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STUDIES ON FLAGELLAR ATPase FROM SEA URCHIN SPERMATOZOA

I. PURIFICATION AND SOME PROPERTIES OF THE ENZYME

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SUMMARY

When the flagella isolated from glycerinated sea urchin spermatozoa were dialyzed against Tris–EDTA solution, a crude extract of flagellar ATPase was obtained. Applying the crude extract to a Sepharose 4B column and a hydroxylapatite column, successively, the specific activity of the enzyme was increased about 7-fold over the crude extract.

A small peak of ATPase, probably an aggregated type, was observed prior to the major peak on the Sepharose 4B column. Several peaks of ATPase activity which appeared on the hydroxylapatite column seemed to be derived from a single form of this enzyme.

Some properties of flagellar ATPase were examined, using the purified preparation. The purified enzyme showed high substrate specificity for ATP. The K_m for ATP was $5 \cdot 10^{-5}$ M. The enzyme was activated by Mg^{2+}, Mn^{2+}, Ni^{2+} and Ca^{2+}, and was inhibited by Cd^{2+}, Zn^{2+}, Hg^{2+} and sulfhydryl reagents. Two pH optima appeared, at 7.0 and 8.5.

Recombination between the solubilized ATPase and the EDTA-treated flagella (outer doublet microtubules) was observed after dialysis against a solution containing either Ca^{2+} or Mg^{2+}. The percentage of recombination was higher in the case of the purified enzyme than of the crude extract.

INTRODUCTION

The investigation to clarify the mechanism underlying flagellar movement at the molecular level started by regarding flagellar movement as a kind of muscular contraction and trying to extract myosin- and actin-like proteins from flagella. ENGELHARDT AND BURNASHEVA[1] first reported extraction of a myosin-like protein, "spermosin", from bull sperm tails. Later, GIBBONS[2] developed a successful method for solubilizing ATPase from Tetrahymena cilia, identified the ATPase protein as the projections (arms) located on the outer doublet microtubules of cilia, and named it "dynein". He found two forms of dynein, which sedimented at 14 S and 30 S in the ultracentrifuge.

Abbreviation: PCMB, *p*-chloromercuribenzoate.

Since then, several reports[3–12] have appeared concerning flagellar or ciliary ATPase. In the case of sea urchin spermatozoa[4,8], only the ATPase corresponding to 14 S dynein was obtained after dialysis of flagella against 1 mM Tris –0.1 mM EDTA (pH 8.2), whereas in the presence of salt[11], *e.g.* 0.6 M KCl, an aggregated form of ATPase probably corresponding to 30 S dynein of Tetrahymena cilia appeared together with the 14 S component. The enzymatic properties of flagellar and ciliary ATPase were not changed by purification and were found to be similar among the preparations from different sources[13,14]. However, there is a possibility that the solubilized ATPase in all these studies was insufficiently purified. It is desirable to obtain a purified enzyme preparation free from protein contaminants, especially microtubule proteins (tubulin), because the ATPase activity of myosin, the muscle counterpart of dynein, is much increased and modified by adding a small amount of actin which resembles tubulin.

The present work deals with a procedure for purifying flagellar ATPase from sea urchin spermatozoa, which seem to be a better material than Tetrahymena cilia for studying the mechanism of flagellar movement because of the ease of obtaining a sufficient amount. We also describe recombination between the once solubilized ATPase and EDTA-treated flagella, mostly the outer doublet microtubules, which has not yet been reported in the case of purified enzyme or 14 S dynein.

MATERIALS AND METHODS

Materials

Spermatozoa were mainly collected from the sea urchin *Pseudocentrotus depressus* after introduction of 0.5 M KCl into the perivisceral cavity. Glycerinated sperm suspension[15] was prepared by mixing spermatozoa suspended in a medium consisting of 0.3 M KCl, 20 mM Tris–thioglycolate buffer (pH 8.3) and 10 mM $MgCl_2$ with an equal volume of glycerin at 4° and stored at –20° until use.

