos & Tielens, 1994; Van Hellemond & Tielens, 1994; Saruta *et al*. 1995). *In vitro*, however, the enzyme fumarate reductase as well as succinate dehydrogenase can catalyse both fumarate reduction and succinate oxidation (Ackrell *et al*. 1992; Van Hellemond *et al*. 1997). Therefore, the demonstration of fumarate reductase activity *in vitro* is no evidence for the presence of a distinct fumarate reductase. On the other hand, the enzyme fumarate

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Table 1. Ratios of fumarate reduction and succinate oxidation activities *in vitro*

(Activities of succinate oxidation and fumarate reduction were determined spectrophotometrically as described in the Materials and Methods section. The activity ratio was calculated as follows: specific activity of succinate oxidation of the sample, divided by the specific activity of fumarate reduction of the sample. Results of 3 independent experiments are shown with standard deviations. The activity ratio of succinate oxidation/fumarate reduction of rat and bovine heart differed significantly from the activity ratios of all other species investigated ($P < 0{\cdot}001$).)

Species	Succinate oxidation/ fumarate reduction activity ratio
Rat (heart)	$14{\cdot}3 \pm 2{\cdot}6$
Bovine (heart)	$16{\cdot}7 \pm 1{\cdot}1$
Mytilus edulis (abductor muscle)	$1{\cdot}39 \pm 0{\cdot}07$
Crassostrea angulata (abductor muscle)	$1{\cdot}25 \pm 0{\cdot}51$
Arenicola marina (body wall muscle)	$0{\cdot}39 \pm 0{\cdot}26$
Fasciola hepatica metacercariae	$0{\cdot}19 \pm 0{\cdot}09$
Fasciola hepatica adult	$0{\cdot}21 \pm 0{\cdot}03$
Haemonchus contortus adult	$0{\cdot}08 \pm 0{\cdot}02$
Schistosoma mansoni sporocysts	$0{\cdot}95 \pm 0{\cdot}15$
Schistosoma mansoni adults	$0{\cdot}74 \pm 0{\cdot}35$

reductase functions preferentially in the direction of fumarate reduction whereas succinate dehydrogenase functions preferentially in the opposite direction, succinate oxidation (Ackrell *et al*. 1992). Therefore, a low ratio of succinate oxidation/fumarate reduction activity *in vitro* is indicative of the occurrence of fumarate reduction *in vivo* (Ackrell *et al*. 1992; Van Hellemond *et al*. 1995).

Mitochondrial fractions of rat and bovine heart, aerobic-functioning tissues that do not reduce fumarate *in vivo*, but depend on Krebs cycle activity and therefore succinate oxidation, oxidized succinate much better than they reduced fumarate (Table 1). On the other hand, mitochondrial fractions or homogenates of organisms that reduce fumarate *in vivo*, like parasitic helminths and lower marine organisms (Van Hellemond *et al*. 1995), reduced fumarate at least as well as they oxidized succinate (Table 1). The activity ratios of succinate oxidation/ fumarate reduction in fractions of *S. mansoni* were significantly lower than those from aerobic functioning organisms that do not reduce fumarate *in vivo* (rat and bovine mitochondria), and comparable to the ratios of organisms that are known to reduce fumarate *in vivo* (Table 1). These results show that schistosomes contain an enzyme capable of efficient fumarate reduction, which suggests that succinate is produced by fumarate reduction in sporocysts.

Furthermore, a dedicated enzyme, that is able to reduce fumarate efficiently, was present both in adults and sporocysts of *S. mansoni*. It is as yet unknown if more than one form of complex II is present in any stage of *S. mansoni*, as was shown for *H. contortus* (Roos & Tielens, 1994) and *A. suum* (Saruta *et al*. 1995).

