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A Ca^{2+}-ACTIVATED ATPase SPECIFICALLY RELEASED BY Ca^{2+} SHOCK FROM *PARAMECIUM TETRAURELIA*

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Deciliation of *Paramecium tetraurelia* by a Ca^{2+} shock procedure releases a discrete set of proteins which represent about 1% of the total cell protein. Marker enzymes for cytoplasm (hexokinase), endoplasmic reticulum (glucose-6-phosphatase), peroxisomes (catalase), and lysosomes (acid phosphatase) were not released by this treatment. Among the proteins selectively released is a Ca^{2+}-dependent ATPase. This enzyme has a broad substrate specificity which includes GTP, ATP, and UTP, and it can be activated by Ca^{2+}, Sr^{2+}, or Ba^{2+}, but not by Mg^{2+} or by monovalent cations. The crude enzyme has a specific activity of 2–3 μmol/min per mg; the optimal pH for activity is 7.5. ATPase, GTPase, and UTPase all reside in the same protein, which is inhibited by ruthenium red, is irreversibly denatured at 50°C, and which has a sedimentation coefficient of 8–10 S. This enzyme is compared with other surface-derived ATPases of ciliated protozoans, and its possible roles are discussed.

Introduction

Ca^{2+} plays a role in several surface-related phenomena in *Paramecium*. Ciliary reversal and consequent backward swimming (the avoiding response) in *Paramecium* are triggered by Ca^{2+} influx through the excitable ciliary membrane [1,2]. *Paramecium* usually swims forward, propelled by the coordinated beating of the thousands of cilia that cover its surface. When an obstacle is encountered, the protozoan avoids it by swimming backwards briefly before resuming forward motion in a different direction. The avoidance reaction is accomplished by a temporary reversal of the direction of the power stroke of the cilia. Ciliary reversal is a consequence of the excitation of the surface membrane, during which Ca^{2+} permeability increases dramatically, allowing Ca^{2+} to flow down its electrochemical gradient into the cell. This Ca^{2+} influx raises the intracellular Ca^{2+} concentration from less than 10^{-7} M to more than 10^{-6} M, and the higher Ca^{2+} concentration triggers an energy-dependent mechanism for reversing the direction of the ciliary beat [1,2]. Normal forward swimming resumes, presumably, when the intracellular Ca^{2+} levels return to their pre-stimulus values.

Paramecium clearly has a system for extruding Ca^{2+} into the medium, where it is normally present (in laboratory media) at 0.1 to 1 mM. The system is inhibited at low temperature [3] and is capable of maintaining a very considerable concentration gradient. Thus, there are at least two mechanisms involved in swimming behavior that depend upon both calcium ion and energy; one responsible for Ca^{2+} extrusion, the other for ciliary reversal. The energy source for the latter is apparently ATP [4] and it is possible that ATP drives the Ca^{2+} extrusion as well.

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Abbreviations: EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid; ATP, adenosine 5'-triphosphate; ADP, adenosine 5'-diphosphate; AMP, adenosine 5'-monophosphate; GTP, guanosine 5'-triphosphate; CTP, cytidine 5'-triphosphate; UTP, uridine 5'-triphosphate; ATPase, adenosine triphosphatase; TEA-Cl, tetraethylammonium chloride; TEMED, *N*,*N*,*N*',*N*'-tetramethylethylenediamine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mops, 4-morpholinepropanesulfonic acid; Pipes, 1,4-piperazinediethanesulfonic acid; PMSF, phenylmethanesulfonyl fluoride.

Another surface phenomenon triggered by Ca^{2+} is the extrusion of trichocysts [5,6], exocytotic vesicles which lie just beneath the *Paramecium* surface membrane. In response to a variety of stimuli, these vesicles fuse with the plasma membrane and expel their contents in the form of a long thin structure. Plattner et al. [7] have used histochemical techniques to demonstrate a Ca^{2+}-ATPase at the site of trichocyst extrusion, and we have recently shown that the Ca^{2+}-binding protein calmodulin is a major component of extruded trichocysts [8].

As part of our efforts to characterize biochemically those components of *Paramecium* responsible for the regulation of swimming behavior and for trichocyst extrusion, we have partially purified and studied the Ca^{2+}-dependent ATPase activities in the protozoan. We report here the properties of a Ca^{2+}-ATPase released under the conditions of Ca^{2+} shock used to deciliate paramecia.

Materials and Methods

Reagents. Electrophoresis grade acrylamide and *N,N'*-methylenebisacrylamide were obtained from BioRad; TEMED was from Sigma. Glyceraldehydephosphate dehydrogenase and 3-phosphoglycerate kinase were from Calbiochem Corp. TEA-Cl was from Eastman. Carrier-free $^{32}P_i$ was from New England Nuclear, and [γ-^{32}P]ATP was made using a modification of the method of Schendel and Wells [9].

Solutions. Dryl's salt solution [10] consisted of: 1 mM Na_2HPO_4, 1 mM NaH_2PO_4, 2 mM sodium citrate, and 1.5 mM $CaCl_2$, with final pH adjusted to 6.9. STEEP solution [11] contained: 0.15 M sucrose, 15 mM Tris, 2.5 mM Na_2EDTA, 11% (v/v) ethanol, and 30 mM KCl, with final pH adjusted to 8.3. STEN solution [12] contained: 0.5 M sucrose, 20 mM Tris-Cl, 2 mM EDTA, 6 mM NaCl, with pH adjusted to 7.5. Solution A, modified from Subbiah and Thompson [13], was: 0.15 M KCl, 4 mM $MgCl_2$, 0.5 mM Na_2EDTA, 2 mM Tris, with pH adjusted to 7.8. Tris-Ca^{2+} wash buffer contained: 1 mM $CaCl_2$, 0.1 mM Na_2EDTA, 1 mM Tris, with pH adjusted to 7.5.

Growing cultures. Paramecium tetraurelia, stock 51 S (non-kappa bearing), was grown at 22–28°C in cerophyl medium [14] supplemented with 1 mg/l of β-sitosterol and previously inoculated with *Enterobacter cloacae*. Cells were maintained in the logarithmic phase of growth by transfer to fresh medium every one or two days. Transfers were done by filtering the culture through four thicknesses of cheesecloth and centrifuging the filtrate in pear-shaped centrifuge tubes at $150 \times g$ in an oil-testing centrifuge (IEC model HN-S) for 2–3 min. The pelleted cells were removed with a Pasteur pipette and put into fresh medium. Enough bacteria were carried over this way to make inoculation of the fresh medium unnecessary.

Deciliation. Cultures were harvested by filtering and centrifuging as for cell transfer. The pelleted cells were resuspended in cold Dryl's buffer, which induced extensive trichocyst firing. Upon subsequent centrifugation the trichocysts formed a fluffy layer on top of the cell pellet, which was removed. The cell pellet was washed until no further trichocysts were released, at which point the cells were resuspended in Dryl's solution (15 ml/ml of pelleted cells). To this was added an equal volume of STEN solution, and the suspension was left on ice for 10 min. Cells were then deciliated by the addition of $CaCl_2$ and KCl to final concentrations of 10 mM Ca^{2+} and 30 mM K^+. Deciliation was allowed to proceed until it was at least 90% complete (5–10 min). The extent of deciliation was monitored by phase contrast microscopy. No blistering or lysis of cells was observed using this procedure.

The deciliated cell suspension was centrifuged at $850 \times g_{max}$ for 2 min in the HNS centrifuge to pellet cell bodies (P-I) and the supernatant fluid was recentrifuged to remove any remaining cell bodies. The supernatant (S-I) was centrifuged at $27000 \times g_{max}$ for 20 min to pellet the cilia (P-II). The resultant ciliary supernatant (S-II) was saved.