Preparation of flagellar ATPase and EDTA-treated flagella

Subsequent procedures were carried out at 0–4°. Isolation of flagella from spermatozoa and solubilization of ATPase from the flagella were performed according to the method of MOHRI[3] with some modification. Glycerinated spermatozoa were collected from 200 ml of glycerinated sperm suspension by centrifugation for 30 min at approximately 12000 x *g*, and suspended in about 150 ml of Ca^{2+}, Mg^{2+}-free artificial sea water. Spermatozoa were again collected by centrifugation for 15 min at 3000 x *g*, suspended in about 150 ml of Ca^{2+}, Mg^{2+}-free sea water, and disrupted with a sonicator (Kubota Ultrasonic Generator KMS-200A). The suspension of broken spermatozoa was centrifuged for 10 min at 3000 x *g* and the precipitate, consisting mostly of sperm heads, was discarded. The flagella were then collected from this supernatant by centrifugation for 15 min at 12000 x *g* and suspended in about 100 ml of 10 mM Tris–HCl buffer (pH 8.3) containing 0.5 mM EDTA and 0.1 % β-mercaptoethanol (Tris–EDTA solution). The suspension of flagella was homogenized with a Teflon homogenizer, dialyzed against 2 l of Tris–EDTA solution for 18 h, and centrifuged for 60 min at 70000 x *g*. The solubilized flagellar ATPase (crude extract) and the EDTA-treated flagella were obtained as the supernatant and the precipitate, respectively. The EDTA-treated flagella thus obtained should contain both outer

doublet microtubules and flagellar membranes[4]. According to our experience, however, the membranes were apt to be lost during glycerination and washing in Ca^{2+}, Mg^{2+}-free sea water. The procedure for purifying the crude extract will be described in RESULTS.

Assay of flagellar ATPase

The standard assays for ATPase activity were performed by incubating at 30° for 10 min in a test tube containing 250 μmoles Tris–HCl buffer (pH 8.3), 40 μmoles $MgCl_2$, 5 μmoles ATP and the enzyme solution in a final volume of 2 ml. The reaction was usually started by the addition of ATP. After 10 min, the reaction was terminated by adding 0.5 ml of 25% cold trichloroacetic acid, and the precipitate, if present, was removed by centrifugation. An aliquot (1 ml) of the supernatant solution was used for analysis of inorganic phosphate according to the method of LOHMANN AND JENDRASSIK[16].

One unit of the enzyme activity was defined as the amount causing liberation of 1 μmole P_i per min under the above conditions.

For determination of K_m, P_i was determined by the method of MURPHY AND RILEY[17], by which it is possible to measure a very small amount. The reaction was initiated by adding an aliquot of enzyme solution to reaction mixtures containing different concentrations of ATP. After 5 min the reaction was stopped by adding H_2SO_4. Each tube was subsequently assayed for P_i.

Assay of alkaline phosphatase

Alkaline phosphatase activity was assayed[18] by incubation in a solution containing 250 μmoles Tris–HCl buffer (pH 8.3), 40 μmoles $MgCl_2$, 5 μmoles *p*-nitrophenylphosphate and the enzyme solution in a final volume of 2 ml. After an appropriate period an aliquot (0.5 ml) of the reaction mixture was pipetted into 4 ml of 0.1 M NaOH in a test tube, and the absorbance was measured at 415 nm.

Determination of protein concentration

Protein was determined by the method of LOWRY *et al.*[19], using bovine serum albumin as the standard, or by measuring absorbance at 280 nm.

Reagents

Hydroxylapatite was prepared by the method of TISELIUS *et al.*[20]. T-7 phage was a generous gift from Dr. T. Nishioka, Tokyo Metropolitan University. Other chemicals were obtained from commercial sources. Distilled water, subsequently deionized to remove traces of metal ions, was used throughout the experiment.

RESULTS

Purification of flagellar ATPase

The crude extract (about 100 ml) obtained as described above was concentrated with a Diaflo membrane filter (PM-10) to 8 ml and dialyzed against Tris–EDTA solution for 18 h, and the precipitate was discarded after centrifugation.

