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Purification and characterization of a tartrate-sensitive acid phosphatase of *Trypanosoma brucei*

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In search for invariant surface proteins in *Trypanosoma brucei* bloodstream forms, acid phosphatase was investigated. Earlier work had shown that part of the cellular phosphatase activity is associated with the flagellar pocket of the parasite. It is demonstrated that *T. brucei* contains at least two membrane-bound enzymes, one is sensitive to the inhibitor L-(+)-tartrate while the other is resistant. The tartrate-sensitive phosphatase was purified to homogeneity by monoclonal antibody affinity chromatography and shown to be a glycoprotein of low abundance (13,000 molecules/cell). It has an apparent molecular weight of 70,000 Da. The usefulness of acid phosphatase as a marker for characterizing the membrane lining the flagellar pocket is discussed.

Trypanosoma brucei; Acid phosphatase; Flagellar pocket; Latency; Cytochemical staining

1. INTRODUCTION

The only well-characterized surface component of the mammalian stage of the parasitic protozoon *Trypanosoma brucei* is the variant surface glycoprotein (VSG), which forms a dense coat over the entire body of the flagellate [1,2]. In search for surface molecules other than VSG the specialized region of the plasma membrane lining the flagellar pocket or reservoir deserves special attention. The pocket is formed by an invagination of the plasma membrane around the arising flagellum. The microtubule-free membrane lining the pocket is considered to be the only site for endocytosis and exocytosis in trypanosomes, comprising only 1 μm^2, i.e. 0.2% of the total cellular membrane area [3,4]. Therefore, the isolation of this specialized part of the plasma membrane is prohibitive unless highly specific markers and affinity reagents are available.

The present investigation was stimulated by the observation that the flagellar pocket harbors an acid phosphatase, an enzyme which in mammalian cells is mainly associated with lysosomes. By incubating glutaraldehyde-fixed trypanosomes in Gomori's staining solution and glucose-6-phosphate or 2-glycerophosphate as substrates, Seed et al. [5] and Langreth and Balber [6] found heavy deposits of lead-phosphate in the flagellar pocket. In addition, acid phosphatase activity was found in lysosomes, in all structures containing endocytosed ferritin and in Golgi cisternae. Furthermore, comparison of phosphatase activity in live cells and detergent lysates suggested that a considerable fraction of the enzyme was readily accessible to substrate in the intact organism [6,7] and, therefore, associated with the cell surface. Finally, McLaughlin [8] found that *T. rhodesiense* contains a tartrate-sensitive and a tartrate-resistant acid phosphatase which this author considers to be distributed between the flagellar pocket and the surface membrane.

In this study, the existence of two different acid phosphatases in *T. brucei* is clearly demonstrated. The tartrate-sensitive activity is purified to homogeneity. Although accessibility experiments confirm that part of this enzyme is located at the surface, its low abundance has prevented the determination of its cellular distribution by a stoichiometric assay such as immunoelectron microscopy.

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Abbreviations: Np-P, *p*-nitrophenylphosphate; MeUmb-P, 4-methylumbelliferyl-phosphate; MeUmb, 4-methylumbelliferone; PBS, phosphate-buffered saline; VSG, variant surface glycoprotein; SDS, sodium dodecylsulfate

2. MATERIALS AND METHODS

2.1. *Estimation of acid phosphatase activity*

Aliquots of enzyme containing fractions were incubated in 1 ml 100 mM acetate-buffer, 0.2% Triton X-100, pH 5.0, in the presence or absence of 0.5 mM Np-P for 60 min at 37°C. After addition of 100 μl 1 M Na_2CO_3, the absorbance of *p*-nitrophenol at 405 nm was estimated and converted to μmoles substrate hydrolyzed using an $\epsilon_{405} = 18.5\ cm^2/\mu mol$. The tartrate-resistant activity was measured in the presence of 40 mM L-(+)-tartrate. The tartrate-sensitive activity corresponds to the difference between the total activity and the tartrate-resistant fraction.

