Effects of Inhibitors on Mitochondrial Adenosine Triphosphatase of *Tetrahymena pyriformis* ST

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Mitochondrial adenosine triphosphatase (ATPase) of the ciliate protozoon *Tetrahymena pyriformis* ST is completely inhibited by antiserum prepared against F_1 -ATPase purified from *Schizosaccharomyces pombe*, and by naturally occurring inhibitor proteins from this yeast and from bovine heart mitochondria. An ATPase inhibitor protein is also present in extracts of *T. pyriformis*. Mitochondrial ATPase of *T. pyriformis* is only partially inhibited by the F_0 -ATPase inhibitors *N,N'*-dicyclohexylcarbodiimide, oligomycin, leucinostatin, triethyltin sulphate and venturicidin, and (at high titres) by the F_1 -ATPase inhibitors Dio-9, efrapeptin, 4-chloro-7-nitrobenzofurazan and spegazzinine. Aurovertin, citreoviridin and quercetin were not inhibitory. Resistance to inhibitors distinguishes this mitochondrial ATPase from all those previously examined.

INTRODUCTION

The adenosine triphosphatases (ATPases) of protozoa have been less well studied than those in yeast and mammalian systems. *Tetrahymena pyriformis* possesses (a) a mitochondrial Mg^{2+} -dependent ATPase (Turner & Lloyd, 1971); (b) a cytosolic Ca^{2+} -dependent ATPase isolated from a post-ribosomal supernatant fraction (Chua *et al.*, 1976); (c) a dynein ATPase (Gibbons, 1966) which has a pH optimum of 8.5–9.0 and is dependent on the presence of Mg^{2+} , Ca^{2+} , Fe^{2+} , Co^{2+} or Ni^{2+} for activity; and (d) a Mg^{2+} -dependent ATPase found in cell homogenates (Conner *et al.*, 1963), the activity of which was only slightly enhanced by Na^+ and K^+ and was not inhibited by the cardiac glycoside, ouabain [Frasch *et al.* (1978) were also unable to find a Mg^{2+} -dependent Na^+/K^+ ATPase in *Trypanosoma cruzi*].

The components of mitochondrial energy conservation show remarkable similarities in most eukaryotic cell types (Lloyd, 1974) but exceptions have been noted for the protozoa. The cytochromes *c* of *T. pyriformis* (Lloyd & Chance, 1972), *Crithidia fasciculata* (Hill *et al.*, 1971) and *Acanthamoeba castellanii* (Edwards *et al.*, 1977) all have unusual difference spectra; in addition, the amino acid sequence of cytochrome *c* of *T. pyriformis* differs considerably from that of all other known eukaryotic cytochromes *c* (Tarr & Fitch, 1976). The mitochondrial ATPase of *Crithidia luciliae* is also exceptional in that it is only partially inhibited by oligomycin and *N,N'*-dicyclohexylcarbodiimide. Activity is not inhibited by either aurovertin or by the naturally occurring ATPase inhibitor (F_1 -inhibitor protein) from beef heart mitochondria (Oppendoes *et al.*, 1976). The mitochondrial ATPase of *C. fasciculata* is inhibited by oligomycin, venturicidin, triethyltin sulphate, *N,N'*-dicyclohexylcarbodiimide, leucinostatin, Dio-9 and quercetin, but not by spegazzinine or by compounds which interact with the β -subunit of F_1 -ATPase (Yarlett & Lloyd, 1981).

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In this paper we show that the mitochondrial ATPase of *T. pyriformis* is unusual in being highly resistant to inhibition by compounds which are potent inhibitors of the mammalian and yeast enzyme complexes.