Column chromatography on Sepharose 4B. The supernatant solution was applied to a Sepharose 4B column (2.8 cm × 105 cm) which had been equilibrated with 10 mM

Tris–HCl buffer (pH 7.5) containing 0.1 mM EDTA and 0.1 mM dithiothreitol. The column was eluted with the same solution at a flow rate of 15 ml/h with a fraction size of 5 ml. An elution profile is shown in Fig. 1. Two peaks (minor and major ones) of ATPase activity were detected. The third protein peak, which lacked enzyme activity, seemed to originate mainly from central pairs of microtubules and also from outer doublet microtubules to some extent. The fractions (Nos. 41–57) constituting the major peak of ATPase activity were combined and the medium of the

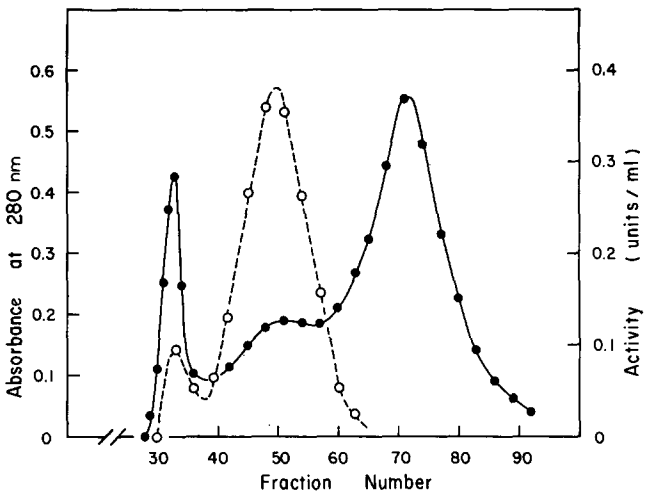

Fig. 1. Column chromatography of the crude extract of flagellar ATPase on Sepharose 4B. Conditions are described in the text. Fraction 34 appears to be void volume determined as the elution volume of T-7 phage. ●—●, $A_{280\ nm}$; ○---○, ATPase activity.

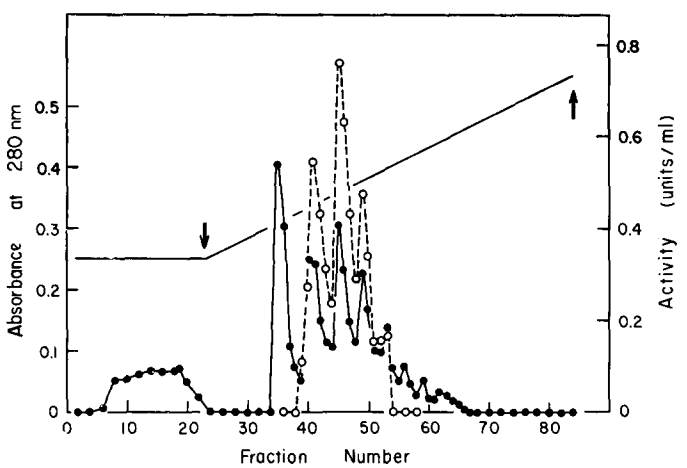

Fig. 2. Column chromatography of the partially purified flagellar ATPase on hydroxylapatite. 75 ml of the active fractions 42–57 eluted from the Sepharose 4B column, in 0.1 M potassium phosphate buffer (pH 7.5) containing 0.05% β-mercaptoethanol, was applied to a hydroxylapatite column, and the column was washed with 50 ml of the same buffer. The other conditions are described in the text. Arrows indicate concentrations of potassium phosphate buffer: ↓, 0.1; ↑, 0.7 M. ●—●, $A_{280\ nm}$; ○---○, ATPase activity.

combined fractions was exchanged by dialysis with 0.1 M potassium phosphate buffer (pH 7.5) containing 0.05 % β-mercaptoethanol.

