TWO TYPES OF ADENOSINE TRIPHOSPHATASE FROM FLAGELLA OF CHLAMYDOMONAS REINHARDI

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Summary Chlamydomonas flagella were found to contain, in addition to dynein, a distinct low molecular weight, calcium specific adenosine triphosphatase. Some properties of this enzyme and of adenylate kinase are reported.

INTRODUCTION

Cilia and simple flagella contain a characteristic array of 2 central microtubules and 9 outer doublets (1), guanine nucleotides bound to tubulin (2) and 10 mM free ATP (3), and energy transducing protein(s) which can be solubilized as heterogeneous adenosine triphosphatases (ATPase), called dynein. Dynein is thought to be localized in sidearms of the "A" tubules (3,4). In Tetrahymena cilia (4) and sea urchin sperm flagella (5) there is some evidence, but no proof, that 14 S and 30 S dynein are aggregates of the same subunit(s), the latter having molecular weights of 260,000 - 290,000 (6). Dynein ATPase is quite specific for ATP and is activated by calcium nearly as well as by magnesium. Calcium ions may participate in the regulation of both microtubule assembly (7) and flagellar motility (8).

Preparatory to a search for dynein-like ATPase associated with brain microtubules, we have examined the ATPases in Chlamydomonas flagella. We report here the presence, in addition to dynein (1), of a soluble low-molecular weight calcium specific ATPase (9).

MATERIALS AND METHODS

Chlamydomonas reinhardi wild type +137c, supplied by Dr. R. P. Levine, was grown at 25 - 28°C in 4.5 liter bottles in high salt inorganic medium (10) aerated at 2 liters/minute with 1 to 3% CO_2 in air. Cultures were synchronized by alternate periods of 10 hours darkness and 14 hours light (400 footcandles). When the cell density reached 2 X 10^6/ml, cells were harvested from 4 bottles

between the 3rd and 5th hours of the light period with a De Laval Model 102 cream separator (11) operated at maximum speed with the inlet aperture half open. The cell slurry was immediately resuspended in 900 ml of 10 mM Tris-HCl, pH 7.95. In our hands this procedure caused detachment of about half the flagella. The dense resuspension was therefore placed on a rotary shaker for 1 hour to allow flagellar regeneration (the results were the same whether regeneration took place in Tris buffer or culture medium). At the end of this time, phase contrast microscopy showed 85 - 90% normal biflagellate cells. Cells were then pelleted by centrifugation at 25°C in a Sorvall GS-3 rotor for 10 minutes at 2,000 X g. In some cases, cells were isolated on a smaller scale, without loss of flagella, by centrifuging the original culture directly. Flagella were detached by the "sucrose-pH 4.5" or, when specified, "STEEP + calcium" procedure of Witman et al. (11), and were isolated by centrifugation to a sucrose interface (11), and then pelleted for 20 minutes at 31,000 x g. Flagella were extracted (11) by overnight dialysis against TED (0.1 mM EDTA and dithiothreitol, 1 mM Tris, pH 8.0); 90% of the ATPase was then not sedimentable in 45 minutes at 105,000 x g.

ATPase was routinely assayed (12) in reaction mixtures containing 27 mM Tris-HCl, pH 7.9, 1.2 mM $MgSO_4$ or $CaCl_2$, 0.15 mM EDTA, and 1 mM ATP. After incubation at 25°C inorganic phosphate was measured by the Fiske-SubbaRow method. Adenylate kinase was assayed as described by Gibbons (12): the formation of ATP from ADP is coupled to TPN reduction in the presence of hexokinase and glucose-6-P dehydrogenase. All assays were shown to be in the range of proportionality to protein concentration and reaction time, and rates are expressed in units of μmoles of product formed per minute.

Sucrose density gradient centrifugation was done according to Martin and Ames (13) using catalase (11.2 S) and malic dehydrogenase (4.0 S) as internal standards. To aid comparison of different experiments, the observed enzyme activities were all adjusted to what they would have been if 1 mg of flagellar extract protein had been applied to the gradient. Protein was measured by the

Lowry method. Sucrose and Tris were Schwarz-Mann Ultra Pure grade. Nucleotides were purchased from P & L Biochemicals, Good buffers from Sigma, and malic dehydrogenase and catalase from Calbiochem.