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2.2. *Separation of two acid phosphatases by isoelectric focusing*

Trypanosoma brucei variant clone 1.4 was isolated from the blood of infected rats by DEAE cellulose column chromatography [9]. A detergent extract from parasite membranes was prepared as described in [10] replacing Triton X-100 by 1% octyl-β-D-glucoside. For preparative isoelectric focusing, 4 g BioLyte (granulated polyacrylamide from Bio-Rad Laboratories) was swollen in 60 ml H_2O overnight. After addition of 1 g octylglucoside, 100 mg arginine-HCl, 0.5 ml Ampholine 5–7, 2 ml Ampholine 7–9 and 2 ml Ampholine 9–11 the volume was adjusted to 100 ml with H_2O. After casting in a flat bed (10.8 × 24.3 cm), the gel was dried at 37°C to a final weight of 50 g. The gel was supplied with 3 ml of trypanosome extract (equivalent to 3×10^9 cells) and run at 15 W (constant) and 1500 V (maximum) on a Multiphor 2117 (Pharmacia-LKB) for 15 h. Fractions containing the tartrate-sensitive phosphatase activity were eluted from the gel and concentrated on an Amicon PM10 membrane.

2.3. *Production of hybridoma cell lines*

BALB/c mice were immunized with partially purified tartrate-sensitive phosphatase reconstituted in liposomes. Cholesterol (4 mg), dicetylphosphate (1 mg), phosphatidylcholine (15 mg) and octylglucoside (60 mg) were dissolved in 2 ml chloroform and dried to a film in a round-bottomed flask by an N_2-stream. The protein solution (see above) was added and the mixture sonicated. Extensive dialysis against PBS (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.2) at 4°C resulted in proteoliposomes which were pelleted by ultracentrifugation. Mice were injected intraperitoneally with proteoliposomes and boosted in 4-week intervals. Spleen cells of mice with high serum antibody levels against the enzyme were fused with the myeloma line P3-NS1-1Ag4-1 as described before [10].

For detection of anti-phosphatase antibodies, protein A-Sepharose 6 MB (50 μl) equilibrated with 100 mM Na-phosphate, 0.1% Triton X-100, pH 8.0, was incubated with mouse serum or hybridoma supernatant in a total volume of 400 μl buffer. After rotating the samples for 60 min at room temperature, the gel was washed 3 times with 400 μl buffer. Subsequently, the gel was rotated with 25 μl trypanosome membrane extract (equivalent to 2.5×10^7 cells) in 400 μl buffer for 60 min at room temperature. After 3 washings with 400 μl 10 mM Na-phosphate, 0.1% Triton X-100, pH 8.0, the gel was incubated with 50 μl 100 mM acetate, 0.1% Triton X-100, 0.2 mg/ml MeUmb-P, pH 5.0, with or without 40 mM L-(+)-tartrate for 1–16 h at room temperature. For screening hybridoma clones, a holder containing forty 0.5-ml Eppendorf tubes was directly inspected for MeUmb formation under a UV lamp. A line designated mAT502 secreting an IgG_1 monoclonal antibody was cloned and established as an ascites in BALB/c mice.

2.4. *Purification of the tartrate-sensitive acid phosphatase*

The monoclonal antibody mAT502 was purified from ascites fluid by chromatography on protein A-Sepharose CL-4B and coupled to activated CH-Sepharose 4B as described by the manufacturer (Pharmacia-LKB). The following purification was performed on an FPLC-system (Pharmacia-LKB) at a flow-rate of 0.15 ml/min at 4°C. A column (0.5 ml) containing 2 mg mAT502 IgG was equilibrated against 100 mM Na-phosphate, 0.1% Triton X-100, pH 7.0, and loaded with a membrane extract of $2.5 \cdot 10^{10}$ trypanosomes using 1% Triton X-100 as detergent. Thereafter, the column was washed with 30 ml of 100 mM Na-phosphate, 0.1% Triton X-100, pH 7.0, and the bound enzyme was eluted with 100 mM glycine-HCl, 0.1% Triton X-100, pH 3.0. Fractions of 1 ml were collected in tubes containing 200 μl 1 M Tris-HCl, 0.1% Triton X-100, pH 8.0. The enzyme-containing fractions were pooled and stored at −70°C. The column could be used for further purifications.

