Adenosine Triphosphatase Activity of *Tritrichomonas foetus*

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(Received 11 January 1979; revised 25 April 1979)

Homogenates of *Tritrichomonas foetus* exhibited a Mg^{2+} -dependent adenosine triphosphatase (ATPase) activity, with a pH optimum in Tris buffers of 8.2 to 8.3. The activity was not sensitive to oxygen. At high concentrations, quercetin and 4-chloro-7-nitrobenzofurazan inhibited ATPase activity in the cytoplasmic extract by 20 and 70%, respectively, whereas oligomycin, venturicidin, triethyltin, leucinosatin, dibutylchloromethyltin chloride, spagazzinine, efrapentin, citreoviridin and sodium azide had no effect and *N,N'*-dicyclohexylcarbodi-imide stimulated the activity somewhat. The activity was localized in a population of small cytoplasmic particles which also contained an acid phosphatase. There was no indication of an association of ATPase with hydrogenosomes. The ATPase activity (or activities) in this aerotolerant anaerobe is different from the ATPases characteristic of mitochondria or of anaerobic bacteria.

INTRODUCTION

Flagellate protozoa of the order Trichomonadida are parasitic aerotolerant anaerobes. Those species that have been studied are distinct from aerobic eukaryotes in that they have no cytologically recognizable mitochondria (Brugerolle, 1972; Daniel *et al.*, 1971; Honigberg *et al.*, 1971; Honigberg, 1978) and no detectable cytochromes (Ryley, 1955; Čerkasovová, 1970; Lloyd *et al.*, 1979), and they do not carry out oxidative phosphorylation (Čerkasov *et al.*, 1978; Müller & Lindmark, 1978). The major organelle of their energy metabolism is the hydrogenosome which produces H_2 under anaerobic conditions (Lindmark & Müller, 1973; Lindmark *et al.*, 1975) and acts as a respiratory organelle in the presence of O_2 (Čerkasov *et al.*, 1978; Müller & Lindmark, 1978). The biological nature and affinities of this organelle are poorly understood. Since adenosine triphosphatases (ATPases) of different organelles and micro-organisms show characteristic properties, it was felt that a study of the ATPase activities present in a trichomonad could contribute to the elucidation of the biological nature of hydrogenosomes.

This paper reports some of the properties of the ATPase in *Tritrichomonas foetus*. No inhibitor binding sites characteristic of mitochondrial ATPase nor the presence of an ATPase similar to that found in *Clostridium pasteurianum* (Clarke & Morris, 1976, 1977) could be detected. The ATPase is not localized in the hydrogenosomes but in a population of small particles which were described earlier as organelles containing certain acid hydrolases (Müller, 1973).

METHODS

Maintenance, growth and harvesting of the organism. *Tritrichomonas foetus* KV₁ (obtained from Dr B. M. Honigberg) was cultured in Diamond's liquid TYM medium without agar, supplemented with 10% (v/v) heat-inactivated horse serum (Diamond, 1957). Stock cultures were subcultured daily and used to inoculate 400 or 800 ml of culture medium (in 500 or 1000 ml Erlenmeyer flasks closed with screw caps).

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Cultures were grown for 24 h at 37 °C and harvested when the population reached 1×10^6 to 2×10^6 organisms ml^{-1} (Müller, 1973). Organisms were harvested at room temperature in a Sorvall RC-3 centrifuge (GS-3 rotor; 500 ml bottles) at 1000 g_{av} for 5 min, and then washed three times in 50 mM-Tris/ H_2SO_4 buffer (pH 6.7) at room temperature.

Preparation of cell extracts and subcellular fractionation. Organisms were resuspended in 3 vol. ice-cold 50 mM-Tris/ H_2SO_4 buffer or in unbuffered 250 mM-sucrose. Cells were broken with 20 to 30 strokes in a Potter-Elvehjem tissue homogenizer fitted with a smooth Teflon pestle rotating at 1000 rev. min^{-1} . Whole cells and nuclei were sedimented in a Sorvall RC-2B refrigerated centrifuge (SS-34 rotor) at 400 g_{av} for 4 min and the supernatant (termed the cytoplasmic extract) was carefully decanted. In certain experiments rigorous precautions were taken to maintain anaerobiosis during the harvesting and disruption of organisms and subsequent preparation of homogenates, by bubbling argon through all buffers and by the inclusion of 150 mM-2-mercaptoethanol.