Column chromatography on hydroxylapatite. The resulting solution was applied to a hydroxylapatite column (1.6 cm × 15 cm), previously equilibrated with the buffer solution used for dialysis. The column was first washed with 50 ml of the buffer, and the active protein was eluted by a linear gradient applied from a mixing bottle containing 150 ml of 0.1 M potassium phosphate buffer (pH 7.5) in 0.05 % β-mercaptoethanol, with a reservoir containing 150 ml of 0.7 M potassium phosphate buffer (pH 7.5) in 0.05 % β-mercaptoethanol. The flow rate was maintained at 20 ml/h and fractions of 5 ml were collected. The potassium phosphate buffer of the fractions was exchanged by means of gel filtration with 10 mM Tris–HCl buffer (pH 8.3) containing 0.05% β-mercaptoethanol. Namely, for enzyme assay and protein determination, 0.3 ml of each fraction was applied to a Sephadex G-25 column (1.5 cm × 6.5 cm) equilibrated with the same buffer and 61–90 drops were collected. An elution pattern of this material is shown in Fig. 2. Multiple peaks of ATPase activity were always observed. Fractions 40–44, 45–48, and 49–50 in Fig. 2 were then pooled separately.

The purification processes and results of a typical preparation are summarized in Table I. The purified enzyme showed a maximum specific activity of 3.55 μmoles P_i/min per mg protein.

TABLE I

SUMMARY OF PURIFICATION OF FLAGELLAR ATPase

Protein concentrations of hydroxylapatite fraction were calculated based on the fact that $A_{280\ nm}$ = 1 corresponded to 0.73 mg protein.

Step	*Total protein (mg)*	*Total activity (units)*	*Specific activity (units/mg)*	*Yield (%)*
Crude extract	166.0	89.6	0.54	100
Sepharose 4B	44.0	41.0	0.92	46
Hydroxylapatite	7.8	24.3	3.12	27
Fraction 40–44	3.1	9.1	2.94	
Fraction 45–48	2.9	10.3	3.55	
Fraction 49–51	1.8	4.9	2.72	

Two peaks of ATPase activity on Sepharose 4B column

The crude extract of flagellar ATPase prepared from another species of sea urchin, *Hemicentrotus pulcherrimus*, was applied to a Sepharose 4B column. ATPase activity was separated into two peaks in this case also (Fig. 3a). When major fractions (Nos. 25–36) were combined, concentrated by ultrafiltration, precipitated with $(NH_4)_2SO_4$ at 60 % saturation and then re-chromatographed on the same column, the ATPase activity appeared at the same position of minor fractions (Fig. 3b). On the other hand, when major fractions, concentrated by ultrafiltration, was directly re-chromatographed on the same column, the ATPase activity appeared in this case at the same position of major fractions (Fig. 3c). From these results it appeared that the flagellar ATPase in major fractions represents a non-aggregated type and changes to an aggregated type (minor fractions) in the presence of high concentration of

$(NH_4)_2SO_4$. Moreover, enzymatic properties of major and minor fractions appearing on Sepharose 4B column (Figs. 1 and 3a), and the aggregated type of ATPase (Fig. 3b) were essentially the same: K_m for ATP was in the order of $3 \cdot 10^{-5}$ M, and the enzyme activity was far more activated by Mg^{2+} than Ca^{2+}. Therefore, the minor fraction appearing on Sepharose 4B column appears to be an aggregated type corresponding to the major fractions which would presumably be a non-aggregated type.

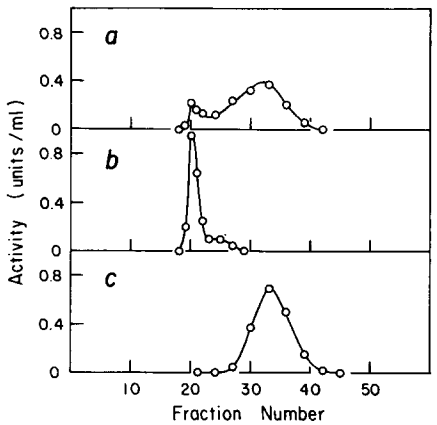

Fig. 3. Column chromatography of the crude extract of flagellar ATPase on Sepharose 4B and re-chromatography of separated fractions. a. The crude extract (about 100 ml) prepared from spermatozoa of *Hemicentrotus pulcherrimus* was concentrated to about 8 ml, dialyzed and applied to a Sepharose 4B column (1.6 cm × 100 cm). 3-ml fractions were collected at a flow rate of 15 ml/h. Fraction 20 appears to be void volume. Other detailed conditions were the same as in Fig. 1. b. Major fractions (Nos. 25–36) in Fig. 3a were concentrated, precipitated with $(NH_4)_2SO_4$ and re-chromatographed on the same column. c. Concentrated major fractions were directly re-chromatographed on the same column without treatment of $(NH_4)_2SO_4$.

