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Accessibility of *Trypanosoma brucei* procyclic glycosomal enzymes to labeling agents of various sizes and charges

J.-P. Patthey and J. Deshusses

Dept of Biochemistry, University of Geneva, 30 quai E. Ansermet, CH-1211 Geneva 4, Switzerland

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The high efficiency of glycolysis in *Trypanosoma brucei* has been attributed to impermeability of the glycosomal membrane to most metabolites. However, the strong stimulation of the glycolytic rate by exogenous metabolites and coenzymes in intact glycosomes is only compatible with their accessibility to the internal space. The accessibility of glycosomal enzymes to protein labeling agents of varying charge and size has been investigated. The results show that the glycosomal membrane is permeable to small molecules of the size of metabolites, but impermeable to larger molecules.

Glycosomal membrane; Latency; Protein labeling; (*Trypanosoma brucei*)

1. INTRODUCTION

A peculiarity of the Trypanosomatidae family, which includes several pathogenic protozoa like *Trypanosoma* and *Leishmania*, is that most of their glycolytic enzymes are localized in a microbody-like particle called the 'glycosome' [1,2].

Glycosomes have been best characterized in *T. brucei brucei*, the agent of the cattle disease 'nagana' in Africa. The long slender bloodstream form of this organism, which lives in the mammalian, lacks functional mitochondria and is completely dependent upon glycolysis for its energy supply. Glycosomal organization is thought to favour the very rapid and efficient glycolysis observed [1], and is therefore a potential target for therapeutic agents. However, in the procyclic form of the parasite living in the tsetse midgut, the glycosome, which has a slightly different organization, may be less important since the mitochondrion is fully active at this stage [3,4].

Electron micrography shows the presence of a single membrane surrounding glycosomes [5]. Its function as a diffusion barrier has been deduced from the increase (4- to 9-times) of the specific activity of glycosomal enzymes, when detergent is added to intact glycosomes [1,3,5,6]. This phenomenon, known as latency, was observed in both bloodstream [7–9] and procyclic [3,4,10] form cells. It was explained by subcellular compartmentalization of glycolysis where ATP, NAD^+(H) and the other phosphorylated intermediates are confined to the glycosome. Specific translocators have been proposed for glucose, DHAP, GP, 3-PGA and P_i^- [1,8]. However, since the addition to intact glycosomes of ATP, PEP, oxaloacetate, NAD^+ [10], F-6-P, ADP, NADH, and the adenylate kinase inhibitor P^1,P^5-di(adenosine-5′) pentaphosphate [9] affects the glycosomal enzymes' activities, these molecules

Correspondence address: J.-P. Patthey, Dept of Biochemistry, University of Geneva, 30 quai E. Ansermet, CH-1211 Geneva 4, Switzerland

Abbreviations: SDS-PAGE, polyacrylamide gel electrophoresis with SDS; DHAP, dihydroxyacetone phosphate; GP, α-glycerophosphate; 3-PGA, 3-phosphoglycerate; PMSF, phenylmethylsulfonyl fluoride; PEP, phospho*enol*pyruvate; F-6-P, fructose 6-phosphate; [1-^{14}C]EAI, ethyl[1-^{14}C]acetimidate; [1-^{14}C]IAI, isethionyl[1-^{14}C]acetimidate

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apparently penetrate inside the particle. These results contradict the model of a glycosome with a relatively impermeable membrane.

In order to clarify the permeability properties of the glycosomal membrane of the procyclic form, we have treated intact glycosomes with protein labeling agents of varying size and charge.

2. MATERIALS AND METHODS

Percoll was purchased from Pharmacia (Uppsala). Enzymobeads were from Bio-Rad (CA, USA), lactoperoxidase (from bovine milk) from Calbiochem-Behring (La Jolla, CA) and glucose oxidase (from *Aspergillus niger*) from Serva (Heidelberg). All other enzymes and coenzymes were obtained from Boehringer (Mannheim). $Na^{125}I$, [1-^{14}C]EAI and [1-^{14}C]IAI were purchased from Amersham (Bucks, England). The other chemicals were obtained from Merck (Darmstadt), Fluka (Buchs, Switzerland) and Serva (Heidelberg).

Procyclic form trypanosomes, stock STIB 366, were cultivated according to [11] and collected at a cell density of 4×10^7/ml.