Fractionation of cilia. The cilia (P-II) were fractionated by a modification of the method of Subbiah and Thompson [13]. Cilia from a 1 ml cell pellet were suspended in 1.9 ml of solution A, with mercaptoethanol omitted. To the suspended cilia was added 0.1 ml of 1% (w/v) Triton X-100 in solution A and the mixture was vortexed 5–10 s to detach membranes. Centrifugation for 5 min at $14000 \times g$ and 4°C gave a pellet (P-III) which contained some intact cilia, axonemes, and trichocysts but very few membrane vesicles. The

supernatant (S-III) was centrifuged at 21500 × *g* and 4°C for 30 min to give a pellet (P-IV) and a supernatant fluid (S-IV). P-IV was predominantly membrane vesicles. P-III and P-IV were suspended in solution A and all fractions were kept on ice. An alternative procedure for extracting the soluble contents of isolated cilia is described in Results.

Extraction of deciliated bodies. Deciliated cell bodies (P-I) were resuspended in 20 mM Tris, 2 mM $CaCl_2$, pH 7.5, and homogenized with 20 up-and-down strokes in a Potter-Elvehjem homogenizer driven by an electric motor. This treatment broke open more than 95% of the cell bodies, as judged by phase contrast microscopy. A sample of this homogenate was kept aside, and the remainder was centrifuged for 15 min at 3900 × *g* in a Beckman JA-20 rotor at 4°C. The resulting pellet (P-V) was resuspended in 20 mM Tris, pH 7.5 and kept at −20°C. The supernatant fluid (S-V) was also stored at −20°C. Protein was determined by the method of Lowry et al. [15], using bovine serum albumin as a standard. A flow diagram of the fractionation is shown in Fig. 1.

Concentration of S-II. S-II was concentrated using an Amicon model 402 filtration cell with a PM10 filter at 20 lbs/inch^2 of N_2, at 4°C. The flow rate under these conditions was about 50 ml/h. The resulting concentrate was dialyzed in the cold overnight against a solution of 0.2 mM $CaCl_2$ in 1 mM Tris-HCl at pH 7.5 then lyophilized and dissolved in 5 mM Tris-HCl, pH 7.5, containing 2 mM $CaCl_2$, and stored frozen.

ATPase assays. The compositions of assay media used in different experiments are described in detail in the Results. Assays were routinely done at 37°C in 3-ml centrifuge tubes to which 0.02 M $CaCl_2$ (0.3 ml), 0.03 M ATP (0.1 ml), 0.02 M Tris-HCl buffer, pH 7.5 (0.5 ml), and water were added to give a final volume of 2.0 ml and final concentrations of 3 mM Ca^{2+}, 1.5 mM ATP, and 5 mM Tris-HCl upon initiation of the assay by addition of enzyme. Where necessary, the ion concentration in the added enzyme solution was taken into account. It made no difference whether the assay was initiated with ATP or enzyme addition. The assay was stopped after 10 min by adding 0.1

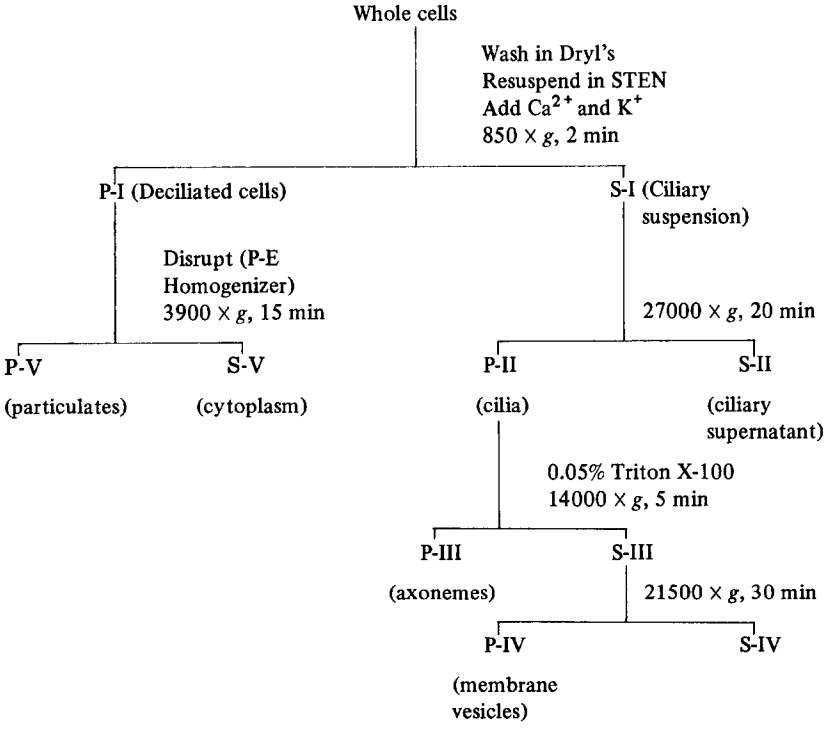

Fig. 1. Flow scheme for cell fractionation.

ml of 6% perchloric acid, vortexing, and chilling the tubes. Aliquots were then assayed for P_i production. In some experiments, ATP labeled with ^{32}P in the γ-position was used, and $^{32}P_i$ production was assayed [16]. In other cases, including all experiments with substrates other than ATP, P_i was measured by the method of Chen et al. [17]. All assays were done in duplicate, and blanks with no enzyme added were always included.

Assays of marker enzymes. Catalase, a marker for peroxisomes, was assayed at 28°C as described [18]; the disappearance of peroxide was measured as a decrease in A_{250}. Sodium azide at 4.4 mM completely inhibited this activity. Acid phosphatase, a lysosomal marker, was assayed at 37°C, with *p*-nitrophenylphosphate as substrate [19]. To 0.4 ml of buffer-substrate solution (50 mM sodium citrate, pH 4.8, containing 5.5 mM *p*-nitrophenylphosphate and 0.1% (v/v) Triton X-100) was added 0.45 ml of enzyme in 20 mM Tris, pH 7.5, 2 mM $CaCl_2$. Assays were quenched after 0 and 15 min by the addition of 1.5 ml 0.1 N NaOH and the A_{405} of each sample was compared with that for a standard curve constructed with *p*-nitrophenol.

Glucose-6-phosphatase, an endoplasmic reticulum marker, was assayed at 37°C by measuring P_i release. To 1 ml of buffer-substrate solution (0.1 M citrate, pH 6.5, containing 80 mM glucose-6-*P*) was added 0.3 ml of enzyme in 20 mM Tris, pH 7.5, 2 mM $CaCl_2$. Assays were quenched at 0, 15, and 30 min by the addition of 2 ml cold 10% trichloroacetic acid, and P_i was assayed by the method of Chen et al. [17]. Lactate dehydrogenase (which is usually a cytoplasmic enzyme but which has been found in the particulate fraction of *Paramecium*) was assayed at 28°C by measuring NADH consumption (A_{340} decrease) during the conversion of pyruvate to lactate as previously described [20]. Hexokinase (a cytosol marker) was assayed at 28°C by measuring the decrease in cresol red absorbance at 560 nm as an indicator of the decrease in pH from ATP hydrolysis during glucose phosphorylation [21]. Interfering ATPase activity was eliminated when necessary by the addition of 4 mM EGTA to the assay to remove Ca^{2+}.

For all of the assays of enzyme markers, conditions were chosen so that the assay was performed in the linear stage of the activity vs. protein curve. One unit is defined as that amount which catalyzes the formation of 1 μmol of product per min at the assay temperature.