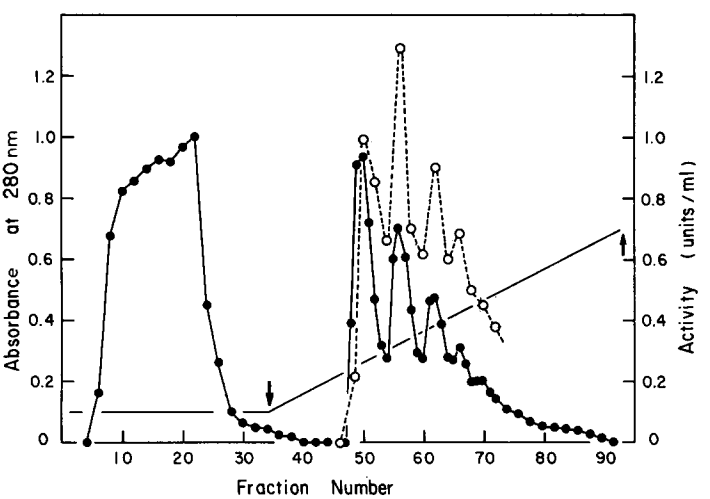

Fig. 4. Column chromatography of the crude extract of flagellar ATPase on hydroxylapatite. 100 ml of the crude extract in 0.1 M potassium phosphate buffer (pH 7.5) containing 0.05% β-mercaptoethanol was applied to a column (1.6 cm × 15 cm), and the column was washed with 100 ml of the same buffer. The other conditions are described in the text. Arrows indicate concentrations of potassium phosphate buffer; ↓, 0.1; ↑, 0.7 M. ●—●, $A_{280\ nm}$; ○---○, ATPase activity.

Multiple peaks of ATPase activity appearing on hydroxylapatite column

The next experiments were performed to investigate whether the appearance of several peaks of ATPase activity on the hydroxylapatite column was due to the presence of more than one form of the ATPase or to some other reason(s).

The crude extract of the ATPase was directly dialyzed against 0.1 M potassium phosphate buffer (pH 7.5) containing 0.05 % β-mercaptoethanol. The dialyzed solution was applied to a 1.6 cm × 15 cm column of hydroxylapatite, equilibrated with the above buffer. The column was washed with 100 ml of the same buffer. A linear gradient elution was performed under the conditions described above. The multiple peaks of ATPase activity were also detected in this experiment (Fig. 4). Fractions 48–53 (HA-1), 54–59 (HA-2), and 60–64 (HA-3) were pooled separately.

The HA-1 fraction was then dialyzed for 18 h against 0.1 M potassium phosphate buffer (pH 7.5) containing 0.05 % β-mercaptoethanol, and the content was applied to a 1.6 cm × 15 cm column of hydroxylapatite equilibrated with the same buffer. The column was washed with 60 ml of the same buffer, and re-chromatography was performed under the same conditions. Multiple peaks appeared in this case also (Fig. 5). Fractions 37–42, 43–46, and 47–50 appeared to correspond respectively to the HA-1, HA-2, and HA-3 fractions obtained in the first run.

These results appear to negate the possibility that the observed peaks were due to the presence of several different forms of the ATPase.

Properties of flagellar ATPase

In the subsequent works, HA-1, HA-2, and HA-3 fractions were used instead of the highly purified enzyme. These fractions had a specific activity of 2–2.5 μmoles P_i/min per mg protein and were free from the third protein peak on Sepharose 4B column. Moreover, any difference in enzymatic properties was scarcely observed among these fractions and the highly purified preparation.