For cell fractionation studies the cells were resuspended in 250 mM-sucrose and broken by the procedure described above. Differential centrifugation, as previously described (Lindmark & Müller, 1973), yielded four fractions: a nuclear fraction (N, at 1600 $g\text{-min}$), a large particle fraction (P_1 , at 3.6×10^4 $g\text{-min}$), a small particle fraction (P_2 , at 7.2×10^6 $g\text{-min}$), and a final supernatant (S). In one experiment the large and the small particle fractions were subfractionated by isopycnic centrifugation in a sucrose gradient using the Beaufay rotor (Leighton *et al.*, 1968). The methods used and the representation of the results were described earlier (Müller, 1973).

Enzyme assays. ATPase (EC 3.6.1.3) was assayed by incubating extracts in a mixture containing 50 mM-Tris/ H_2SO_4 buffer, 2 mM-ATP and 6 mM- MgSO_4 at pH 8.3 in a total volume of 1 ml at 30 °C. After the reaction had been stopped by adding 100 μl of 50% (w/v) trichloroacetic acid, the protein precipitate was sedimented by centrifugation and P_i was determined in the supernatant fluid by the method of Fiske & SubbaRow (Leloir & Cardini, 1957). Corrections for enzyme and substrate blanks were made as a routine and results are mean values from triplicate determinations (S.D. $< \pm 3\%$). In experiments where the effect of freezing and thawing and of detergents on the activity was studied, the cytoplasmic particles were osmotically protected by inclusion of 250 mM-sucrose in all solutions. Assays for acid phosphatase (EC 3.1.3.2) using *p*-nitrophenylphosphate as substrate, β -*N*-acetylglucosaminidase (EC 3.2.1.30), NADH dehydrogenase (NADH: oxygen oxidoreductase) and malate dehydrogenase (decarboxylating) were as previously described (Müller, 1973; Lindmark & Müller, 1973). Protein was determined by an automated Lowry procedure with bovine serum albumin as standard (Leighton *et al.*, 1968). Enzyme units (U) are defined as the amount of enzyme necessary to release 1 μmol product min^{-1} under the assay conditions.

Chemicals. ATP (disodium salt), oligomycin and quercetin (3,3',4',5,7-pentahydroxyflavone) were from Sigma; citreoviridin from Cambrian Chemicals, Beddington Farm Road, Croydon; *N,N'*-dicyclohexylcarbodi-imide and venturicidin from BDH; 4-chloro-7-nitrobenzofurazan (Nbf-Cl) from Aldrich Chemical Co., Wembley, Middx; efrapetin (antibiotic A23871) and leucinostatin from Dr R. Hamill, Lilly Research Laboratories, Minneapolis, Minn., U.S.A.; triethyltin sulphate and dibutylchloromethyltin chloride from Dr D. E. Griffiths, Department of Molecular Sciences, University of Warwick, Coventry; and spgazzinine, a dihydroindole alkaloid, from Division de Química Organica Superior, Universidad Nacional de la Plata, La Plata, Argentina. All inhibitors were dissolved in ethanol.

RESULTS

In Tris/maleate or Tris/ H_2SO_4 buffers the ATPase displayed optimum activity at pH 8.2 to 8.3. The specific activity of ATPase at the pH optimum in Tris/ H_2SO_4 at 30 °C was 25.5 ± 5.6 mU (mg protein) $^{-1}$ (mean value $\pm$ s.d. for determinations on six different extracts). The pH dependence and specific activity of the enzyme were not altered if the homogenates were prepared under anaerobic conditions in the presence of 2-mercaptoethanol. The enzyme was Mg^{2+} -dependent. Replacement of Mg^{2+} by Ca^{2+} decreased activity to 64%, and Na^+ or K^+ , added singly or together, gave less than 17% of the activity observed with Mg^{2+} (Table 1).

Of the 12 compounds tested as ATPase inhibitors, only Nbf-Cl and quercetin gave significant inhibition (Table 2). The concentrations used were greatly in excess of the I_{50} values previously determined for the mitochondrial ATPase of yeast (Lloyd & Edwards, 1976, 1977). At the optimum pH, *N,N'*-dicyclohexylcarbodi-imide [20 μg (mg protein) $^{-1}$] stimulated activity by 21% whereas slight inhibition was observed at pH values below 6.3.

Activity of ATPase of cytoplasmic extracts prepared in 250 mM-sucrose was increased

Table 1. *Effect of cations on ATPase activity of T. foetus*

A cytoplasmic extract was dialysed for 24 h against 50 mM-Tris/HCl buffer (pH 8.7) in 250 mM-sucrose. The assay for ATPase was as described in Methods except that Tris/HCl was used instead of Tris/H₂SO₄ buffer. Cations or EDTA were added at 20 mM.

Addition	Relative activity
None	8
EDTA	4
Mg ²⁺	100
Ca ²⁺	64
K ⁺	17
Na ⁺	13
K ⁺ + Na ⁺	14

Table 2. *Effect of inhibitors on ATPase activity of a cytoplasmic extract of T. foetus*

Inhibitors dissolved in ethanol were added to the enzyme preparation and pre-incubated for 10 min before starting the reaction. The specific activity of the extract was 33 mU (mg protein)⁻¹. Tests and controls contained 2% (v/v) ethanol.