RESULTS

Extracts, prepared by a procedure which solubilized 90% of the flagellar ATPase and 1/3 to 1/2 of the protein, reproducibly contained ATPase which had a specific activity of 1.2 - 2.3 units/mg in the presence of Ca^{++}, but only 0.4 - 0.6 in the presence of Mg^{++}. Fractionation on a Bio-Gel A-15m column (Fig. 1) revealed at least 2 peaks of dynein ATPase (fraction number 47 - 65), which was activated by either Mg^{++} or Ca^{++}, and in most other properties resembled dynein as previously described (1,5,12). However, there was also a symmetrical peak of ATPase in low molecular weight fractions (80 - 90), activated only by Ca^{++}. The Ca-ATPase was stable for weeks at 0°C, and had a single pH optimum, determined with a variety of buffers, of 7.9. Activity was maximal with Ca^{++} concentrations 1 to 3 times the ATP concentration and dropped sharply on either side of this range; Mg^{++} inhibited when added in addition to Ca^{++}. The K_m for ATP was 4×10^{-4} M when the Ca:ATP ratio was kept constant at 2. Salts were inhibitory: 50% inhibition by 0.21 M KCl. There was no activity when Ca^{++} was replaced with actin + Mg^{++}, or K^{+} + EDTA, under conditions activating myosin ATPase (the muscle proteins were gifts of Drs. E.Eisenberg and R. Adelstein), and oligomycin and ouabain were not inhibitory. GTP, ITP, CTP and UTP were hydrolyzed at 1/3 the rate of ATP. Nucleoside diphosphates could not be tested because adenylate kinase was present in the same fraction. Nucleoside monophosphates, inorganic pyrophosphate, and p-nitrophenylphosphate were not hydrolyzed.

Sucrose gradient fractionation of extracts (Fig. 2) indicated molecular weights corresponding to about 12 S and 18 S for dynein and 3.0 S for Ca-ATPase. Adenylate kinase assay showed 2 peaks (Fig. 2); the faster sedimenting component was well separated from Ca-ATPase after longer centrifugation time. The heterogeneity of adenylate kinase of Tetrahymena cilia ($\leq$ 10 S) was

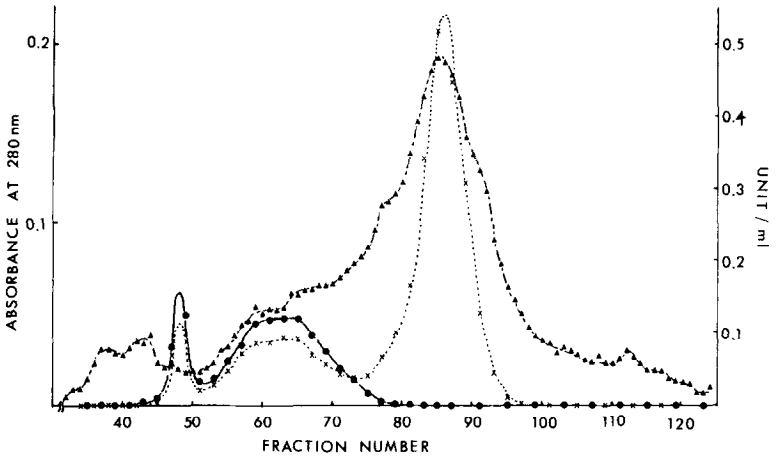


Fig. 1. ATPase activity in the presence of Mg^{++} (—●—), or Ca^{++} (····X····), and protein (-- ▲ --, absorbance at 280 nm) in fractions of flagellar extract eluted from a Bio-Gel A-15m column. Seven mg of protein was applied to a 3 x 45 cm column, equilibrated and eluted at 4°C with 5 mM Tris-HCl, pH 7.8, containing 0.5 mM EDTA, 0.1 mM dithiothreitol, and 5 mM KCl. The flow rate was 22 ml/hr and 2 ml fractions were collected.

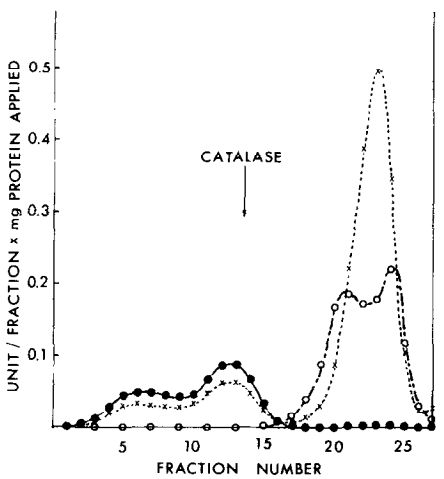
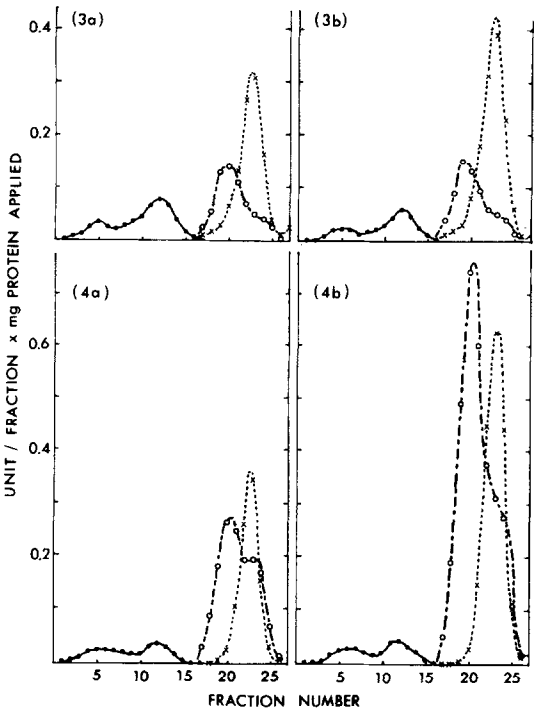