Quinone composition in various stages of S. mansoni

For efficient reduction of fumarate *in vivo* not only a distinct enzyme complex but also distinct quinones are necessary to couple electron transport to the reduction of fumarate. Electron transfer from succinate to cytochrome *c* is dependent on ubiquinone, whereas in eukaryotes efficient electron transfer from NADH to fumarate is dependent on rhodoquinone (Van Hellemond *et al*. 1995). Therefore, if sporocysts of *S. mansoni* are capable of producing either CO_2 via the Krebs cycle or succinate via fumarate reduction, depending on the conditions, then these sporocysts should contain not only ubiquinone but rhodoquinone as well.

We analysed the quinone content in *S. mansoni* to investigate the presence of rhodoquinone. Although all investigated stages of *S. mansoni* contained rhodoquinone-10, the sporocysts especially contained a substantial amount of rhodoquinone compared with the amount of ubiquinone (Table 2). The relative amount of rhodoquinone present in *S. mansoni* sporocysts is comparable to that in other facultative anaerobic-functioning eukaryotes, like oysters, mussels, lugworms and fresh water snails, that produce succinate during anoxic conditions (Van Hellemond *et al*. 1995). Furthermore, the rhodoquinone concentration in sporocysts is sufficiently high to propose that the amount of succinate produced (16 nmol/h/mg protein (Tielens *et al*. 1992)) is formed via fumarate reduction, as adults of *F. hepatica*, which are almost completely dependent on fumarate reduction (100 nmol/h/mg protein (Tielens, Van den Heuvel & Van den Bergh, 1984)), contain not more than twice that amount of rhodoquinone (460 pmol/mg protein) (Van Hellemond *et al*. 1996). Therefore, sporocysts of *S. mansoni* contain a substantial amount of rhodoquinone, not only compared with the amount of ubiquinone but also relative to the amount of succinate produced during anaerobic conditions.

S. mansoni sporocysts contain the essential components for succinate production via fumarate reduction as they contain an enzyme dedicated to fumarate reduction together with the presence of a substantial amount of rhodoquinone, which is necessary for efficient fumarate reduction in eukaryotes (Van Hellemond *et al*. 1995). Further-

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Table 2. Quinones in *Schistosoma mansoni*

(Quinones were extracted, isolated and quantified as described in the Materials and Methods section. Results of 3 independent experiments are shown with standard deviations. The rhodoquinone content of miracidia and sporocysts differed significantly from that in adults ($P < 0{\cdot}05$) and the rhodoquinone content of sporocysts differed significantly from that in cercariae ($P < 0{\cdot}05$).)

Stage	Ubiquinone-10 (pmol/mg protein)	Rhodoquinone-10 (pmol/mg protein)	Rhodoquinone (% of total quinone)*
Adults	310±90	14±5	4±1
Miracidia	630±130	160±36	21±4
Sporocysts	720±330	240±90	25±5
Cercariae	780±190	85±11	10±2

* Values represent the percentage rhodoquinone of the total quinones (rhodoquinone + ubiquinone).

more, the catalytic properties of complex II of the electron-transport chain and the rhodoquinone concentration in *S. mansoni* sporocysts were similar to those in other fumarate reducing eukaryotes, which strongly suggests that in *S. mansoni* sporocysts succinate is produced by fumarate reduction, comparable to succinate production in most other parasitic helminths.