Non-denaturing polyacrylamide gels. The electrophoresis gels and buffer were prepared as described by Clarke [22]. Gels were run in the cold at 1 mA per tube until the Bromphenol blue tracking dye reached the bottom of the gel tubes, which usually took about 2 h. The gels were then removed and either stained for protein with Coomassie brilliant blue (stained 2 h with 0.1% Coomassie brilliant blue in MeOH/HOAc/H_2O (50:7:43, v/v) and destained in 7.5% acetic acid in H_2O) or assayed for ATPase activity using a modification of the method of Abrams and Baron [23]. Gels were incubated in a solution of 1.5 mM ATP and 3 mM $CaCl_2$ in 5 mM Tris-HCl, pH 7.5, at 38°C, usually for 15 min. The gels were then washed once with water and immersed in a solution at 45°C of 10% ascorbate and 0.42% ammonium molybdate in 0.5 M H_2SO_4 (1:6, v/v) until blue bands showing the position of enzyme were clearly visible. Gels were rinsed quickly with distilled water and immediately scanned at 740 nm with a Gilson gel scanner.

SDS-polyacrylamide gel electrophoresis. All SDS-polyacrylamide gels were linear gradients of 7.5–15% acrylamide with a 3% stacking gel as described [24]. Samples were first lyophilized and then dissolved in sample buffer containing 10% glycerol, 5% 2-mercaptoethanol, 3% SDS, 62.5 mM Tris, pH 6.8, and 0.01% Bromphenol blue. Samples were heated at 100°C for 2 min and loaded onto the gel. Gels were run at 20 mA. Molecular weight standards (all from Sigma Chemical Co., St. Louis, MO) were as follows: conalbumin 86000), bovine serum albumin (68000), catalase (60000), ovalbumin (43000), α-chymotrypsinogen A (28000), myoglobin (17000), and dansylated cytochrome *c* (15000).

Results

Distribution of ATPase among subcellular fractions

When washed paramecia were deciliated by the STEN procedure [12], about 20% of the total Ca^{2+}

-activated ATPase activity of whole cell extracts was released into the soluble fraction (S-II, Fig. 1), and a smaller amount of Ca^{2+}-ATPase remained associated with isolated cilia (P-II) (Table I). Deciliated bodies also contained soluble Ca^{2+}-ATPase activity which represented about 20% of the total Ca^{2+}-ATPase of whole cell extracts. The distribution of Mg^{2+}-ATPase among these three fractions was different from that for Ca^{2+}-ATPase; more Mg^{2+}-ATPase was associated with cilia and cytoplasm than was found in the ciliary supernatant (S-II) fraction (Table I).

The Ca^{2+}-ATPase activity of all three fractions was abolished in the presence of EDTA or EGTA, and ruthenium red also inhibited all three fractions, although not to the same extent (data not shown). GTP was also hydrolyzed by these fractions; the activity with GTP was higher than with ATP for the cytoplasm and ciliary supernatant, but lower for isolated cilia (Table I).

The specific activity of the Ca^{2+}-ATPase in the ciliary supernatant (S-II ATPase) (2–4 units/mg) was much higher than that of cytoplasm (0.06 units/mg) or of cilia (0.3 units/mg), and we therefore focused on the S-II ATPase. Fraction S-II, which contained only 1–10 μgm protein/ml, was concentrated 10- to 50-fold by ultrafiltration (Methods). Essentially all of the ATPase activity and at least 75% of the protein were recovered in the concentrated material.

Selective release of Ca^{2+}-ATPase during deciliation.

The high specific activity of Ca^{2+}-ATPase in S-II suggested specific release of this enzyme. We determined the distribution of several other enzyme markers among the cell bodies, cilia, and soluble (S-II) fraction to confirm that the Ca^{2+}-ATPase was preferentially released during deciliation. After deciliation, about 1% of total cell protein was found in S-II; about 2% was in cilia, and the remainder was in deciliated cell bodies. Catalase, hexokinase, and lactate dehydrogenase, which in other eucaryotes are cytoplasmic enzymes, were found primarily in cell bodies after deciliation; less than 2% release of each into fraction S-II occurred in four separate experiments (Table II). Similarly, greater than 98% of the acid phos-

TABLE I

SUBCELLULAR DISTRIBUTION OF Ca^{2+}-ATPase AND Mg^{2+}-ATPase

Ca^{2+}- or Mg^{2+}-dependent ATPase activities were assayed as described in Methods, except that Ca^{2+} (or Mg^{2+}) was present at 4 mM, and ATP (or GTP) at 2 mM. The fractions shown here correspond to those described in Fig. 1. These data represent average values for three independent experiments, normalized to a total of 1 g of protein in whole cells, the equivalent of about 10^8 cells. n.d., not determined.

Fraction	Protein (mg)	Ca^{2+}-ATPase				Mg^{2+}-ATPase [c]	
		Total units	Spec. act. (units/mg)	RuR [a] inhib. (%)	GTPase [b] / ATPase	Total units	Spec. act. (unit/mg)
Whole cells	(1000)						
P-I, deciliated cells	970						
S-V, cytoplasm	530	30	0.06	72	3.0	5	0.009
P-V, particulate	440	70	0.16	n.d.	n.d.	n.d.	
S-II, ciliary supernatant	7	30	4.3	72	1.7	1.2	0.17
P-II, cilia	17	5	0.3	35	0.8	2.3	0.14
P-III, axonemes	7	3.5	0.5	n.d.	n.d.	1.05	0.15
P-IV, vesicles	2	2.0	1.0	n.d.	n.d.	0.5	0.25
S-IV, Triton extract	4	2.0	0.5	n.d.	n.d.	0.4	0.10

[a] Ruthenium red (RuR) was included in the assay at 0.01 mM.

[b] To determine the relative activity with GTP and ATP as substrate, GTP was substituted for ATP in the assay mixture.

[c] Mg^{2+} (4 mM) replaced Ca^{2+} in the assay mixture.

TABLE II

SUBCELLULAR DISTRIBUTION OF SEVERAL MARKER ENZYMES AND Ca^{2+}-ATPase

Fraction	Protein		Acid phosphatase		Glucose-6-phosphatase	
	mg	%	Units	%	Units	%
Deciliated cell extract	970	97 ±0.5	57	99.8 ± 0.2	53	99.8 ± 0.2
cytoplasm	530	53	5.2	9	6	11
particulate	440	44	38	67	28	53
Ciliary supernatant (S-II)	7	0.7 ± 0.2	0	0	0.02	0.03 ± 0.07
Cilia	17	1.7 ± 0.4	0.15	0.2 ± 0.2	0.12	0.22 ± 0.1

Each enzyme was assayed in each fraction in three independent experiments; the values given are averages of the 3. Data are normalized to 1 g of total protein in whole cells, the equivalent of about 10^8 cells. Activity is expressed as total units recovered in a

phatase (a lysosomal marker), glucose-6-phosphatase (endoplasmic reticulum marker), and catalase (a peroxisomal marker) were localized in cell bodies after deciliation. To exclude the possibility that the ciliary supernatant contained a powerful inhibitor of the marker enzymes, we mixed ciliary supernatant with cytoplasm and found no inhibition of cytoplasmic activities (data not shown). Thus, deciliation did not induce the release of cytoplasmic contents or intracellular organelles, but rather, resulted in the specific release of Ca^{2+}-ATPase; when only 1% of protein was released into the S-II fraction, up to 20% of the Ca^{2+}-ATPase activity was released. The pattern of peptides seen on SDS-polyacrylamide gels of ciliary supernatant fractions from several independent preparations (Fig. 2a) were very similar, further suggesting that this fraction is a discrete collection of proteins, and not merely the result of partial cell lysis. SDS gels of whole cell homogenates (centrifuged to remove particulate material) showed a broad smear of protein very different from the pattern of the ciliary supernatant fraction, and there was clearly a strong proteolytic activity in this cytoplasmic fraction which contributed to the smearing. We detected a small amount of proteolytic activity in the ciliary supernatant with azocasein as substrate, but the peptide pattern in Fig. 2a was stable with storage, and bovine serum albumin added to the ciliary supernatant was not degraded during storage; neither was the SDS-gel pattern altered by the presence of serum albumin and the protease inhibitor PMSF (Fig. 2b, tracks 4 and 5).

