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Ultrastructural and biochemical detection of biotin and biotinylated polypeptides in *Schistosoma mansoni*

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Abstract

Biotinylation is proposed for the identification of surface proteins in *Schistosoma mansoni* using the streptavidin-HRP conjugate for the detection of labeled polypeptides. However, control samples also showed several endogenous biotinylated polypeptides. In an attempt to determine the possibility of nonspecific binding between the streptavidin-HRP conjugate and polypeptides from *S. mansoni*, the conjugate was blocked with biotinamidecaproate-N-hydroxysuccinimide ester (BcapNHS) before biotin-streptavidin blotting. No bands were detected on the nitrocellulose sheet, demonstrating the specific recognition of biotin by the streptavidin present in the conjugate. Whole cercariae and cercarial bodies and tails showed several endogenous biotinylated polypeptides. The biotin concentration was 13 µg/190,000 cercariae. Adult worms presented less endogenous biotinylated polypeptides than cercariae. These results may be due to changes in the environment from aerobic to anaerobic conditions when cercarial bodies (schistosomula) are transformed into adult worms and a decrease in CO_2 production may occur. Cercariae, cercarial bodies and adult male worms were examined by transmission electron microscopy employing an avidin-colloidal gold conjugate for the detection of endogenous biotin. Gold particles were distributed mainly on the muscle fibers, but dispersed granules were observed in the tegument, mitochondria and cytosol. The discovery of endogenous biotin in *S. mansoni* should be investigated in order to clarify the function of this vitamin in the parasite.

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- *Schistosoma mansoni*

Introduction

Schistosoma mansoni is one of the most prevalent parasitic helminths that infect humans. Snails of the genus *Biomphalaria* are the intermediate host of this parasite, releasing cercariae, the infective larvae for humans, into fresh water. During penetration through the skin, the cercariae lose their tails and the glycocalyx, originating the schistosomula, that later will be transformed into the adult worms. At the same time, changes on the surface and ultrastructural and metabolic changes occur to adapt the worms to the new environment.

S. mansoni survival is dependent on the

degradation of glucose obtained from the endogenous glycogen reserve. Evidence for the presence of gluconeogenesis in *S. mansoni* was not observed until recently, when the activity of four gluconeogenic enzymes was demonstrated in adult worms of *S. mansoni* (1). Two of those enzymes were carboxylases which must contain biotin as prosthetic group in their structures. The presence of endogenous biotin has been shown in several prokaryotes (2-4), fungi and yeast (5,6) and in birds and mammals (7,8). However, no reports are available about this vitamin in helminths.

In the present study, we identified endogenous biotinylated polypeptides in cercariae and adult worms of *S. mansoni* using a streptavidin-HRP conjugate, and the presence of endogenous biotin in cercariae, cercarial bodies and adult worms was detected by transmission electron microscopy.

Material and Methods

Parasites

S. mansoni cercariae of the São Lourenço da Mata (SLM) strain were kindly supplied by Dr. J.F. Gonçalves, Sector of Malacology, Centro de Pesquisas Aggeu Magalhães, FIOCRUZ, Recife, PE. Adult worms were obtained from ether-anesthetized infected mice by heart perfusion 40-45 days after infection with approximately 150 cercariae per animal. Cercarial bodies and tails from *S. mansoni* were separated by vortexing (9), followed by isolation on a Percoll gradient from 10 to 50% (10).

Sample solubilization

Cercariae, cercarial bodies, cercarial tails and adult worms of *S. mansoni* were homogenized separately in 0.25 M Tris-HCl buffer, pH 6.8, containing 4% (w/v) sodium dodecyl sulfate (SDS) and 10% (v/v) 2-mercaptoethanol. After homogenization, the samples were kept at 95°C for 45 min and the digested material was used for electrophoresis and blotting. All solubilized samples were treated with 10% (w/v) trichloroacetic acid for 15 min at 70°C. The samples were then kept on ice for 30 min and centrifuged at 1,000 *g* for 15 min at room temperature. The precipitates were resuspended in 0.5 ml phosphate buffered saline (PBS) (0.14 M NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, and 8.2 mM Na_2HPO_4), pH 7.4, for the protein assay (11).