Inhibitor	Concn [μ g (mg protein) ⁻¹]	Activity (% of control)
<i>N,N'</i> -Dicyclohexylcarbodi-imide	72	132
Oligomycin	182	94
Venturicidin	36	104
Triethyltin sulphate	36	92
Leucinostatin	36	107
Dibutylchloromethyltin chloride	72	99
Spegazzinine	18	95
Quercetin	182	82
Efrapeptin	36	103
Nbf-Cl	182	23
Citreoviridin	18	104
Sodium azide (in H ₂ O)	2 mM	93

Fig. 1. Effect of Triton X-100 on activities of ATPase (▲) and acid phosphatase (●) in cytoplasmic extracts of *T. foetus*. The final concentration of protein in the assay was 490 μ g ml⁻¹.

by about 20% by three cycles of freezing and thawing, without any further increase if the procedure was repeated up to 15 cycles. A 50% increase of activity was observed in the presence of low concentrations [200 μ g (mg protein)⁻¹] of the non-ionic detergent Triton X-100, but larger amounts of the detergent elicited a significant decrease in activity (Fig. 1). Acid phosphatase activity similarly was not unmasked fully by freezing and thawing, but could be fully activated by Triton X-100 at $\geq 500 \mu$ g (mg protein)⁻¹.

The distributions of malate dehydrogenase (decarboxylating), a marker enzyme for

Fig. 2. Distribution of enzymes in a homogenate of *T. foetus* after differential centrifugation. Relative specific activities of the enzymes are plotted against the cumulative percentage of protein recovered in each fraction. The direction from left to right corresponds to increasing centrifugal field. The left-hand block (N) of each histogram represents the nuclear fraction and the right-hand block represents the final supernatant (S). The large particle fraction (P₁; stippled) and the small particle fraction (P₂; shaded) were subfractionated by isopycnic centrifugation (Fig. 3). Percentage recoveries: ATPase (114), acid phosphatase (95), β-N-acetylglucosaminidase (59), malate dehydrogenase (decarboxylating) (80), NADH dehydrogenase (84) and protein (74).

hydrogenosomes (Müller, 1973; Lindmark & Müller, 1973), and of acid phosphatase and β-N-acetylglucosaminidase, markers for the smaller and larger hydrolase-containing particles, respectively, showed, after either the differential (Fig. 2) or isopycnic (Fig. 3) centrifugation, distributions similar to those reported earlier (Müller, 1973). Over 90 % of the NADH dehydrogenase was associated with the final supernatant, indicating a low contamination of the particulate fractions with the non-sedimentable portion of the cytoplasm. More than 90 % of the total ATPase was associated with the particulate fractions, with highest relative specific activity in the small particle fraction (Fig. 2). The distribution patterns of ATPase observed after differential (Fig. 2) and isopycnic (Fig. 3) centrifugation were similar to those of acid phosphatase, suggesting that the major subcellular site of these two enzymes is a well-defined population of hydrolase-containing smaller particles. No evidence could be found for an association of ATPase with hydrogenosomes. Isopycnic centrifugation clearly separated from the hydrogenosomes the ATPase activity recovered in the large particle fraction after differential centrifugation.

Although the fractionation results did not suggest the association of ATPase with hydrogenosomes, the properties of the enzymic activity observed in the large particle fraction were studied to obtain further information on the possibility of an ATPase being present in these organelles. The optimum pH value for ATPase in the large particle fraction, as in the cytoplasmic extracts, was 8.3; stimulation of activity was observed when *N,N'*-dicyclohexylcarbodi-imide [$95 \mu\text{g (mg protein)}^{-1}$] was included in the assay at all pH values between 5.2 and 8.6. Quercetin [$240 \mu\text{g (mg protein)}^{-1}$] gave up to 59 % inhibition over this pH range; azide had no effect. At pH 8.3, *Nbf-Cl* [$450 \mu\text{g (mg protein)}^{-1}$] gave 70 % inhibition and triethyltin sulphate [$90 \mu\text{g (mg protein)}^{-1}$] 50 % inhibition. All other compounds tested with ATPase activity of cytoplasmic extracts were without significant inhibitory effect on the ATPase activity of the large particle fraction. *N,N'*-Dicyclohexylcarbodi-imide [up to $95 \mu\text{g (mg protein)}^{-1}$] did not inhibit ATPase in any of the fractions.