Among the peptides present in the ciliary supernatant was the 'immobilization antigen', a peptide of about 250 kDa which is the major protein of ciliary membranes [25]. Centrifugation of the ciliary supernatant for 1 h at $100000 \times g$ removed this peptide, as well as several others also characteristic of the ciliary membrane. It seems likely that very small vesicles of ciliary membrane contaminated the ciliary supernatant when only a $27000 \times g$ centrifugation was used to pellet cilia. The high speed centrifugation did not sediment the Ca^{2+}-ATPase, and the supernatant from this treatment was enriched for a peptide of about 68 kDa (Fig. 2c).

To compare the polypeptides of the ciliary supernatant with the soluble polypeptides of isolated cilia, we extracted cilia by vortexing them for 2 min in 1 mM Tris, 0.1 mM EDTA, pH 8.3 [25]. Centrifugation at $48000 \times g_{max}$ yielded a pellet containing the particulate fractions of cilia (membrane vesicles, axonemes, and incompletely demembranated cilia). The Tris-EDTA extract contained a subset of ciliary proteins similar to, but not identical with, the proteins of the ciliary supernatant (compare tracks 2 and 3 of Fig. 2b), suggesting that at least some of the proteins of the ciliary supernatant may have originated from the intraciliary space.

Metal ion dependence of S-II ATPase

This fraction contained about 2 mM Ca^{2+} remaining from the deciliation step, which after

Catalase		Lactate dehydrogenase		Hexokinase		Ca^{2+}-ATPase	
Units	%	Units	%	Units	%	Units	%
27,000	98 ±2.1	5.4	98 ±2.5	2,900	97 ±1.9	100	74 ±4.9
18,000	67	2.8	52	1,500	52	30	22
3,500	13	0.7	13	2,000	69	70	52
250	0.9±0.9	0	0	45	1.5±1.9	30	22.2±7.3
180	0.6±1.1	0.12	2.2±2.5	55	1.9±0.07	5	3.7±2.4

given fraction, and as percentage of total cell activity recovered in each fraction. In all cases, 1 enzyme unit represents the formation of 1 μmole of product per min under the assay conditions described in Methods.

dilution of the enzyme into the assay mixture represented about 10 μM total Ca^{2+}. With no added divalent cation, this fraction had a low but measurable basal activity (Table III), which was further reduced by the addition of either EDTA or EGTA. This inhibition by chelating agents was reversible; addition of an excess of Ca^{2+} after 2 min of incubation with EGTA resulted in partial or complete recovery of activity.

The addition of 2 mM Ca^{2+}, Sr^{2+}, or Ba^{2+} stimulated the basal ATPase rate by factors of about 35, 20, and 9, respectively (Table III). Mn^{2+} gave no more than a 3-fold stimulation, and the activity with Mg^{2+}, Ni^{2+}, Co^{2+}, Na^{+}, K^{+}, or $Na^{+}+K^{+}$ was not measurably above the basal level. Ni^{2+} or Mg^{2+}, when added in amounts equimolar with Ca^{2+}, inhibited the Ca^{2+}-stimulated ATPase by 70–75% (Table III), but

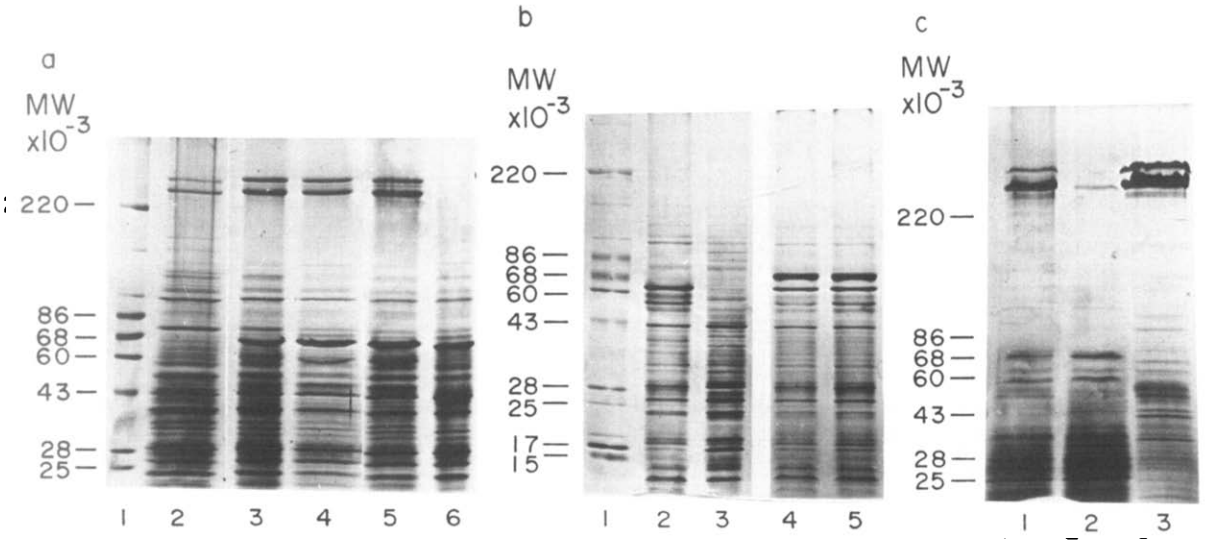

Fig. 2. SDS-polyacrylamide gel electrophoresis of S-II fractions. These gels are 7.5–15% linear gradients run as described in Methods. (a) Molecular weight standards in lane 1; lanes 2–6 are ciliary supernatants from five independent cell harvests. (b) Molecular weight standards (lane 1); lanes 2 and 3 are the ciliary supernatant (S-II) and the Tris-EDTA extract (see text) of cilia from the same cell harvest. Lanes 4 and 5: S-II fractions from the same cell harvest, prepared in the absence (4) and presence (5) of PMSF. Bovine serum albumin (M_r 68000) was added to these samples as an internal marker for proteolytic activity. (c) Lane 1 is the S-II fraction; lanes 2 and 3 are the supernatant and pellet, respectively, from a $125000 \times g_{av}$ centrifugation for 1 h of the S-II fraction in lane 1.

TABLE III

METAL ION ACTIVATION OF THE ATPase ACTIVITY IN S-II

ATPase activity was determined in a standard assay mixture containing 0.02 units (about 5 μg) of S-II ATPase and the indicated addition(s). ATP concentration in assay: 1 mM.

Addition	Concentration	ATP hydrolysis (relative to control)
None	–	(1.0)
EDTA	0.5 mM	0.3
EGTA	0.5	0.3
Ca^{2+}	2.0	35
Ba^{2+}	2.0	9
Sr^{2+}	2.0	20
Mg^{2+}	2.0	2
Ca^{2+} and Mg^{2+}	2.0 (each)	10
Mn^{2+}	2.0	3
Cd^{2+}	2.0	3
Co^{2+}	2.0	1
Ni^{2+}	2.0	0
Ni^{2+} and Ca^{2+}	2.0 (each)	8
Na^{+}	2.0	1
K^{+}	2.0	1
$Na^{+}+K^{+}$	2.0 (each)	1

when present at one-tenth the Ca^{2+} concentration, these ions had little or no inhibitory effect (data not shown).