Sample biotinylation

A total of 2,000 cercariae, 1,500 bodies or 4,500 tails were washed with cold sterile PBS. Each sample was incubated with 0.22 mM biotinamidecaproate-N-hydroxysuccinimide ester (BcapNHS) (Calbiochem Boehring Diagnostics Hoechst, La Jolla, CA) in 250 µl PBS for 5 min at room temperature (12). The reaction was stopped by the addition of 500 µl 2.2 mM L-lysine in PBS.

Detection of biotinylated polypeptides

Solubilized samples (30 µg protein) were submitted to SDS-PAGE (13) and the proteins were transferred to nitrocellulose sheets at a constant current of 100 mA for 50 min at room temperature. The nitrocellulose membrane was incubated overnight at 4°C with 3% (w/v) skim milk in 10 mM Tris buffer, pH 7.2, containing 0.15 M NaCl and 0.5% (v/v) Triton X-100 (TBS-T), washed several times with TBS-T, and incubated with a streptavidin-HRP conjugate (1:4,000 dilution) at room temperature. The membranes were washed with TBS-T and incubated with 10 mM 3,3',5,5'-tetramethylbenzidine, 18 mM sodium dioctyl-sulfosuccinate in citrate-phosphate buffer (50 mM citric acid and 90 mM disodium phosphate), pH 5.0, containing 25% (v/v) ethanol, until visualization of the bands.

Electron microscopy

For transmission electron microscopy, the cercariae, cercarial bodies and adult worms were fixed overnight in 2% (v/v) glutaraldehyde, 4% (v/v) p-formaldehyde and 5 mM $CaCl_2$ in 0.1 M cacodylate buffer, pH 7.4, at 4ºC, postfixed with 1% (w/v) OsO_4, containing 5 mM ferricyanide, for 2 h at 4ºC, dehydrated in acetone and embedded in EPON 812. Sections were cut with a diamond knife, picked up on copper grids, dipped in a colloidon solution and dried. The grids were kept in PBS, pH 7.4, containing 5 mM mannose, 5 mM galactose and 5 mM fucose, for 1 h, washed with distilled water and then incubated with an avidin-colloidal gold conjugate in TBS-T for 2 h at 37ºC. After washing with TBS-T and distilled water, the grids were dried and incubated with uranyl acetate and lead citrate. The samples were examined with a JEOL 100 CX-II electron microscope.

Results

Cercarial bodies and tails of *S. mansoni* were separated on a 20-30% Percoll gradient (v/v) for the tails and 40-50% (v/v) gradient for the bodies.

Figure 1 shows the biotinylated polypeptides of cercariae, cercarial bodies and tails labeled or not with BcapNHS and identified by the streptavidin-peroxidase conjugate. Comparison of the polypeptides from labeled and unlabeled cercariae (Figure 1b and c) demonstrated the presence of several additional bands in the labeled cercariae. Polypeptides from cercarial bodies and tails isolated from labeled cercariae (Figure 1d and f) did not differ from the unlabeled samples (Figure 1e and g). However, the tails presented more biotinylated polypeptides than the bodies. When the bodies and tails were labeled after separation and isolation on the Percoll gradient, a different distribution of the biotinylated polypeptides was observed on the nitrocellulose sheets (Figure 1h and i) compared with the samples obtained from the labeled cercariae, including polypeptides larger than 118 kDa and smaller than 27 kDa.