Fig. 3. Distribution of enzymes after isopycnic centrifugation of the large particle fraction (left) and the small particle fraction (right) represented in Fig. 2 by the stippled and shaded areas, respectively. Density-frequency plots are shown. The scales of the ordinates have been adjusted so that the area under each histogram is proportional to the percentage of total enzyme activity present in the particle fractions used as starting material; however, the ordinate has been expanded threefold for protein, to improve clarity. The dashed lines represent the relative frequencies of components distributed uniformly through the gradients; they therefore delimit areas equal to those of the corresponding histograms. Relative frequency is given by the equation (de Duve, 1967):

$$\text{Relative frequency} = \frac{\text{Enzyme units in a fraction}}{\text{Total enzyme units recovered}} \times \frac{1}{\text{Percentage density increment in that fraction}}$$

Percentage recoveries for the two gradients: ATPase (116 and 75), acid phosphatase (76 and 107), malate dehydrogenase (decarboxylating) (65 and 78), protein (83 and 97).

DISCUSSION

Electron microscopy of *T. foetus* has revealed many different subcellular structures, but no organelles recognizable as mitochondria (Honigberg *et al.*, 1971; Müller, 1973; Honigberg, 1978). The most conspicuous membrane-bounded organelles are the hydrogenosomes, the subcellular sites of pyruvate oxidation, which differ in several major characteristics from mitochondria (Müller, 1973; Lindmark & Müller, 1973; Čerkasov *et al.*, 1978; Müller & Lindmark, 1978) but whose biological nature is not fully elucidated. It is now well

established that some eukaryotic micro-organisms lack differentiated mitochondria under certain environmental conditions or in certain stages of their life cycle. This is observed in anaerobically grown, facultatively anaerobic yeasts and in the bloodstream forms of members of the *Trypanosoma brucei* group. However, these organisms still retain mitochondrial ATPase activity sensitive to oligomycin (Cartledge & Lloyd, 1973; Opperdoes *et al.*, 1977), to *N,N'*-dicyclohexylcarbodi-imide (Cartledge & Lloyd, 1973), as well as to a number of other mitochondrial ATPase inhibitors (T. G. Cartledge & D. Lloyd, unpublished observations). In contrast to these organisms, the ATPase activity of *T. foetus* was inhibited only by Nbf-Cl and quercetin, which, although potent inhibitors of the F_1 portion of mitochondrial ATPase (Lloyd & Edwards, 1977), are likely to be less specific in their action than the antibiotic inhibitors oligomycin, citreoviridin, leucinostatin and efrapeptin. Quercetin is also known to inhibit the Na^+, K^+ -ATPase of plasma membranes (Racker, 1976). Mitochondrial ATPases from mammals, yeast (Lloyd & Edwards, 1977) and from the protozoon *Acanthamoeba castellanii* (S. W. Edwards & D. Lloyd, unpublished observations) all show similar sensitivities to inhibitors. Thus the present observations further confirm the absence from *T. foetus* of enzymes typical of the inner mitochondrial membrane (Lloyd *et al.*, 1979).

The similarity between several of the reactions involved in the generation of H_2 from pyruvate in *T. foetus* (Lindmark & Müller, 1973) and in obligately anaerobic bacteria of the genus *Clostridium* (Uyeda & Rabinowitz, 1971) has led to the speculation that the hydrogenosome may have evolutionary affinities with these prokaryotes. An oxygen-sensitive ATPase in *C. pasteurianum* has a transport function (Riebeling *et al.*, 1975; Clarke & Morris, 1977), and although the enzyme is not inhibited by a number of inhibitors, it is sensitive to inhibition by *N,N'*-dicyclohexylcarbodi-imide and Nbf-Cl (Clarke & Morris, 1976, 1977). Thus the ATPases of *T. foetus* do not include an enzyme resembling either the mitochondrial or clostridial enzymes in their inhibitor binding sites.

Our results demonstrate that there is no Mg^{2+} -activated ATPase in the hydrogenosomes, suggesting that transport processes participating in the function of this organelle (Čerkasovová & Čerkasov, 1976) are not linked directly to an ATP-dependent proton translocation mechanism.

The subcellular location of the ATPase of *T. foetus* was found to be the population of smaller subcellular particles, which have previously been shown to contain acid phosphatase and also β -glucuronidase, but which lack several other hydrolases that are confined to larger hydrolase-containing organelles in this organism (Müller, 1973). The functional role of these organelles and that of the trichomonad ATPase await further investigations.

We wish to thank Dr J. McLaughlin for stimulating discussion and Miss N. Dick and Mr D. Fishel for expert technical assistance. D. L. was Guest Investigator at The Rockefeller University (June to July 1978) and held a Leverhulme Travelling Fellowship. This research was supported by U.S. Public Health Service Grant AI11942 from the National Institute of Allergy and Infectious Diseases.

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