The rate of ATP hydrolysis increased with increasing Ca^{2+} concentration over the range of 0.5–3.0 mM, with ATP constant at 1.5 mM ATP (Fig. 3). Half-maximal rates occurred at about 1.5 mM Ca^{2+}, and the highest rate of hydrolysis was achieved when the ratio of Ca^{2+} to ATP was approx. 2 (Fig. 3).

Specificity for nucleotide substrate

At a given concentration (1 mM), several nucleoside triphosphates were hydrolyzed at rates similar to those for ATP (Table IV); rates were highest for the purine nucleoside triphosphates (GTP, ATP and dATP), but UTP was also hydrolyzed at about two-thirds the rate for ATP. CTP was a relatively poor substrate, and neither ADP nor AMP was measurably hydrolyzed. No hydrolysis of glucose-6-*P* occurred under our assay conditions. At ATP concentrations below 0.1 mM, activity increased with increasing ATP concentration, but it appeared to level out between 0.1 and 1 mM ATP (Fig. 4).

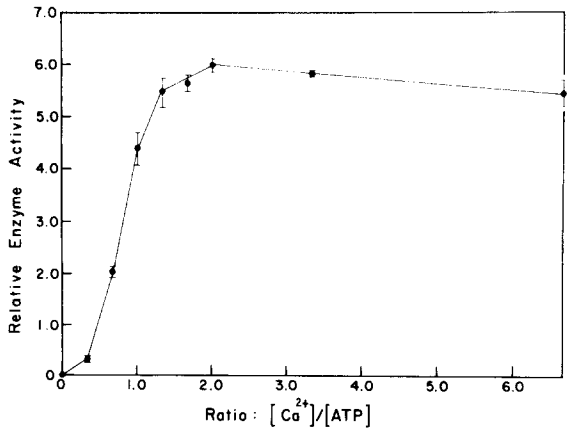

Fig. 3. Effect of Ca^{2+} concentration on ATP hydrolysis by ciliary supernatant (S-II). Final concentrations of 1.5 mM ATP and 3 μg/ml of S-II ATPase were incubated in a standard assay with the concentrations of Ca^{2+} shown for 10 min. For this figure and for Figs. 4–9, the points are averages of three independent experiments, with error bars indicating one standard deviation. The enzyme used here and in Figures 4–9 had a specific activity of 2 units/mg.

Dependence of ATP hydrolysis upon pH and buffer

In Tris buffer, the rate of hydrolysis of ATP was greatest at pH 7.5, but there was measurable activity from pH 6.3 to 8.5 (Fig. 5). At pH 7.5, the optimum concentration of Tris was 5 mM; 50 mM Tris gave activities 20% lower. Other buffers at pH

TABLE IV

RELATIVE SUBSTRATE SPECIFICITY OF S-II CaATPase

A standard assay of P_i production was performed in a mixture containing 0.02 units (5 μg) of S-II. Ca^{2+} was present at 2 mM in all assays. Activity is expressed relative to that with ATP as substrate.

Substrate (1 mM)	Relative activity
ATP	(100)
dATP	95
GTP	130
UTP	70
CTP	15
AMP	1
ADP	1
Glucose-6-*P*	0

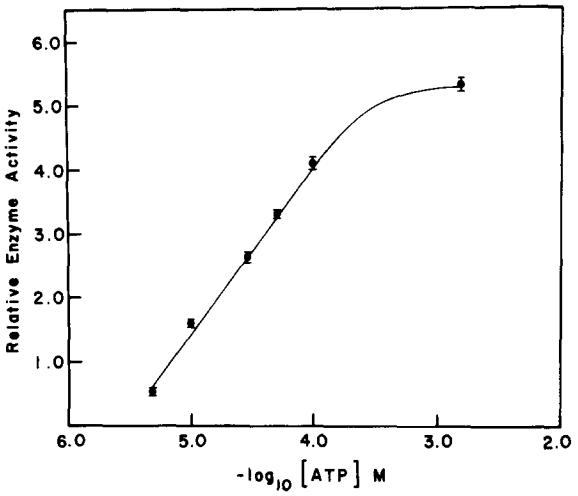

Fig. 4. Effect of ATP concentration upon ATP hydrolysis by ciliary supernatant (S-II). Ca^{2+} was held constant in the standard assay solution, and ATP concentration was varied from 5 μM to 1.5 mM. Bovine serum albumin (50 μg/ml) was added to stabilize the S-II ATPase, which was present at 3 μg/ml.

7.5 and 5 mM (Mops, Hepes, and Pipes) gave rates about one-half as great as those observed in 5 mM Tris.

Thermal stability of the ATPase

The Ca^{2+}-ATPase activity in S-II was stable at room temperature for hours, but at higher temperatures it lost activity rapidly (Fig. 6). We have

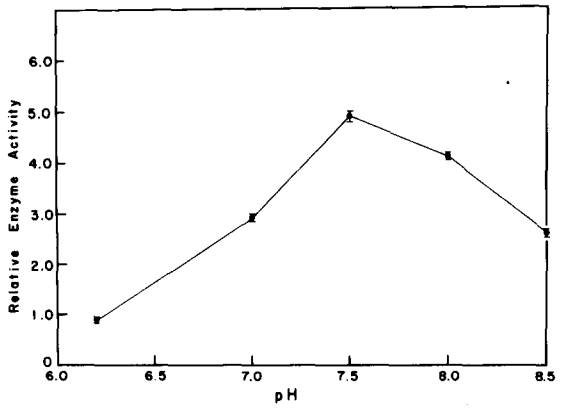

Fig. 5. Effect of pH upon ATP hydrolysis by ciliary supernatant (S-II). An assay mixture containing 3 mM $CaCl_2$, 1.5 mM ATP, and S-II ATPase (3 μg/ml) was buffered with 20 mM Tris-HCl at each pH shown, for a 10 min incubation at 37°C.

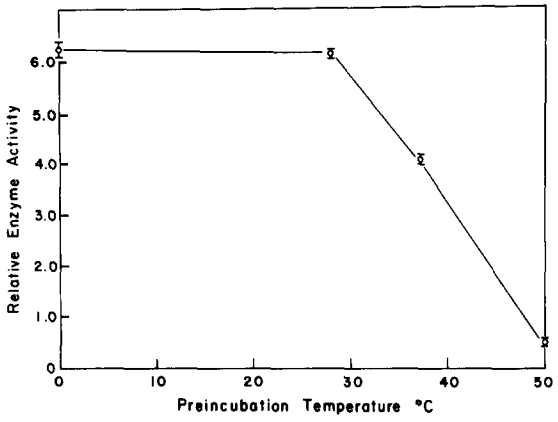

Fig. 6. Thermal inactivation of S-II ATPase. Aliquots of S-II ATPase in 10 mM Tris (pH 7.5) and 3 mM $CaCl_2$ were preincubated for 1 h at the indicated temperature before being assayed for ATPase in a standard assay.

stored the enzyme at protein concentrations of about 0.05 mg/ml in Tris buffer containing 20% glycerol (v/v) at −20°C for months without significant loss of activity; in fact, we have occasionally observed an increase of activity of more than 50% upon storage.

Inhibition of ATP hydrolysis by NaCl, Ruthenium red and Mersalyl

Addition of NaCl to the assay mixture reversibly inhibited ATP hydrolysis; 0.1 M NaCl inhibited 30%, and at 0.5 M NaCl, virtually complete inhibition occurred. Ruthenium red, a potent inhibitor of Ca^{2+}-ATPases from other sources, strongly inhibited the *Paramecium* enzyme; at 8 μM Ruthenium red, ATPase activity was reduced by 50%, and 0.1 mM Ruthenium red gave complete inhibition (Fig. 7). Mersalyl (Salyrgan) was also an inhibitor, although not as potent as Ruthenium red; 50% inhibition occurred at about $5 \cdot 10^{-4}$ M mersalyl.