Fig. 2. Sucrose gradient fractionation of activities in flagellar extracts of Mg-ATPase (—●—), Ca-ATPase (····X····), and adenylate kinase (-- O --). Flagellar extract (about 200 µg protein) was layered on 5 ml of a 5 to 20% sucrose gradient in the same buffer used for gel filtration. The gradient was centrifuged for 5 hours in a Spinco SW 65 rotor at 60,000 rpm at 1°C. Eleven drops were collected per fraction.

attributed to aggregation (12), but it seems at least as likely that there are 2 different enzymes in Chlamydomonas flagella: one of these might mediate transphosphorylation of tubulin-bound guanine nucleotides (2).

Further evidence that the Ca-ATPase was in fact of flagellar origin was



Figs. 3,4. Activities of Mg-dynein (—●—), 3.0 S Ca-ATPase (····X····) and adenylate kinase (--O--) in sucrose gradient fractions of flagellar extracts prepared from: cells harvested without deflagellation (Fig. 3) and isolated at once (3a) or after 3.5 hours in dense suspension (3b); cells completely deflagellated (Fig. 4) and allowed to regenerate flagella in dense suspension for 1 hour (4a) or 2 hours (4b).

obtained as follows. Flagella were isolated at an earlier stage of growth (8×10^5 cells/ml) and were detached by 2 procedures (see METHODS). Negligible Ca-ATPase was found in extracts obtained by dialysis of cells deflagellated by sucrose-pH 4.5, with or without repeated exposure of cells to this pH. When small amounts of deflagellated cells were added to flagella and dialyzed together there was no change in Ca-ATPase; this also argues somewhat against Ca-ATPase being a proteolytic digestion product of dynein. The latent Ca-ATPase in chloroplasts (14,15) resembles our enzyme in some ways but is larger and more specific for ATP. Also our flagella, though often slightly green, contained < 0.05% of the cellular chlorophyll, and the specific activity of the ATPase in Chlamydomonas chloroplast fragments prepared according to Sato et al. (15) was only 0.14 μmole/min x mg protein, whereas in flagellar extracts it was > 1.0.

The fact that others have not detected flagellar Ca-ATPase could be related to 2 changes in our procedure resulting from the partial deflagellation caused by harvesting: a) some of the flagella used for the experiments of Figs. 1 - 2 were newly regenerated and b) the cells were kept for an hour in abnormally crowded suspension with limitation of light and gas exchange. The effects of (a) and (b) on Ca-ATPase activity were therefore examined separately. Fig. 3 shows the results obtained when cells were harvested without deflagellation and flagella were isolated immediately (Fig. 3a; manipulations unavoidably required that cells be in crowded suspension for 40 minutes), or after 3.5 hours on a shaker (Fig. 3b). Preliminary results show that the latter conditions caused some increase in Ca-ATPase activity, but the enzyme is clearly also present under conditions used by other investigators (Fig. 3a). In the experiment of Fig. 4 150 ml of a dense suspension was completely deflagellated by shearing for 5 minutes at top speed with a Virtis-23 homogenizer, and new flagella were harvested after regeneration during 1 or 2 hours. Ca-ATPase was increased somewhat more in newly regenerated flagella at 2 hours (Fig. 4b, results were similar after 4 hours) than in the experiment of Fig. 3. The amount of enzyme per flagellum increased even more because, although flagella had grown to essentially full length (12 μ) at 1 hour, total and extractable flagellar protein apparently doubled between 1 and 2 hours. It is interesting that the adenylate kinase(s) increased specifically only in the case of newly regenerated flagella, and seemed to do so sequentially, the increase of the slower sedimenting activity preceding that of the faster (Fig. 4). We are currently trying to purify and separate the adenylate kinase enzyme(s) from Ca-ATPase.

The 3 S Ca-ATPase does not resemble known calcium transport enzymes. It could be a subunit of dynein or a product of partial proteolytic digestion, or a distinct energy transducer. Histochemical localization of ATPase (p. 222 in reference 1) shows activity near the central microtubules as well as the outer dynein arms.

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