Glycosomes were isolated essentially as described in [5], except that the cells were disrupted by nitrogen cavitation as in [3]. The integrity of glycosomes was verified by measuring the latency of hexokinase. Latency was calculated as described [10] and measured as in [12]. Glycosomal preparations with less than 80% hexokinase latency were discarded. Protein content was determined by the method of Bradford [13]. All manipulations were carried out at 0°C in an isotonic 170 mM sucrose, 50 mM phosphate buffer (pH 7.2), supplemented with 1 mM EDTA, 0.1 mM PMSF, unless otherwise specified.

Glycosomes were used for labeling immediately after preparation. For radioiodination with Enzymobeads [14], glycosomes (40 μg protein) were incubated for 20 min at 25°C with 0.6 mCi $Na^{125}I$, 5.6 μmol D-glucose and 50 μl of an Enzymobead vial prediluted in 0.5 ml of the experimental buffer. The reaction was stopped by addition of 0.05% NaN_3 and the Enzymobeads removed by low-speed centrifugation.

Radioiodination of glycosomes (90 μg protein) with soluble 2 IU lactoperoxidase and 70 IU glucose oxidase was performed at 4°C in the presence of 40 μmol D-glucose and 150 μCi $Na^{125}I$ for 25 min in a final volume of 500 μl. The reaction was stopped with 0.05% NaN_3 and 0.95 μM $Na_2S_2O_3$. Proteins were then precipitated with 5% trichloroacetic acid in the presence of 0.2 mg/ml of lysozyme as carrier protein. The protein pellets were washed once with trichloroacetic acid and resuspended by neutralizing with NaOH.

Labeling with [1-^{14}C]EAI and [1-^{14}C]IAI was performed by addition of the reagents in solid form to the glycosome suspension because of their instability in aqueous solution. Various ratios of reagent to glycosomes were used (from 0.70 to 0.01 μCi reagent/μg protein). Incubations were performed for 30 min at 37°C in a total volume of 240 μl, at pH 8.0. The reaction was stopped by addition of 60 μl of a solution of 25 mM glycine.

Parallel experiments were also run simultaneously in the presence of 0.5% (w/v) Triton X-100 to render all the proteins accessible to the reagents. The labeled samples were analyzed by 10% acrylamide SDS-PAGE [15] and stained with Coomassie brilliant blue or silver [16]. Autoradiographies (^{125}I) or fluorographies (^{14}C) of the gels were then produced.

3. RESULTS AND DISCUSSION

Glycosomes obtained from *T. brucei* disrupted by nitrogen cavitation were purified according to Opperdoes et al. [5]. Their protein pattern in an SDS gel (fig.1) is identical to that published for pure glycosomes [9,17]. All manipulations were done in an isotonic buffer in order to preserve the integrity of the isolated glycosomes. Their integrity was assayed by the exclusive glycosomal localization of hexokinase in *T. brucei* [1,3,4,6]. Only preparations showing a minimal 80% latency for hexokinase were analyzed. This is the value reported for intact glycosomes contaminated by only a minor fraction of damaged particles [3,4,6,7,9]. Control experiments showed that the latency was unaffected during the labeling procedure. It should be noted that glycosomes are relatively insensitive to osmotic strength variation, as most of the published values of glycosomal enzyme latency were determined in low osmotic strength assay media [12]. Furthermore, the latency determined in glycosomes subjected to osmotic shock was found to be unmodified (not shown).