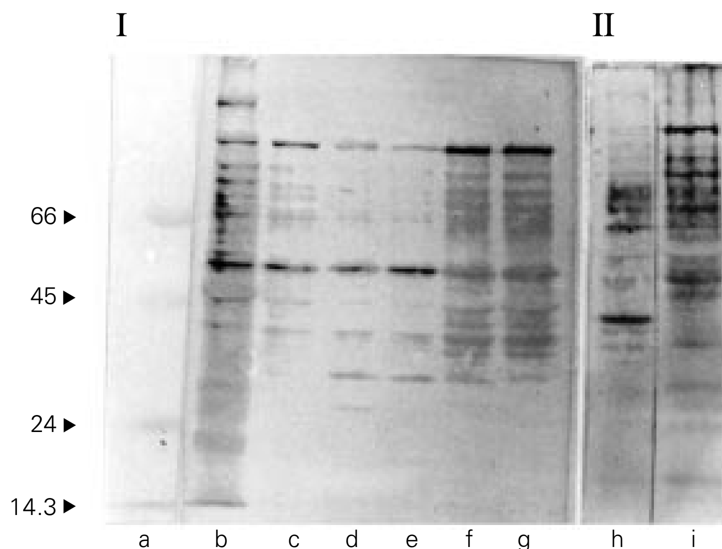

Figure 1 - Detection of biotinylated polypeptides on nitrocellulose sheets using a streptavidin-HRP conjugate. *a*, Molecular mass standards; *b*, cercariae labeled with BcapNHS; *c*, unlabeled cercariae; *d*, cercarial bodies from labeled cercariae; *e*, unlabeled bodies; *f*, cercarial tails from labeled cercariae; *g*, unlabeled tails; *h*, cercarial bodies and *i*, tails labeled with BcapNHS after isolation on a Percoll gradient.

Bands were not detected on the nitrocellulose sheets in the samples containing endogenous biotinylated polypeptides incubated with HRP or streptavidin-HRP conjugate treated with BcapNHS, followed by incubation with an appropriate substrate.

Detection of the endogenous biotinylated polypeptides in male and female worms of *S. mansoni* by the streptavidin-HRP conjugate revealed three bands with apparent molecular masses of 59, 66 and 84 kDa in males, and very faint bands in females (data not shown).

Biotin and biocitin were used as standards for the analysis of these compounds in cercariae. The result obtained with biotin was 13 µg and the result obtained with biocitin was 14.5 µg per 190,000 cercariae.

Transmission electron microscopy of all samples showed the presence of endogenous biotin in *S. mansoni.* In the cercariae, gold particles were mainly distributed on muscle