METHODS

Maintenance, growth and harvesting of organisms. *Tetrahymena pyriformis* strain ST (obtained from Dr Y. Suyama, Department of Biology, University of Pennsylvania, Philadelphia, U.S.A.) was maintained and grown as previously described (Lloyd *et al.*, 1971) in a medium containing 2% (w/v) proteose peptone (Difco), 0.1% yeast extract (Difco), 0.5% glucose, $5 \mu\text{g FeCl}_3 \cdot 6\text{H}_2\text{O ml}^{-1}$ and 0.1% antifoam RD emulsion (Hopkin & Williams, Chadwell Heath, Essex, U.K.), adjusted to pH 7.2 with 5 M-KOH (Dickinson *et al.*, 1976). In this medium cell populations grew with a mean cell generation time of 182 min (range 18 min in 15 experiments) to a cell density of 8.0×10^5 (range 0.8×10^5) organisms ml^{-1} . Organisms were harvested from cultures (4 l) at a density of 2.2×10^5 (range 1.4×10^5) organisms ml^{-1} as previously described (Lloyd *et al.*, 1971).

Disruption of organisms and preparation of mitochondria. Harvested cells were washed once in a buffer consisting of 3.2 M-sucrose, 10 mM-EGTA, 24 mM-Tris/HCl pH 7.2, and resuspended in this buffer at 4 °C at 20–30 mg protein ml^{-1} . The cells were disrupted by homogenization at 4 °C in a chilled 50 ml glass homogenizer fitted with a PTFE plunger (Jencons Scientific, Hemel Hempstead, Herts. U.K.) Cell breakage was followed microscopically until about 70% breakage was achieved. Mitochondria were prepared by differential centrifugation as previously described (Turner *et al.*, 1971).

Preparation and gel filtration of submitochondrial particles. Mitochondrial fractions (3–5 ml samples containing 10–15 mg protein ml^{-1}) were treated with ultrasound for 30 s (20 kHz, 500 W; MSE sonicator, power setting 5) to produce a suspension of submitochondrial particles. Gel filtration of submitochondrial particles was done by passage through a Sephadex G-50 column (10 cm $\times$ 1 cm) eluted with preparation buffer. The particles were eluted in the void volume (4.6 ml).

Preparation of ATPase inhibitor proteins. Suspensions of *T. pyriformis* submitochondrial particles were heated (90 °C for 5 min) and then cooled on ice and centrifuged (38 000 g for 20 min). The supernatant containing the inhibitor protein was purified by filtration through an XM-100 membrane filter (Amicon, Lexington, Mass., U.S.A.) under N_2 (1.37×10^5 Pa) at 4 °C (removing about 70% of proteins of mol. wt > 20000) and concentrated by removing about 50% of the liquid by filtration through a PM-10 membrane filter (Amicon) under N_2 (3.44×10^5 Pa) at 4 °C. ATPase inhibitor proteins from beef heart mitochondria and *Schizosaccharomyces pombe* mitochondria were prepared by the same technique but purification by membrane filtration was omitted. Inhibitor peptides from all three sources were shown to be single peptides of mol. wt < 15000 by sodium dodecyl sulphate gel electrophoresis.

Immunological assay of ATPase. Antiserum raised by injecting a rabbit with 25 mg F_1 -ATPase prepared from submitochondrial particles from *S. pombe* (Beechey *et al.*, 1975) was kindly prepared by Dr J. Edwards (Sully Hospital, South Glamorgan, Wales).

Enzyme assays. ATPase (EC 3.6.1.3) was routinely assayed by two methods. (a) Proton release was measured using a Pye model 79 pH meter fitted with an EIL combination micro-pH-electrode (Chance & Nishimura, 1967). The output of the pH meter was connected via an amplifier to a 50 mV potentiometric recorder. Calibration was done by adding known volumes of 100 mM-HCl. Extract was added to a stirred medium (2 ml) at 29 °C containing 4 mM-ATP, 4.5 mM-MgSO₄ and 10 mM-Tris/HCl pH 7.15 with a trace of carbonic anhydrase to prevent pH drift caused by absorption of atmospheric CO₂. (b) Extract was incubated at 29 °C in a medium (1 ml) containing 4 mM-ATP, 4.5 mM-MgSO₄ and 50 mM-Tris/HCl pH 7.15. After the reaction was stopped by addition of 0.1 ml 50% (w/v) trichloroacetic acid, the protein was removed by centrifugation at 1200 g min and the supernatant was assayed colorimetrically for P_i by the method of Fiske and Subba-Row (1925). Corrections for enzyme and substrate blanks were done as routine. All enzyme assays were performed within 7 h of preparation of extracts.

