Adenosine Triphosphatase Activity of *Tritrichomonas foetus*

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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, leucinostatin, dibutylchloromethyltin chloride, spegazzinine, efrapeptin, 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**

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Cultures were grown for 24 h at 37 °C and harvested when the population reached 1 × 10⁶ to 2 × 10⁶ 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, for 5 min, and then washed three times in 50 mM-Tris/H₂SO₄ 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/H₂SO₄ 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 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-min), a large particle fraction (P₁, at 3-6 × 10⁶ g-min), a small particle fraction (P₂, at 7-2 × 10⁶ g-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 Beaufoy 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/H₂SO₄ buffer, 2 mM-ATP and 6 mM-MgSO₄ 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 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. < ±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'-diclohexylcarbodi-imide and venturicidin from BDH; 4-chloro-7-nitrobenzofurazan (Nbf-Cl) from Aldrich Chemical Co., Wembley, Middx.; efrapeptin (antibiotic A23871) and leucinostatin from Dr R. Hamill, Lilly Research Laboratories, Minneapolis, Minn., U.S.A.; triethyltin sulphate and dibutyllethylmethylin chloride from Dr D. E. Griffiths, Department of Molecular Sciences, University of Warwick, Coventry; and spegazzinine, a dihydroindole alkaloid, from Division de Quimica Organica Superior, Universidad National de la Plata, La Plata, Argentina. All inhibitors were dissolved in ethanol.

RESULTS

In Tris/maleate or Tris/H₂SO₄ buffers the ATPase displayed optimum activity at pH 8-2 to 8-3. The specific activity of ATPase at the pH optimum in Tris/H₂SO₄ at 30 °C was 25.5 ± 5.6 mU (mg protein)⁻¹ (mean value ± 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 Mg²⁺-dependent. Replacement of Mg²⁺ by Ca²⁺ decreased activity to 64%, and Na⁺ or K⁺, added singly or together, gave less than 17% of the activity observed with Mg²⁺ (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 I₅₀ values previously determined for the mitochondrial ATPase of yeast (Lloyd & Edwards, 1976, 1977). At the optimum pH, N,N'-diclohexylcarbodi-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 cytoplasmatic 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/H$_2$SO$_4$ buffer. Cations or EDTA were added at 20 mM.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>8</td>
</tr>
<tr>
<td>EDTA</td>
<td>4</td>
</tr>
<tr>
<td>Mg$^{2+}$</td>
<td>100</td>
</tr>
<tr>
<td>Ca$^{2+}$</td>
<td>64</td>
</tr>
<tr>
<td>K$^+$</td>
<td>17</td>
</tr>
<tr>
<td>Na$^+$</td>
<td>13</td>
</tr>
<tr>
<td>K$^+$ + Na$^+$</td>
<td>14</td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Conc. [µg (mg protein)$^{-1}$]</th>
<th>Activity (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N,N'-Dicyclohexylcarbodi-imide</td>
<td>72</td>
<td>132</td>
</tr>
<tr>
<td>Oligomycin</td>
<td>182</td>
<td>94</td>
</tr>
<tr>
<td>Venturicidin</td>
<td>36</td>
<td>104</td>
</tr>
<tr>
<td>Triethyltin sulphate</td>
<td>36</td>
<td>92</td>
</tr>
<tr>
<td>Leucinostatin</td>
<td>36</td>
<td>107</td>
</tr>
<tr>
<td>Dibutylichloromethyltin chloride</td>
<td>72</td>
<td>99</td>
</tr>
<tr>
<td>Spegazzinine</td>
<td>18</td>
<td>95</td>
</tr>
<tr>
<td>Quercetin</td>
<td>182</td>
<td>82</td>
</tr>
<tr>
<td>Efrapeptin</td>
<td>36</td>
<td>103</td>
</tr>
<tr>
<td>Nbf-Cl</td>
<td>182</td>
<td>23</td>
</tr>
<tr>
<td>Citreoviridin</td>
<td>18</td>
<td>104</td>
</tr>
<tr>
<td>Sodium azide (in H$_2$O)</td>
<td>2 mM</td>
<td>93</td>
</tr>
</tbody>
</table>

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 µg 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 µg (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 µg (mg protein)$^{-1}$.

The distributions of malate dehydrogenase (decarboxylating), a marker enzyme for
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 μg (mg protein)⁻¹] was included in the assay at all pH values between 5.2 and 8.6. Quercetin [240 μg (mg protein)⁻¹] gave up to 59% inhibition over this pH range; azide had no effect. At pH 8.3, Nbf-Cl [450 μg (mg protein)⁻¹] gave 70% inhibition and triethyltin sulphate [90 μg (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 μg (mg protein)⁻¹] did not inhibit ATPase in any of the fractions.

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).
**ATPase of Tritrichomonas**

**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 (Čerkasová & 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 \(\beta\)-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.

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**REFERENCES**


ATPase of Tritrichomonas