Incubation time. To determine the proper incubation time, the reactions of

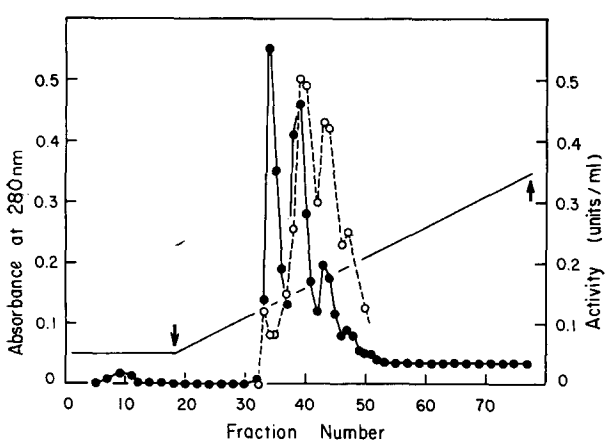

Fig. 5. Re-chromatography of the purified flagellar ATPase (HA-1) on hydroxylapatite. 30 ml of fractions 48–53 in Fig. 4 in 0.1 M potassium phosphate buffer (pH 7.5) containing 0.05% β-mercaptoethanol was applied to a column (1.6 cm × 15 cm), and the column was washed with 60 ml of the same buffer. The other conditions are described in the text. Arrows indicate concentrations of potassium phosphate buffer: ↓, 0.1; ↑, 0.7 M. ●—●, $A_{280\,nm}$; ○---○, ATPase activity.

mixtures containing different concentrations of enzyme solution were stopped at different intervals and the amounts of inorganic phosphate liberated were plotted against incubation time, as shown in Fig. 6a. The amounts of inorganic phosphate liberated in incubation time 5 or 10 min were found to be proportional to the amounts of enzyme added (Fig. 6b). Accordingly, the incubation time was set at 10 min.

pH optimum. Fig. 7 shows the dependence of ATPase activity on pH. The activity showed a broad plateau between about pH 6.0 and 9.0, with small optima near pH 7.0 and 8.5.

Stability. In contrast to ciliary ATPase[6] whose activity was lost by 15% per day in Tris–EDTA–5 mM KCl at 0°, the activity of the purified flagellar ATPase was safely retained after standing for at least two weeks at 0–2° in the presence of 0.05% β-mercaptoethanol in 10 mM Tris–HCl buffer (pH 8.3). In the absence of β-mercaptoethanol, however, 60% of the activity was lost after one week under the same conditions.

Activators. The enzyme was activated by several divalent cations including Mg^{2+}, Mn^{2+}, Ni^{2+}, and Ca^{2+}, as shown in Table II. Under the conditions tested, no appreciable effects were observed with Fe^{2+}, Co^{2+} and Ba^{2+} at $2.5 \cdot 10^{-3}$M, while Cd^{2+} and Zn^{2+} inhibited the ATPase activity. In the presence of Mg^{2+}, the ATPase activity was slightly affected by various concentrations of KCl. A maximum of 20% activation over the level in the absence of K^+ was observed around 0.3 M.

As shown in Fig. 8, the optimal concentration for Mg^{2+} was 10 mM. On the other hand, it was 2.5 mM in the case of Mn^{2+}.

Effect of heavy metals and sulfhydryl reagents. As shown in Fig. 9, the enzyme activity was inhibited by metallic cations and sulfhydryl reagents in the presence of Mg^{2+}. The concentrations of the inhibitors required to give 50% inhibition were $5 \cdot 10^{-2}$, $2 \cdot 10^{-2}$, $2 \cdot 10^{-5}$, $3 \cdot 10^{-6}$, $2 \cdot 10^{-6}$, $2 \cdot 10^{-6}$ and $1.5 \cdot 10^{-6}$ M for iodoacetoamide, iodoacetic acid, Zn^{2+}, Cd^{2+}, *p*-chloromercuribenzoate (PCMB), *N*-ethylmaleimide and Hg^{2+}, respectively. In the case of *N*-ethylmaleimide, when tested at pH 7.5, it was $4 \cdot 10^{-5}$ M.