Protein was measured by the Lowry method with bovine serum albumin as standard.

Chemicals. ATP, carbonic anhydrase, quercetin (3,3',4',5,7-pentahydroxyflavone) ouabain (octahydrate), EGTA, and oligomycin (approx. composition 65% oligomycin A, 20% oligomycin B, 15% oligomycin C) were from Sigma. Bovine serum albumin powder was from Armour Pharmaceutical Co., Eastbourne, Sussex, U.K. Sephadex G-50 was from Pharmacia. *N,N'*-Dicyclohexylcarbodiimide and venturicidin (approx. 4:1 mixture of venturicidin A and B) were from BDH. Citreoviridin (C7026) was from Cambrian Chemicals, Beddington Farm Road, Croydon, London. Dio-9 was from Koninklijke Nederlandsche Gist and Spiritus fabriek, Delft, The Netherlands: a solution of Dio-9 containing 1 mg ml^{-1} gives a value of $A_{303} - A_{400} = 0.1$ (Guillory, 1964); on this basis the commercial product was found to be only 40% pure. 4-Chloro-7-nitrobenzofurazan was from Aldrich Chemical Co., Gillingham, Dorset, U.K. Aurovertin, triethyltin sulphate and dibutylchloromethyltin chloride were gifts from Dr D. E. Griffiths and Dr T. G. Cartledge, Department of Molecular Sciences, University of Warwick,

Coventry, U.K. Leucinostatin (antibiotic A20668A) and efrapeptin (antibiotic A23871) were gifts from Dr R. Hamill of Lilly Research Laboratories, Indianapolis, Ind., U.S.A. Spegazzinine, a dihydroindole alkaloid, was a gift from the División de Química Orgánica Superior, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, La Plata, Argentina.

RESULTS AND DISCUSSION

ATPase assays with submitochondrial particles at various pH values (Fig. 1*a*) indicated the presence of three pH optima, at pH 6.3, 7.15 and 7.35. The optima at pH 6.3 and pH 7.15 correspond with those determined for the F_1 -ATPase activity of mitochondria purified on density gradients (Turner & Lloyd, 1971) and are different from the pH optima for dynein ATPase (Gibbons, 1966) and soluble ATPase (Chua *et al.*, 1976). The inclusion of vanadate (as an impurity in ATP from Sigma) in reaction mixtures inhibits any contribution to ATPase activity from sarcoplasmic reticulum Ca^{2+} -ATPase, plasma membrane Ca^{2+} / Mg^{2+} -ATPase, plasma membrane Mg^{2+} -ATPase and Na^+/K^+ -ATPase but not from mitochondrial ATPase or plasma membrane Ca^{2+} -ATPase (O'Neal *et al.*, 1979). Insensitivity to 10 mM-ouabain also suggested that the ATPase activity was not due to a Na^+/K^+ -ATPase. Inhibition of ATPase activity by F_1 inhibitor proteins from different cell types and by antiserum prepared against F_1 -ATPase further confirmed that the ATPase is mitochondrial (Table 1).

The optimum Mg^{2+} :ATP ratio, at 4 mM-ATP (Fig. 1*b*) at both the pH 6.3 and the pH 7.15 optima, was 1:1.25 for preparations of both mitochondria and submitochondrial particles. The optimum at pH 7.15 was used in all further work.