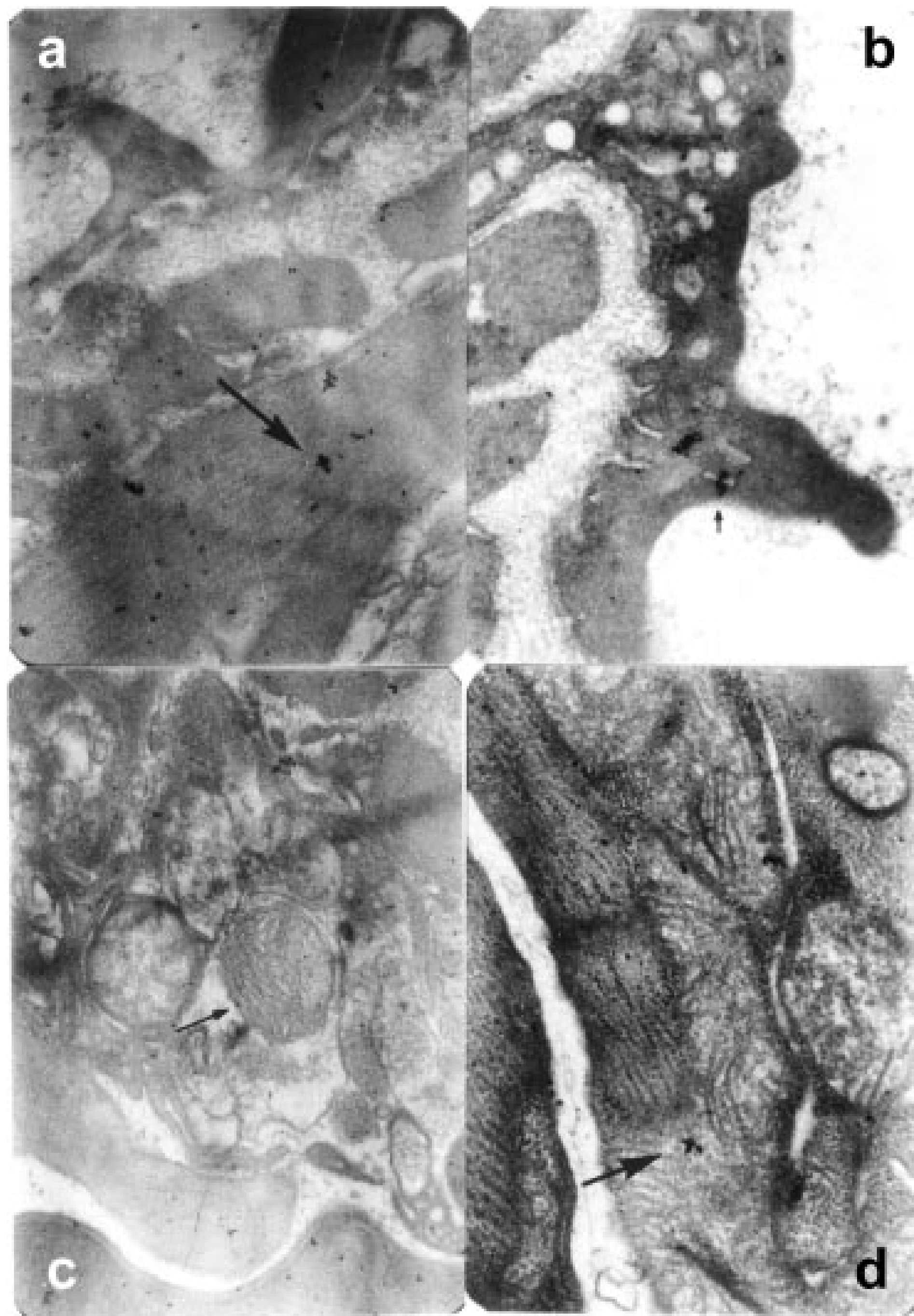

Figure 2 - Transmission electron microscopy showing endogenous biotin in *Schistosoma mansoni*. An avidin-colloidal gold conjugate was used for the detection of the endogenous biotin. *a*, Muscle fibers of cercariae; *b*, spine in a cercarial body; *c*, mitochondria of cercariae; *d*, tissue of male adult worms. Magnification: 40,000X. Arrows point to gold particles.

fibers (Figure 2a), but dispersed granules were observed in the tegument and mitochondria (Figure 2c). Cercarial bodies accumulated dense granules at the base of the spines (Figure 2b). In adult male worms, gold particles were concentrated in areas close to the surface of the worms (Figure 2d).

Discussion

Over the last few years, derivatives of biotin have been used to label biological macromolecules (14-16), including surface proteins of parasites. The profile of surface proteins from *Brugia malayi* labeled with N-

hydroxysuccinimide-biotin (NHS-biotin) did not differ from that obtained with the radioiodination procedure (17). The biological function of *Trypanosoma cruzi* was preserved after labeling its surface proteins with sulfo-N-hydroxysuccinimide-biotin (18). In the present study, the surface of *S. mansoni* cercariae was labeled with BcapNHS in an attempt to identify antigenic proteins, without the risk involving radioactive isotopes. However, control samples presented a large number of endogenous biotinylated polypeptides.

The bands from cercarial bodies and tails obtained from labeled cercariae did not differ from those obtained from unlabeled samples, suggesting the loss of cercarial surface polypeptides from the glycocalyx during the isolation of bodies and tails, since many other bands were seen in the whole labeled cercariae. The different profile of biotinylated bands from bodies and tails labeled after their separation on a Percoll gradient may include proteins present on the external membrane of the schistosomula. The incubation with BcapNHS for 2, 4, 6 and 8 min did not alter the number of bands on the nitrocellulose sheets (data not shown), suggesting that, under the conditions of these experiments, the derivative was not internalized by the worms.

It is well known that tails consume more oxygen than bodies (19) and the transition from aerobic to anaerobic metabolism occurs only in the cercarial bodies (20). On the other hand, biotin is present in carboxylases, enzymes that catalyze the transfer of CO_2 to substrates in the cells. In the host blood, the cercarial bodies find a lower oxygen tension and probably utilize anaerobic metabolism, avoiding O_2 consumption and CO_2 production. Under these conditions the synthesis of biotin-containing enzymes may be reduced in the parasite. The decrease in the relative amount of biotinylated polypeptides in adult worms in comparison with the cercariae may be due to an adaptive metabolic process.

Transmission electron microscopy revealed the presence of biotin in *S. mansoni* mainly in the muscle fibers of cercariae and cercarial bodies, but aggregates of gold particles were also observed at the base of the spines. The function of this vitamin in the parasite is unclear and needs to be investigated.

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