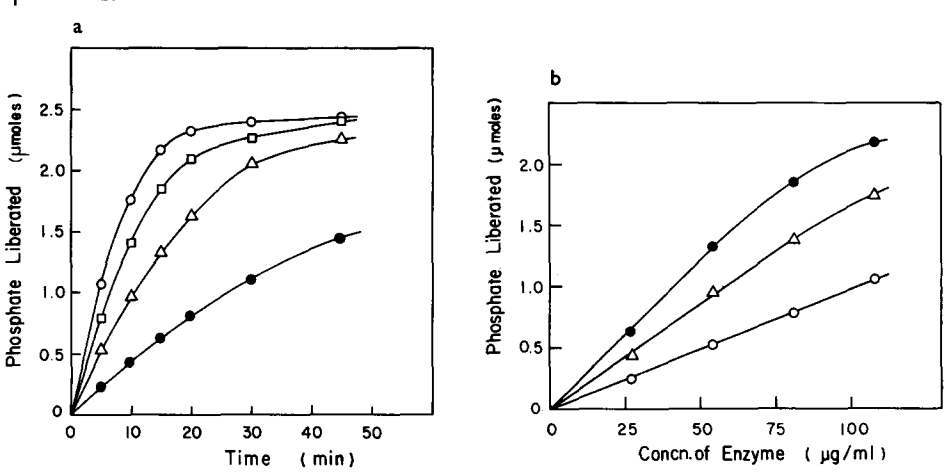

Fig. 6. a. Enzyme activity as a function of incubation time. The concentration of the enzyme solution (HA-2) was 27 (●), 54 (△), 81 (□) or 108 (○) μg protein per ml. b. Liberation of inorganic phosphate from ATP as a function of the amount of enzyme after three different incubation times (○, 5; △, 10; ●, 15 min). The values were calculated from the data shown in Fig. 6a.

In low concentrations, however, these inhibitors increased the activity of the ATPase to some extent (5–15%). For instance, a very rapid decrease in enzyme activity was observed when the PCMB concentration was raised from 10^{-6} to $5 \cdot 10^{-6}$ M, at which concentration the inhibition was complete, whereas a small activating effect of 10% was observed when the PCMB concentration was decreased from $2 \cdot 10^{-7}$ to 10^{-7} M (see Fig. 9). In this connection it must be noticed that activation of ATPase activity of myosin by PCMB is first described by KIELLEY AND BRADLEY[21].

TABLE II

EFFECT OF DIVALENT CATIONS ON FLAGELLAR ATPase ACTIVITY

The assay solution contained 125 mM Tris–HCl buffer (pH 8.3), 2.5 mM ATP and divalent cations (a, $2.5 \cdot 10^{-4}$; b, $2.5 \cdot 10^{-3}$ M) as indicated. The concentrations of the enzymes, HA-1 and HA-2 fractions were 11.2 and 32 μg protein per ml, respectively.

Divalent cations	*Concn. (M)*	*Activity (units/mg)*	
		HA-1	*HA-2*
None		0.21	0.23
Mg^{2+}	a	0.75	1.01
	b	1.15	1.70
Mn^{2+}	a	0.98	1.17
	b	1.10	1.40
Ni^{2+}	a	0.21	0.23
	b	0.43	0.45
Ca^{2+}	a	0.23	0.23
	b	0.32	0.29
Cd^{2+}	a	0.16	0.10
	b	0.16	0.04
Zn^{2+}	a	0.22	0.18
	b	0.15	0.05

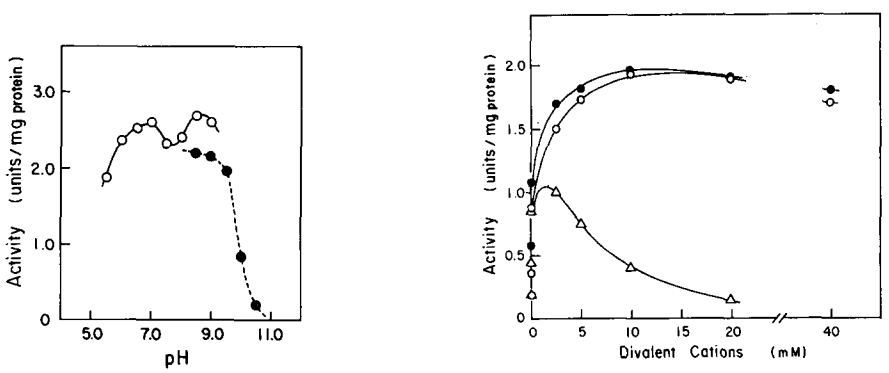

Fig. 7. Enzyme activity as a function of pH. The assay solution contained 2.5 mM ATP, 20 mM $MgCl_2$ and 65 mM buffer (○—○, Tris–maleate; ●---●, glycine–NaOH). The concentration of the enzyme (HA-2) was 4.6 μg protein per ml.