The specific activities of ATPase in preparations of mitochondria, submitochondrial particles and submitochondrial particles after gel filtration were in the ratio 1:1.7 (S.D. 0.45):2.25 (S.D. 0.5) (11 experiments) when assayed by proton release.

None of the agents known to inhibit the enzyme complex at F_0 in yeast and mammalian mitochondria (Lloyd, 1974) gave complete inhibition, even at high titres (Table 1). *N,N'*-Dicyclohexylcarbodiimide was the most inhibitory. All preparations were 10- to 100-fold less sensitive to inhibitors than those from the yeast *Schizosaccharomyces pombe* (Lloyd & Edwards, 1976). Similarly, the F_1 -ATPase inhibitors Dio-9, efrapeptin, 4-chloro-7-nitrobenzofurazan and spegazzinine gave only partial inhibition, and aurovertin, citreoviridin and quercetin were without effect. That similar results were obtained in all cases with mitochondria and submitochondrial particles indicates that it is unlikely that limited accessibility to inhibitor-binding sites leads to the small extent of inhibition observed. Gel filtration to remove any endogenous natural inhibitor did not markedly increase the maximum extent of inhibition attainable (except for that by oligomycin and *N,N'*-dicyclohexylcarbodiimide). Thus it seems unlikely that prior occupancy by the natural inhibitor of binding sites specific for added inhibitors can explain resistance to inhibition.

The mitochondrial ATPase of *T. pyriformis* is thus similar in some respects to that of *Crithidia fasciculata* (Yarlett & Lloyd, 1981), which lacks some inhibitor-binding sites associated with the β -subunit of F_1 -ATPase. It is however, strikingly different from the enzyme complex of *Acanthamoeba castellanii*, which possesses a full complement of inhibitor-binding sites, and is similar in many respects to the ATPases of yeast and mammals (S. W. Edwards & D. Lloyd, unpublished results).

Comparison of the inhibitor-binding properties of ATPase of mitochondria-enriched fractions of *T. pyriformis* with other proton-translocating ATPases is facilitated by reference to data collected by Linnett & Beechey (1979). Resistance to aurovertin distinguishes the mitochondrial ATPase of *T. pyriformis* from that of beef heart and rat liver mitochondrial ATPases and from the ATPase activity of *Rhodospirillum rubrum* chromatophores, but not from chloroplast ATPase, which is also resistant to aurovertin; citreoviridin inhibits beef heart mitochondrial ATPase and ATPase activity of *R. rubrum* chromatophores in a similar

Table 1. *Effect of inhibitors on the ATPase activity of mitochondria and submitochondrial particles of T. pyriformis*

Uninhibited activities were within the range 20–90 nmol ATP hydrolysed min^{-1} (mg protein) $^{-1}$. Titration curves for each inhibitor were obtained by adding small known volumes of inhibitor solutions (in ethanol, except for efrapentin, leucinostatin and the naturally occurring inhibitors) to the ATPase reaction mixture [proton release method (a), or P_i release method (b)], at pH 7.15. Ethanol (1%, v/v) gave 20% inhibition of ATPase; solvent concentrations never exceeded this value and corrections were employed for its contribution to total inhibition at each addition. I_{50} and I_{100} values are expressed as μg inhibitor (or inhibitor protein) per mg protein necessary to give 50% or 100% inhibition, respectively, of total inhibitor-sensitive ATPase. I_{max} values were determined by addition of excess inhibitor and are expressed as a percentage of total ATPase activity. Treatment of submitochondrial particles by gel filtration is described in Methods.