Adults of *S. mansoni* contained only marginal amounts of rhodoquinone (Table 2), which correlates with the absence of succinate production in adults. Furthermore, part of the rhodoquinone present in adults might be used to supply the eggs (and hence the miracidia) with rhodoquinone, although no significant differences in rhodoquinone content between males and females were observed (not shown). On the other hand, the free-living stages of *S. mansoni*, miracidia and cercariae, contained a substantial amount of rhodoquinone (Table 2), although these stages do not produce succinate even in the absence of oxygen (Van Oordt *et al*. 1989; Tielens *et al*. 1991*a*). Therefore, the presence of rhodoquinone and a dedicated enzyme complex are apparently not the only pre-requisites for efficient fumarate reduction *in vivo*. Most probably, cercariae contain rhodoquinone as a remnant of the period in the snail before shedding, as quinones have a half-life of approximately 100 h (Thelin, Schedin & Dallner, 1992; Van Hellemond *et al*. 1996). Miracidia, on the other hand, probably contain rhodoquinone to ensure that immediately after transformation to sporocysts this stage contains a sufficiently large amount of rhodoquinone to survive anoxia within the snail, as rhodoquinone synthesis occurs at a slow rate (Van Hellemond *et al*. 1996). The presence of rhodoquinone in miracidia of *S. mansoni* can therefore be considered to be a pre-adaptation for the possibly anoxic periods occurring in the next host.

Synthesis of rhodoquinone in S. mansoni

It had been suggested that rhodoquinone was synthesized from ubiquinone in the protozoan *Euglena gracilis* (Powls & Hemming, 1966) and therefore, in theory, parasitic helminths could synthesize rhodoquinone from ubiquinone obtained from the host. However, Foster *et al*. (1993) demonstrated that ubiquinone is synthesized *de novo* in *S. mansoni*. In addition, it was recently shown that *F. hepatica* adults synthesize rhodoquinone *de novo* as well, as the number of isoprenoid units attached to the quinones of the parasite differed from that in the host and, furthermore, labelled mevalonate was incorporated in rhodoquinone (Van Hellemond *et al*. 1996). Due to technical limitations, rhodoquinone synthesis from labelled mevalonate could not be demonstrated in *S. mansoni*, because the rate of rhodoquinone synthesis in adults was too low, whereas the availability of the stage with an expected higher synthesis rate (sporocysts) was too limited. However, our HPLC analysis demonstrated that next to ubiquinone rhodoquinone is also synthesized *de novo* by schistosomes. The length of the hydrophobic side-chain of rhodoquinone was the same as that of ubiquinone, and contained exclusively 10 isoprenoid units (Table 2), whereas their host (hamster) contains both ubiquinone with 9 or 10 isoprenoid units, in a ratio of 6:4, respectively (not shown). This rhodoquinone was apparently not made by modification of ubiquinone obtained from the host, but was synthesized *de novo*, just as in *F. hepatica* (Van Hellemond *et al*. 1996). In *S. mansoni* especially, sporocysts contained, and thus synthesized, high amounts of rhodoquinone (Table 2).

Differences in metabolism between sporocysts and adults

Although schistosomes contain all the genetic information necessary for fumarate reduction, adult schistosomes do not use this pathway, but instead use another fermentation pathway, lactate production. This use of an energetically less efficient but much simpler pathway confirms that if substrates are plentiful as in the bloodstream of the final host, parasites have a very opportunistic way of living

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(Tielens, 1994). Apparently, food supply in the invertebrate host is more restrictive. Interestingly, adults of *S. mansoni* do contain several essential components for succinate production via fumarate reduction, like PEPCK (Tielens *et al*. 1991*b*) and a complex II that is capable of reducing fumarate (Table 1). On the other hand, rhodoquinone is present only in very low amounts in adults of *S. mansoni*, which suggests that rhodoquinone biosynthesis is down-regulated in the adult stage of *S. mansoni*. The difference in rhodoquinone content during the life-cycle of *S. mansoni* might cause the observed absence of succinate production in adults and the occurrence of succinate production via fumarate reduction in sporocysts.

In conclusion, *S. mansoni* sporocysts contain rhodoquinone (essential for efficient fumarate reduction *in vivo* in eukaryotes) and they produce succinate by fumarate reduction. Therefore, the energy metabolism of schistosomes does not differ in principle from most other parasitic helminths, which are known to rely heavily on fumarate reduction. On the other hand, adults of *S. mansoni* do not use this pathway and excrete only lactate as fermentation product, which correlates with their marginal rhodoquinone content.

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