3. RESULTS

3.1. T. brucei *contains two membrane-bound acid phosphatases*

Using the chromogenic substrate, *p*-nitrophenylphosphate, the phosphatase activity of a cell lysate of bloodstream forms was determined in the presence or absence of 40 mM L-(+)-tartrate. Only 43% of the total activity was sensitive to the inhibitor suggesting the presence of at least two different enzymes. Upon isoelectric focusing, the activity was separated into two peaks corresponding to a tartrate-sensitive enzyme focusing at a pH ≈ 6.5 and a tartrate-resistant enzyme concentrated at a pH ≈ 8.0 (Fig. 1). Both enzymes were largely membrane bound. About 83% of the total activity in a cell lysate was associated with the particulate fraction and upon phase separation of cells in Triton X-114 [11], 94% of the tartrate-sensitive and 68% of the tartrate-resistant enzyme were recovered in the detergent phase.

3.2. *Purification and properties of the tartrate-sensitive acid phosphatase*

The tartrate-sensitive enzyme eluted from a preparative isoelectric focusing gel had a specific activity of a 0.902 μmol min^{-1} mg $protein^{-1}$, about 70-fold higher than in a cell lysate (yield 21%). This partially purified preparation was reconstituted into proteoliposomes which were used for immunization of mice. Taking these mice as spleen cell donors, several hybridoma cell lines reacting with the tartrate-sensitive enzyme were obtained. The line secreting the IgG_1 monoclonal antibody mAT502 was used to purify the enzyme in one step over 3000-fold in a yield of 35% (Table I). Importantly, none of the tartrate-resistant activity was retained by the affinity column while all the tartrate-sensitive enzyme was bound.

The tartrate-sensitive enzyme purified by affinity chromatography was homogenous as judged by SDS-polyacrylamide gel electrophoresis (approx. M_r = 70,000, cf. Fig. 2). The enzyme had a K_m of 0.4 mM for Np-P, showed a broad pH-optimum around pH 5.0, and was inhibited by 50% in the presence of 15 μM L-(+)-tartrate while the tartrate-resistant activity was not inhibited at a concentration as high as 100 mM. Both

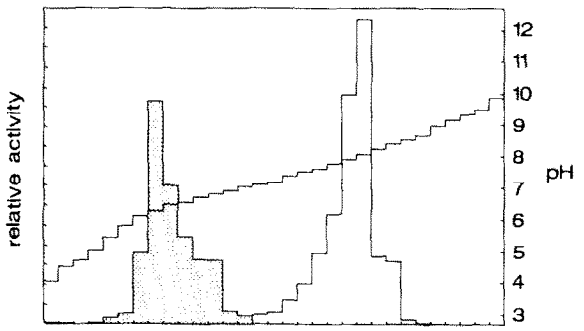

Fig. 1. Preparative isoelectric focussing of a detergent extract of membranes from *T. brucei*. The shaded area shows the distribution of the tartrate-sensitive phosphatase activity; the non-shaded area depicts the tartrate-resistant activity.

Table I

Purification of tartrate-sensitive acid phosphatase

	Protein (mg)	Activity (μmol/min)	Specific activity (μmol/min/mg protein)	Yield (%)
Cell lysate[a]	120	1.61	0.0134	100
Detergent extract from membranes	33	1.24	0.0376	77
Eluate of mAT502 affinity column	0.013	0.565	43.4	35

[a]corresponding to 2.5×10^{10} bloodstream trypanosomes.

enzymes appear to be glycosylated because they were bound to Concanavalin A-Sepharose and 30% of the activity could be recovered by elution with α-methylmannoside.