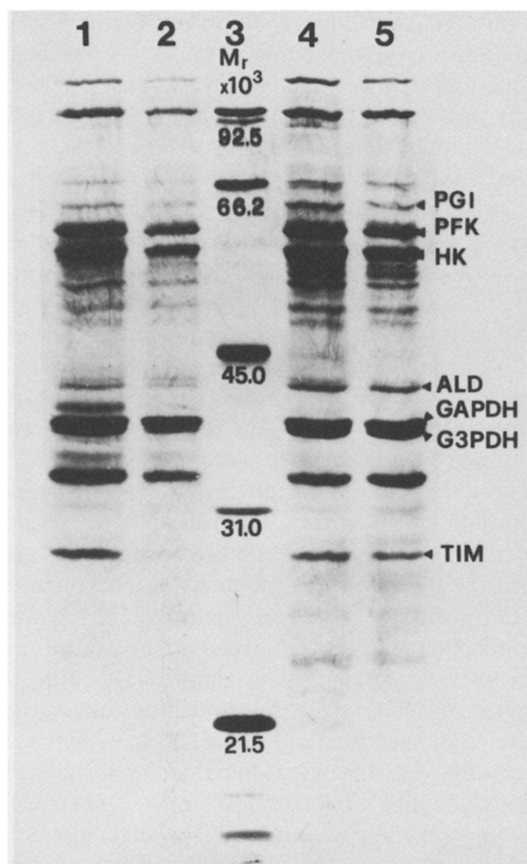

Fig.1. SDS-PAGE of silver-stained glycosomal proteins. The glycosomes were ^{125}I-iodinated using Enzymobeads, in the absence (lanes 2,5) or presence (lanes 1,4) of 0.5% (w/v) Triton X-100. Each lane contains 8 μg proteins. M_r standards (BioRad) (lane 3) and recognized glycosomal enzymes are indicated: PGI, glucosephosphate isomerase; PFK, phosphofructokinase; HK, hexokinase; ALD, aldolase; GAPDH, glyceraldehyde-phosphate dehydrogenase; G3PDH, glycerol-3-phosphate dehydrogenase; TIM, triosephosphate isomerase.

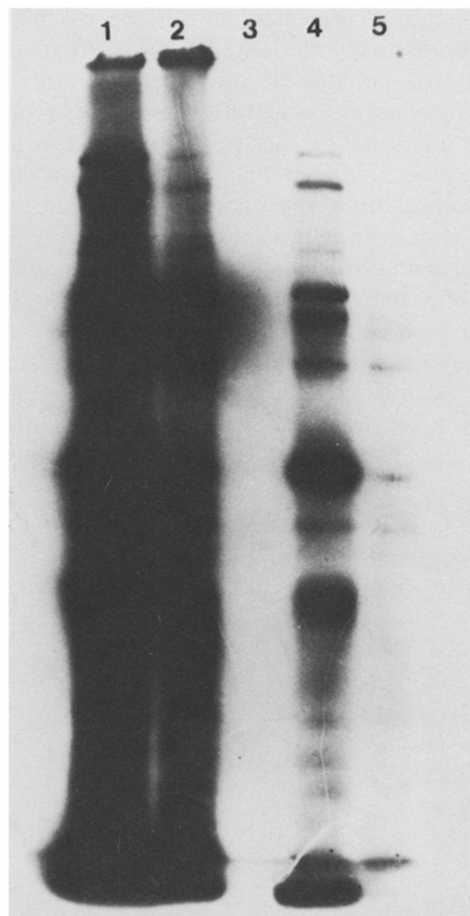

Fig.2. Autoradiography of ^{125}I-labeled glycosomal proteins. The gel is the same as shown in fig.1. Glycosomes were treated with 3.8 μCi ^{125}I per μg protein (lanes 1,2) or 1.9 μCi/μg (lanes 2,5), in the absence (lanes 2,5) or presence (lanes 1,4) of 0.5% (w/v) Triton X-100.

Autoradiographic analysis of an SDS gel shows a low level of protein ^{125}I-iodination in intact glycosomes by the Enzymobead system [14] (fig.2, lanes 2,5). Iodination was much more efficient with glycosomes treated with detergent (fig.2, lanes 1,4). This indicates that iodinating enzymes of Enzymobeads are not able to reach efficiently the glycosomal proteins, unless the membrane is disrupted by the detergent. The low degree of labeling observed in the absence of detergent (fig.2, lanes 2,5) is probably a consequence of labeling a minor fraction of glycosomes whose integrity was damaged during preparation [1,4,9]. Identical results were obtained with soluble lactoperoxidase (M_r 78000) and glucose oxidase (M_r 160000) (not shown). No iodination was detected when lactoperoxidase was omitted from the reaction mixture which excludes iodination mediated by endogenous enzymes.