Selection of standard conditions for measuring ATP hydrolysis

All assays were carried out at 37°C, in a reaction mixture buffered at pH 7.5 with 5 mM Tris-HCl. Concentrations of Ca^{2+} and ATP were 3 mM and 1.5 mM (unless otherwise indicated), to provide the optimal ratio of Ca^{2+} to ATP (2:1) and to provide minimally saturating substrate levels.

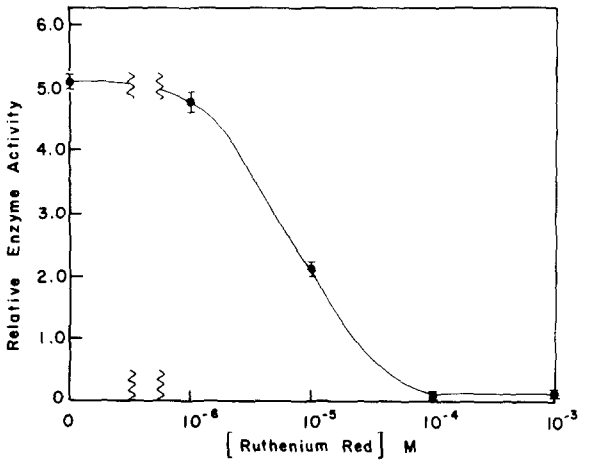

Fig. 7. Inhibition of S-II ATPase by Ruthenium red. To a standard assay mixture containing 3 μg/ml S-II ATPase was added Ruthenium red at the indicated concentrations.

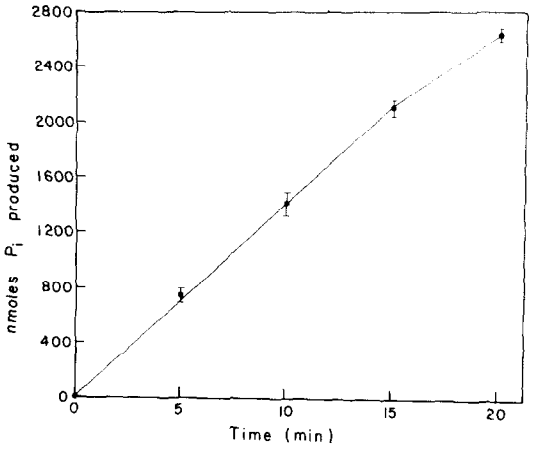

Fig. 9. Time course of S-II ATPase action. A standard assay mixture containing 1.5 mM ATP was incubated with 3 μg S-II ATPase for the indicated times. The last time point (20 min) represents about 80% hydrolysis of the substrate.

When very dilute protein solutions were being assayed, the addition of bovine serum albumin (50 μg/ml) to the assay gave slightly higher and more reproducible rates of hydrolysis (Bovine serum albumin had no ATPase activity).

Under these conditions, the hydrolysis of ATP was linear with respect to enzyme concentration (Fig. 8), and with respect to time up to the point at which 80% of the initial substrate had been hydrolyzed (Fig. 9).

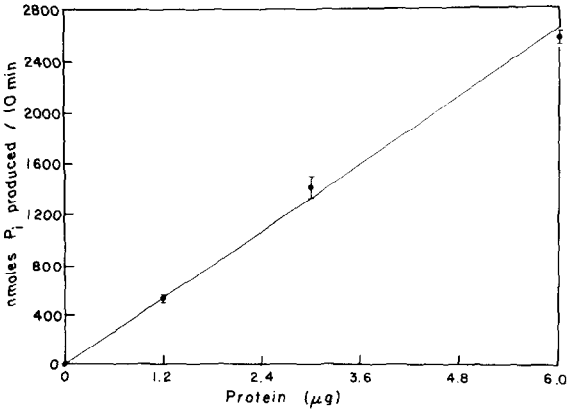

Fig. 8. Proportionality of ATP hydrolysis with added enzyme. The indicated volumes of a concentrated S-II ATPase preparation (1.1 mg/ml) were added to 2 ml of standard assay mixture, containing 1.5 mM ATP, for a 10 min assay at 37°C. The last point represents about 90% hydrolysis of substrate.

Evidence that ATPase, GTPase, and UTPase reside in the same molecule

ATPase activity from concentrated S-II sedimented through a glycerol gradient as a broad peak with a shoulder (Fig. 10), and GTPase and UTPase coincided with ATPase. The ratios of the three activities were roughly constant across the peak. By comparison with the position of appropriate markers, we estimate a sedimentation coefficient of 8–10 S for the peak fraction. To explore further the possibility that there were several distinct nucleoside triphosphatases in S-II, we studied the loss of ATPase, GTPase, and UTPase activity after preincubation at 45, 50, and 55°C. The enzymatic activities decayed exponentially with time, and the decay curves with GTP and UTP were indistinguishable from those with ATP; at 45°C, the half-time for enzyme loss was 45–60 min, at 50°C it was 12 min, and at 55°C it was less than 4 min, with complete loss of activity in 1 h.

Electrophoresis in non-denaturing polyacrylamide gels

Electrophoresis under non-denaturing conditions (Materials and Methods) resolved the ATPase in S-II into several discrete bands which could be stained for ATPase activity. The most prominent band in short incubations with ATP was a rela-

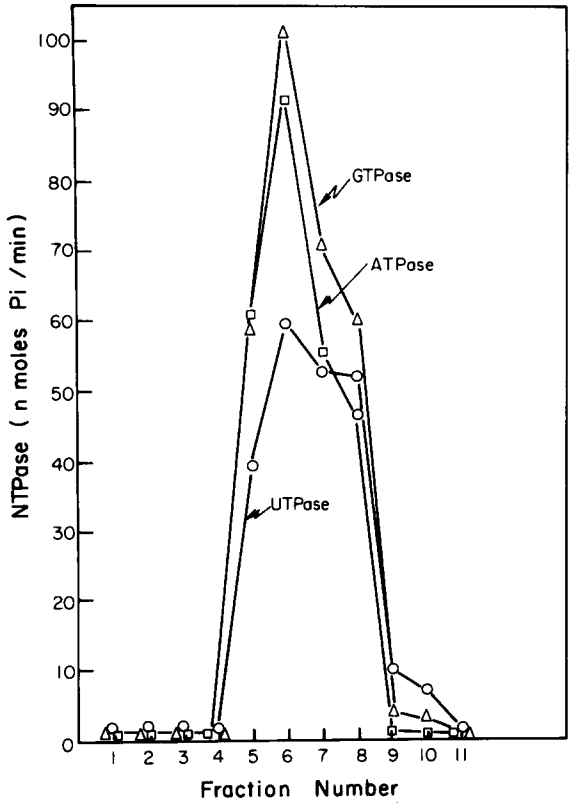

Fig. 10. Sedimentation of S-II ATPase through glycerol gradient. About 0.05 units of S-II ATPase in 0.1 ml were layered onto a 12 ml glycerol gradient, then centrifuged for 16 hours at 4°C in an SW rotor at 41000 rpm. Fractions (30 drops) were collected from the bottom of the tube and assayed for ATPase, GTPase, and UTPase as described in Methods.