Inhibitor	Mitochondria						Submitochondrial particles					
				Untreated						Treated		
	I_{50}	I_{100}	$I_{\text{max}}(\%)$	I_{50}	I_{100}	$I_{\text{max}}(\%)$	I_{50}	I_{100}	$I_{\text{max}}(\%)$	I_{50}	I_{100}	$I_{\text{max}}(\%)$
Agents acting on F_0 (assay a)												
<i>N,N'</i> -Dicyclohexylcarbodiimide	2	16	23	1	17	45	5	50	60			
Oligomycin	ND	1.4	27	ND	14.5	33	ND	0.045	57			
Leucinostatin	ND	33	26	ND	1	12	ND	1.5	13			
Triethyltin sulphate	3	33	37	3	30	40	7	88	44			
Venturicidin	5	66	21	5	66	25	11	111	26			
Agents acting on F_1 (assay a)												
Dio-9			0	ND	4	15						
Efrapentin	0.1	22	20	0.05	22	20	0.7	7.6	30			
4-Chloro-7-nitrobenzofurazan	4	16	25	ND	ND	ND	0.5	5.5	11			
Spegazzine	ND	14	30	ND	14	43	4.5	44	25			
Other inhibitors (assay b)												
F_1 -ATPase antibody	ND	ND	100	ND	ND	100	ND	ND	100			
F_1 inhibitor	300	900	60	300	900	60	300	900	60			
F_1 inhibitor (from <i>T. pyriformis</i>)												
F_1 inhibitor (from <i>S. pombe</i>)	20	200	100	40	80	95	20	250	90			
F_1 inhibitor (from bovine heart mitochondria)	50	200	100	40	200	100	200	500	100			

ND, Not determined.

Fig. 1. Effects of pH and Mg^{2+} concentration on ATPase activity of *Tetrahymena pyriformis*. (a) Submitochondrial particles ($2.0 \text{ mg protein ml}^{-1}$) were incubated at 29°C in buffer containing 4 mM-ATP , 4.5 mM-MgSO_4 and 50 mM-Tris . Tris/acetate buffer was used for the pH range $6.0\text{--}6.6$, and Tris/HCl for the range $6.6\text{--}8.0$. ATPase activity was measured by P_i release; 100% activity represents $67 \text{ nmol } P_i \text{ released min}^{-1} (\text{mg protein})^{-1}$. (b) Submitochondrial particles ($1.0 \text{ mg protein ml}^{-1}$) (○, ●) and mitochondria ($2.0 \text{ mg protein ml}^{-1}$) (△, ▲) were incubated at 29°C in buffer containing 4 mM-ATP and 50 mM-Tris , with a range of Mg^{2+} concentrations. The pH was maintained at 7.15 (open symbols) or 6.3 (filled symbols). ATPase activity was measured by P_i release. Activity is expressed as a percentage of the maximum at 4.5 mM-Mg^{2+} for submitochondrial particles incubated at pH 7.15 , i.e. $72 \text{ nmol } P_i \text{ released min}^{-1} (\text{mg protein})^{-1}$.

manner to aurovertin; quercetin inhibits purified soluble ATPase and membrane-bound ATPase from beef heart mitochondria, spinach chloroplasts and *Escherichia coli* whilst *T. pyriformis* mitochondrial ATPase is totally resistant to these inhibitors. Complete inhibition of beef heart mitochondrial ATPase, spinach chloroplast ATPase, and *E. coli* ATPase by 4-chloro-7-nitrobenzofurazan contrasts with the incomplete inhibition observed in the present study. Efrapeptin, an antibiotic thought to react at, or close to, the active site of F_1 -ATPase (Lardy *et al.*, 1975), completely inhibits beef heart [$I_{50} = 1.2 \mu\text{g} (\text{mg protein})^{-1}$] and yeast mitochondrial F_1 -ATPases and *E. coli* ATPase (at high titres), whereas chloroplast ATPase is only partially inhibited ($I_{\text{max}} = 60\text{--}70\%$). Leucinostatin, organotin derivatives and venturicidin do not inhibit soluble F_1 -ATPase, but they are more potent inhibitors of rat liver and beef heart membrane-bound mitochondrial ATPases than they are of *T. pyriformis* mitochondrial ATPase.

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