Smaller labeling reagents were also used. [1-^{14}C]EAI (M_r 123.5) has been shown to permeate erythrocytes and to label all proteins including hemoglobin, while labeling by [1-^{14}C]IAI (M_r 169)

is restricted to the external surface of the cells because of its charge [18]. To define the reaction conditions, experiments on rat erythrocytes were performed and the penetration properties reported in the literature for both agents were confirmed (not shown). In similar reactions on intact glycosomes, both the charged and non-charged reagents in spite of possible differences in reactivity were found to label all the proteins equally with or without detergent (fig.3). These results indicate that the presence of a charge does not prevent penetration of IAI into the glycosome. From the extent of labeling detected in experiments involving various ratios of the reagents to protein, we can

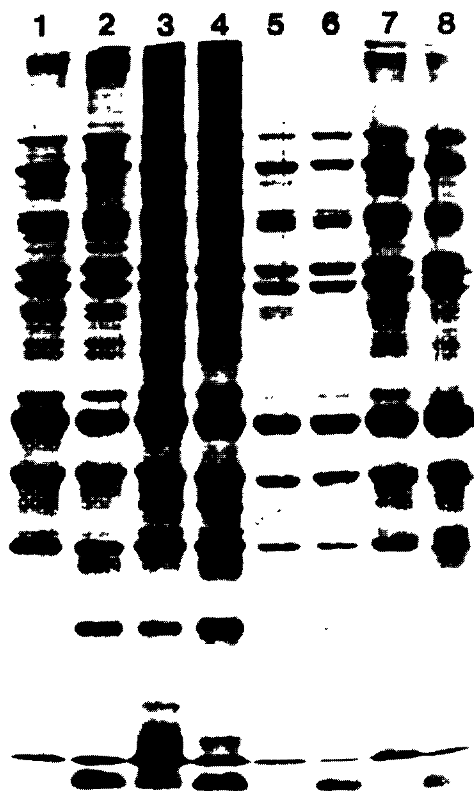

Fig.3. Fluorography of glycosomes labeled with [1-^{14}C]EAI in the absence (lanes 1,5) or presence (lanes 2,6) of 0.5% (w/v) Triton X-100. Glycosomes labeled with [1-^{14}C]IAI in the absence (lanes 3,7) or presence (lanes 4,8) of 0.5% (w/v) Triton X-100 are also shown. Ratio of protein to labeling reagent: 7.2 μg/μCi (lanes 1,2), 16.5 μg/μCi (lanes 3,4), 14.4 μg/μCi (lanes 5,6), 33.0 μg/μCi (lanes 7,8).

exclude the possibility that only proteins from a population of damaged glycosomes are reacting.

One can deduce that the glycosomal particle is freely permeable to low-M_r molecules regardless of their charge. Restriction of permeability is observed with larger molecules (e.g. lactoperoxidase, M_r 78000). Addition of exogenous ATP, PEP, NAD^+ and oxaloacetate strongly stimulates glucose metabolism in intact procyclic glycosomes [10]; this effect is only compatible with the penetration of these substances and is in agreement with the labeling experiments described here. A similar absence of permeability restriction was suggested for ADP, F-6-P and GP in bloodstream form cells' glycosomes [9].

These results suggest that the latency phenomenon in glycosomes should be reinterpreted. Latency of a specific glycosomal enzyme is generally measured by coupled reactions to an NAD^+ redox change, detected by spectrophotometry. The test involves the addition of appropriate exogenous enzymes [12]. In the absence of detergent these exogenous enzymes are excluded, because of their size, from the glycosome by the glycosomal membrane. The claimed latency effect arises from an inefficient coupling reaction in intact glycosomes, since the product of the reaction under test is further transformed by the performing glycosomal multienzyme complex, before diffusing out of the particle and reaching the exogenous enzymes.

Although the distribution and specific activities of glycosomal enzymes have been reported to differ between the bloodstream and procyclic forms [17], the major features of the glycosomes seem to be similar, including latency [3,4], suggesting comparable permeability properties in both forms. The highly efficient glycolysis in the LS bloodstream form of *T. brucei* [19] is presumably a consequence of the structure of the glycosome, but a porous membrane as we find in the procyclic form could not be responsible for high intraglycosomal concentrations of intermediary metabolites, as proposed by some authors [8,9]. The high glycolytic rate measured in glycosomes must rather be due to the reduction of the transient time of the metabolites within the enzymatic complex [20], resulting from the tight packing of enzymes [21], where protein concentration has been estimated at 340 mg/ml [5].

In conclusion, the role of the glycosomal membrane of the procyclic form cells appears not to be in the restriction of the metabolite traffic in and out of the particle, but to be structural, perhaps in providing an organized matrix for the several enzymes involved in a chain of reactions. As a consequence the diffusion time for intermediary metabolites to reach the next enzyme of the chain is reduced, resulting in a marked increase in glycolysis efficiency.

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