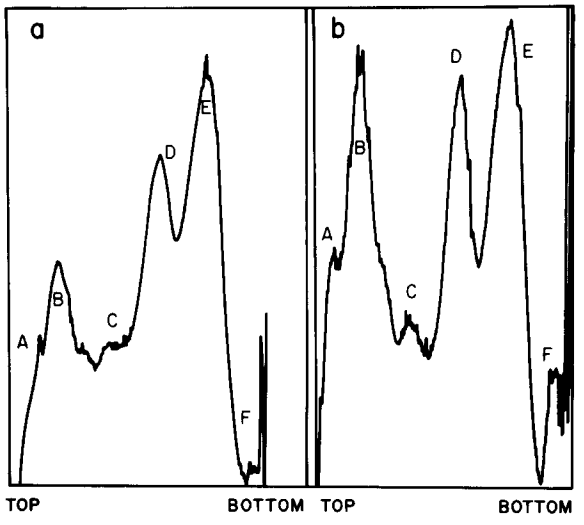

Fig. 11. Native acrylamide gels stained for ATPase activity. S-II ATPase was subjected to polyacrylamide gel electrophoresis under non-denaturing conditions (Methods) in tubes 10 cm long. Individual gels were stained for ATPase as described in Methods for either 20 min (left) or 90 min (right), then scanned with a visible photometer at 760 nm.

tively fast-moving species (E in Fig. 11), but two slower-migrating species were also detectable after short incubations (B and D), and their prominence increased with longer incubations, which also revealed three more weakly-staining bands, A, C, and F, Fig. 11. All six of these bands were greatly diminished in intensity when the ATPase staining was done in the presence of EDTA, or when Mg^{2+} was included in the assay mixture instead of Ca^{2+} (data not shown). Furthermore, at least the three major bands (B, D, E) were stained when ATP was replaced with either UTP or GTP, and the relative intensities of these three bands were similar for gels stained for ATPase, GTPase, or UTPase; each of the three bands of activity resolved on native gels was capable of hydrolyzing each of the three nucleotides. No activity was detected on the gels with ADP or AMP for substrate, nor was glucose-6-*P* hydrolyzed (data not shown).

Discussion

From which cell compartment do the proteins of the ciliary supernatant originate?

The distribution of marker enzymes among cilia, deciliated bodies, and ciliary supernatant suggests that the ciliary supernatant is a discrete fraction, not merely a small portion of the cytoplasm released by deciliation. At least 20% of the Ca^{2+}-ATPase was released but 1% or less of the marker enzymes were released into the ciliary supernatant. Furthermore, the SDS-polyacrylamide gel electrophoresis pattern of the ciliary supernatant did not resemble that of cytoplasmic contents. It seems likely that proteins in this discrete fraction originate in one of three cellular regions: the intraciliary space, extruded trichocysts, or the surface membrane.

When cilia are removed by the STEN procedure, the detached cilia apparently do not reseal to form closed structures (Ramanathan, R., unpub-

lished data). Soluble proteins in the intraciliary space are therefore probably released upon deciliation. A number of proteins which are loosely-associated with isolated cilia may be released by vortexing in Tris-EDTA solution, and the peptide composition of this soluble fraction is similar to that of the ciliary supernatant (Fig. 2b), suggesting a common origin for the two fractions.

Deciliation generally results in the firing of at least some trichocysts, and Plattner et al. [7] have reported that there is Ca^{2+}-ATPase activity in the cell surface at the point of trichocyst fusion during exocytosis. Our washing procedure is deliberately designed to induce trichocyst firing before the deciliation step so as to reduce the contamination of cilia or ciliary supernatant by trichocysts. The major protein component of extruded trichocysts is a family of peptides of mol. wt. 17000, which are not prominent components of our ciliary supernatant [8,25,26]. However, one major polypeptide of the ciliary supernatant ($M_r = 68000$) is the same size as a minor peptide associated with trichocysts. It therefore seems possible that some of the proteins of the ciliary supernatant are derived from extruded trichocysts; it is also possible that certain proteins are located both in cilia and in trichocysts.

Finally, it is possible that the Ca^{2+}-ATPase is an extrinsic membrane protein which under the conditions of STEN deciliation is released as a soluble form. When paramecia were washed less extensively than usual with Dryl's buffer before deciliation, about the same amount of protein was released into the ciliary supernatant upon deciliation, but much less ATPase was found in that fraction, and correspondingly more remained associated with cilia (Riddle, L., (1977) M.S. Thesis, University of Wisconsin-Madison). Andrivon reported that the amount of Ca^{2+}-ATPase associated with isolated *Paramecium* cilia depended upon the method of deciliation employed. With a calcium-shock procedure three times more Ca^{2+}-ATPase was associated with cilia than when they were prepared by a Mn^{2+} shock (Andrivon, C., (1978) Ph.D. thesis, University of Clermont-Ferrand, France). Andrivon did not assay the ciliary supernatant for Ca^{2+}-ATPase, but the Mn^{2+} deciliation may have released more enzyme into that fraction. The ciliary Ca^{2+}-ATPase studied by Andrivon is associated with the ciliary membrane (Brugerolle et al. [27]).

TABLE V

COMPARISON OF SURFACE-ASSOCIATED ATPases OF SOME CILIATED OR FLAGELLATED PROTOZOANS

Organism	*Paramecium tetraurelia*	*Paramecium tetraurelia*	*Paramecium tetraurelia*	*Paramecium caudatum*
Reference	This paper	28	27	29
Sub-cellular localization	Ciliary supernatant	Cilia (Triton extract)	Ciliary membrane	Pellicle (Triton extract)
Ion activation	Ca > Sr > Ba ≫ Mg	Ca ≫ Mg	Ca ≫ Mg	Ca = Sr > Ba ≫ Mg
K_m (ATP)	–	–	–	3 μM
pH optimum	7.5	–	–	6
Specific activity	2–4 μmol·min^{-1}·mg^{-1}	0.6 μmol·min^{-1}·mg^{-1} [a]	0.3–1.0 μmol·min^{-1}·mg^{-1}	0.5 μmol·min^{-1}·mg^{-1} (0.03) [b]
Nucleotide specificity	GTP > ATP > UTP ≫ CTP	ATP	ATP	CTP > UTP > ATP = ITP > ADP
Molecular weight	–	70000	70000	–
Sedimentation coefficient	8–10 S	–	–	9 S

[a] This specific activity refers to the purified enzyme.

[b] The number in parentheses refers to pellicle-bound enzyme; the other data are for Triton-solubilized enzyme.

Relationship between Ca^{2+}-ATPase of the ciliary supernatant and other ATPases of the cilium or cell surface

There are at least six other reports of divalent cation-dependent ATPases detected in the cilia or the surface of *Paramecium* (Table V). Doughty [28] used affinity chromatography to purify from Triton extracts of whole cilia an ATPase activity that was stimulated by Ca^{2+} but only poorly by Mg^{2+}. The purified enzyme gave a single polypeptide of 70 kDa in SDS gels, and its specific activity was about 0.8 unit/mg. Brugerolle et al. [27] characterized an ATPase associated with purified ciliary membrane. This enzyme was stimulated by Ca^{2+} but not by Mg^{2+}; it had about the same specific activity as that described by Doughty; and its subunit molecular weight was 70000. Noguchi et al. [29] described a Ca^{2+}-dependent ATPase associated with the pellicle (surface membrane of cell bodies) of *Paramecium caudatum*. This enzyme, like those of Doughty and of Brugerolle et al., could be solubilized by extraction with Triton X-100, and Noguchi et al., showed that certain properties of the enzyme, including its K_m for ATP, the degree of stimulation by various divalent cations, and its pH dependence, changed somewhat upon solubilization. The specific activity of the partially-purified enzyme was the same as that reported by Doughty, and by Brugerolle et al. The subunit molecular weight was not reported, but the solubilized ATPase had a sedimentation coefficient of about 9. Bilinski et al. [30] have also characterized a Ca^{2+}-ATPase associated with the pellicle, with properties similar to those described by Noguchi et al. These three enzymes do not differ greatly and may be identical. All are clearly different from a fourth ciliary ATPase, dynein, which was studied in *Paramecium* by Hayashi and Takahashi [31] and by Doughty [33]. Dynein from *Paramecium* had properties similar to those of dynein from other sources including *Tetrahymena*; it was associated with axonemes, sedimented in two forms (14 S and 30 S), had a specific activity of 1–3 units/mg, and was stimulated by Mg^{2+} and only poorly by Ca^{2+}. Its subunit molecular weight (from SDS gels) was 340000–390000.