3.3. *Cellular localization*

Numerous attempts to localize the tartrate-sensitive enzyme by immunofluorescence or immunoelectron microscopy using the monoclonal antibody were unsuccessful. In unfixed cells incubated in 150 mM acetate, pH 5.0, about half of the total phosphatase activity was accessible to Np-P confirming previous work [6,7]. Interestingly, 42 or 56% of the resistant or sensitive activity, respectively, were accessible when the two enzymes were differentiated by their sensitivity to L-(+)-tartrate. Although fixation partially inactivated both activities, the degree of latency was not dramatically altered (not shown). Therefore, these experiments suggest that approximately half of both the tartrate-sensitive enzyme and the tartrate-resistant enzyme(s) are located at the cell surface, the rest being intracellular. Another argument for a similar cellular distribution of the enzymes yielded isopycnic sucrose gradient centrifugation experiments of the postnuclear supernatant of cell extracts. Both activities banded as overlapping broad peaks centered at a density of 1.15 g/cm^3 (data not shown, compare [7 and 12]).

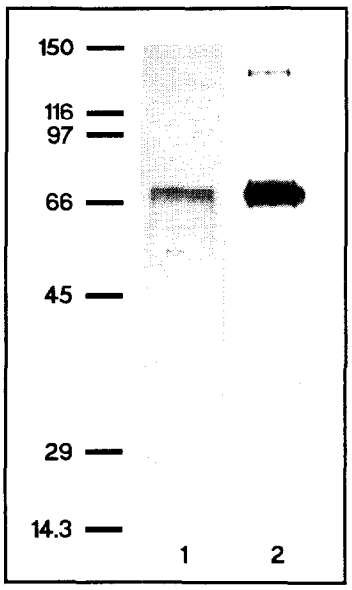

Fig. 2. SDS-polyacrylamide gel electrophoresis of affinity purified tartrate-sensitive phosphatase in the presence (lane 1) or absence (lane 2) of mercaptoethanol stained with silver. The minor component in lane 2 at an approx. M_r 140,000 may be a phosphatase dimer. The molecular weight of standard proteins in kDa is indicated.

4. DISCUSSION

Attempts to purify the acid phosphatase of *T. brucei* led to the finding that there is one tartrate-sensitive and at least one tartrate-resistant enzyme. As judged by the criterion of Triton X-114 phase partitioning all of the former protein can be expected to be an integral membrane component while only 68% of the latter activity has amphipathic properties. While the enzymes showed a very similar behavior on ion exchange columns, their different isoelectric points readily allowed their separation. Using the partially purified tartrate-sensitive phosphatase as an immunogen and a convenient screening assay, an anti-enzyme monoclonal antibody was obtained which allowed a one-step purification.

The objective of this study was to use antibodies against the acid phosphatase as a tool for the eventual isolation of the membrane lining the flagellar pocket which can be expected to harbor a number of interesting and non-variant trypanosomal proteins. The tartrate-sensitive enzyme turned out to be present in only about 13,000 molecules per cell. Considering that the glycosylphosphatidylinositol-specific phospholipase C (30,000 molecules/cell) was at the borderline of detectability by immunoelectron microscopy [10,12], the determination of the cellular distribution of the phosphatase by this stoichiometric assay appears to be prohibitive. In analogy to studies on a lysosomal phosphatase in mammalian cells [13], this unfavorable situation may be improved by overexpression of the gene(s) coding for the phosphatase(s).

Based on information from previous studies [5–8,14] acid phosphatase activity appears to be confined to the flagellar pocket and to intracellular compartments (tubules, vesicles, Golgi stacks) which directly communicate with the flagellar pocket, while, in contrast to mammalian cells, the activity in lysosomes is relatively low [15]. We have confirmed these observations on the electron microscopic level using Ce^{3+} as a capture heavy metal ion [16] but this technique could not be used in the presence of inhibitor because tartrate forms a tight complex with Ce^{3+} preventing any deposit formation (unpublished experiments). The latency experiments suggest that the distribution of the tartrate-sensitive and tartrate-resistant versions of the enzyme

may be similar. Upon cell breakage a spectrum of phosphatase-containing vesicles will be formed comprising probably not more than 1% of the total cellular membrane area, which band in isopycnic sucrose gradients over a broad density range together with most other organelles and membranes with the exception of the well-separated glycosomes. Unless additional techniques are developed, it appears very difficult to separate the minute amount of flagellar pocket membrane from the mixture of most other cellular membranes. Finally, although enzymatic activity is present in the flagellar pocket, no secreted acid phosphatase activity was detectable in the medium of cultured bloodstream forms.

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