Finally, Van Wagtendonk and Vloedman [32] reported that the crude immobilization antigen of *Paramecium*, a surface glycoprotein of about 250 kDa, had ATPase activity. We (Browning, J. and

Paramecium tetraurelia 30	*Paramecium tetraurelia* 33	*Paramecium caudatum* 31	*Tetrahymena pyriformis* 34	*Tetrahymena pyriformis* 36	*Chlamydomonas reinhardii* 37
Pellicle	Axonemes	Axonemes (dynein)	Axonemes (dynein)	Cytosol	Flagella
Ca>Mg	Mg>Ca	Mg	Mg>Ca	Ca=Ba>Mg	Ca≫Mg
–	–	24 μM (30 S) 20 μM (14 S)	11 μM (30 S) 35 μM (14 S)	2.5 mM	0.4 mM
7.8	–	–	8.5–9.0	–	7.9
0.3–0.9 μmol·min^{-1}·mg^{-1}	–	0.3–1.0 μmol·min^{-1}·mg^{-1}	1.3 μmol·min^{-1}·mg^{-1} (30 S) 3.5 μmol·min^{-1}·mg^{-1} (14 S)	0.08 μmol·min^{-1}·mg^{-1}	1–2 μmol·min^{-1}·mg^{-1}
ATP	ATP>GTP	ATP	ATP≫GTP, CTP ITP, UTP	ATP>GTP>CTP> UTP	ATP>GTP, CTP, ITP, UTP
–	500000 [c]	340000–390000	600000 [c]	89000	–
–	–	14 S, 30 S	14 S, 30 S	–	3 S

[c] These numbers are almost certainly overestimates of the molecular weight of dynein.

Nelson, D.L., unpublished data) were unable to detect ATPase activity in our preparations of immobilization antigen.

The published data are consistent with the presence, in cilia, of at least two ATPases: the Mg^{2+}-dependent activity of dynein and the membrane-bound Ca^{2+}-ATPase of Doughty, of Brugerolle et al., and of Noguchi. The properties of the enzyme we have described here are similar to those of this membrane-bound enzyme, but as we isolate it, it is not membrane bound. It seems possible that the enzyme is an extrinsic membrane protein which is solubilized by our deciliation procedure, but not by other procedures.

What is the function of the Ca^{2+}-ATPase?

This Ca^{2+}-ATPase appears to originate from the cell surface, or from some organelle associated with the cell surface. There are several surface-related functions in *Paramecium* which probably involve Ca^{2+}- and energy-dependent reactions. Ca^{2+} clearly controls the direction of the ciliary beat in *Paramecium* [1,2], and ATP is apparently required for the Ca^{2+}-dependent ciliary re-orientation, which occurs independently of ciliary beating [4]. Ciliary reversal is triggered when intracellular Ca^{2+} levels exceed 1 μM [4]. Normally, intracellular Ca^{2+} is maintained well below this level by an energy-dependent Ca^{2+}-extrusion mechanism [3], which by analogy with other Ca^{2+} pumps might be expected to have Ca^{2+}-ATPase activity. A third surface phenomenon believed to be Ca^{2+}-dependent is the exocytotic fusion of trichocysts, vesicular structures just beneath the *Paramecium* surface, and the subsequent explosive extrusion of their contents [5,6]. There is electron microscopic evidence for a Ca^{2+}-dependent ATPase at the site of fusion of trichocysts and surface membrane [7], and a variety of agents which alter transmembrane Ca^{2+}-fluxes affect trichocyst 'firing' in vivo. When unfired trichocysts are isolated in solutions containing EGTA, the addition of Ca^{2+} causes the explosive extrusion in vitro [38]. Several mutants defective in the synthesis or firing of trichocysts were found by Bilinski et al. [30] to have pellicular Ca^{2+}-ATPase with K_m and V_{max} slightly different from that of wild-type cells. Satir et al. [39] have recently claimed that trichocyst firing is inhibited by the calmodulin antagonist trifluoperazine.

A fourth possible role for a Ca^{2+}-ATPase was recently raised by Blum et al. [40], who found that under certain conditions of extraction, the dynein of the ciliary axoneme of *Tetrahymena* was Ca^{2+}-sensitive. Doughty [33] has made a similar observation with *Paramecium* dynein. Dynein is an ATPase which interacts with tubulin to cause the sliding motion responsible for the ciliary power stroke, and is generally found to be a Mg^{2+}-dependent ATPase [41].

Finally, Bloodgood et al. [42] have described a directed motion of certain of the proteins of the surface of *Chlamydomonas* flagella (from base to tip) which is probably energy-dependent and could therefore involve a flagellar ATPase. The possibility of such motion in *Paramecium* ciliary membranes has not been investigated.

The Ca^{2+}-dependent ATPase which we have described here may play a role in one of these cellular functions, or it may be unrelated to any of these.

We have reported [43] that ciliary reversal in *Paramecium* is blocked by phenothiazines, drugs which interfere with calmodulin-regulated, Ca^{2+}-dependent functions. The same drugs, at the same concentrations, strongly inhibit the S-II ATPase (Ref. 43; Levin, A.E. and Nelson, D.L., unpublished data). If the Ca^{2+}-ATPase were a general feature of organisms in which the ciliary or flagellar beat is regulated by Ca^{2+}, one might expect a similar enzyme to be present in those organisms. *Tetrahymena pyriformis*, the ciliary beat of which is regulated by Ca^{2+}, contains a potent, soluble, Ca^{2+}-dependent ATPase activity which has been purified from whole cells [36]; its localization within cells or cilia has not been studied. *Chlamydomonas reinhardii* has two flagella, the waveform of which is regulated by Ca^{2+}, and a soluble, Ca^{2+}-dependent ATPase has been isolated from flagella of this organism [37].

Our ciliary supernatant contains little or no dynein, as judged by its absence in SDS-polyacrylamide gels (Fig. 2). Making reasonable estimates from these gels of the maximal amount of protein in the ciliary supernatant which could be dynein, we calculate that if the Ca^{2+}-ATPase activity is associated entirely with this small amount of dynein, its specific activity must be at least 2

orders of magnitude higher than the published values (~4 units/mg) for purified *Tetrahymena* dynein. We have prepared dynein from *Paramecium* cilia and have found that the sedimentation behavior of Ca^{2+}-ATPase activity is quite different from that of *Paramecium* dynein, which showed two peaks with $s_{20,w}$ of about 15 and 30 S (Stumpf, S. and Nelson, D.L., unpublished data). We therefore feel it is very unlikely that our Ca^{2+}-ATPase is a Ca^{2+}-dependent form of ciliary dynein such as that described by Blum et al. [40] and by Doughty [33].

To determine definitively the role of the several Ca^{2+}-ATPases which have been described, it will be important to learn their relationships to each other and their localization within the cell. We hope to accomplish both of these goals by using antibody raised against the purified ciliary supernatant enzyme. It may also be enlightening to compare the Ca^{2+}-ATPase of wild-type cells with mutants defective in Ca^{2+}-dependent swimming behavior [44] or in trichocyst development or firing [45–47].

Acknowledgements

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