Fig. 8. Enzyme activity as a function of concentration of activators. The assay solution contained 125 mM Tris–HCl buffer (pH 8.3), 2.5 mM ATP and different concentrations of $MgCl_2$ (○), $MgSO_4$ (●), or $MnSO_4$ (△). The concentration of the enzyme (HA-2) was 27 μg protein per ml.

Substrate specificity. The flagellar ATPase showed a high substrate specificity for ATP (Table III). Under the conditions tested, the ATPase hydrolyzed ADP, GTP, and CTP at only about 5–8 % of the rate of ATP. Inorganic pyrophosphate, *p*-nitrophenylphosphate, and UTP were not hydrolyzed. These results suggest that the purified enzyme was contaminated with neither inorganic pyrophosphatase nor alkaline phosphatase. The activity of adenylate kinase was negligible in the purified preparation.

K_m for substrate. The K_m values for ATP, calculated from the Lineweaver–Burk plot (Fig. 10), were found to be $5 \cdot 10^{-5}$ M in both the HA-1 and HA-2 fractions.

Recombination of solubilized ATPase with EDTA-treated flagella

The solubilized flagellar ATPase, either the crude or the purified preparation,

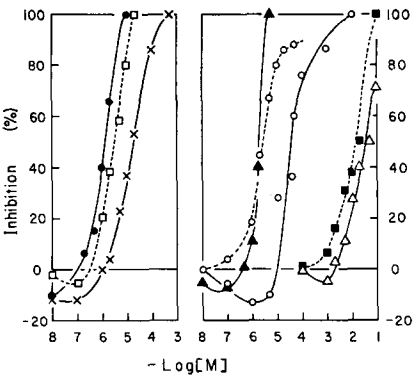

Fig. 9. Effect of heavy metals and sulfhydryl reagents on ATPase activity. The assay solution contained 125 mM Tris–HCl buffer (pH 8.3), 20 mM $MgCl_2$, 2.5 mM ATP and various concentrations of metallic cations or sulfhydryl reagents as shown. β-Mercaptoethanol contained in the enzyme solution was removed by dialysis. In the case of iodoacetic acid, iodoacetoamide and *N*-ethylmaleimide, the activity was assayed after preincubation for 20 min at room temperature. The sulfhydryl reagents were all freshly prepared. The concentration of the enzyme (HA-2) was 27 μg protein per ml. △—△, iodoacetoamide; ■---■, iodoacetic acid; ○—○, *N*-ethylmaleimide at pH 7.5; ○---○, *N*-ethylmaleimide at pH 8.3; ▲—▲, PCMB; ×—×, $ZnCl_2$; □---□, $CdSO_4$; ●—●, $HgSO_4$.

TABLE III

LIBERATION OF INORGANIC PHOSPHATE FROM VARIOUS SUBSTRATES BY FLAGELLAR ATPase AS A FUNCTION OF INCUBATION TIME

The assay solution contained 250 μmoles Tris–HCl buffer (pH 8.3), 40 μmoles $MgCl_2$ and 5 μmoles various substrates as shown, in a final volume of 2 ml. The concentration of the enzyme (HA-2) was 40 μg protein per ml.

Substrate	P_i (*μmole*) *Incubation time (min)*		
	5	*10*	*15*
ATP	0.205	0.405	0.590
ADP	0.015	0.035	0.035
GTP	0.015	0.025	0.035
CTP	0.015	0.025	0.045
UTP	0.000	0.000	0.000

when mixed with EDTA-treated flagella, was again incorporated into the flagellar fraction in the presence of divalent cations.

The EDTA-treated flagella, suspended in 10 mM Tris–HCl buffer (pH 8.3) containing 0.1 % β-mercaptoethanol, were homogenized with a Teflon homogenizer. Samples A, B, and C, containing 2 ml of the ATPase fraction (crude extract), 2 ml of the EDTA-treated flagellar fraction, and 2 ml of ATPase *plus* 2 ml of the EDTA-

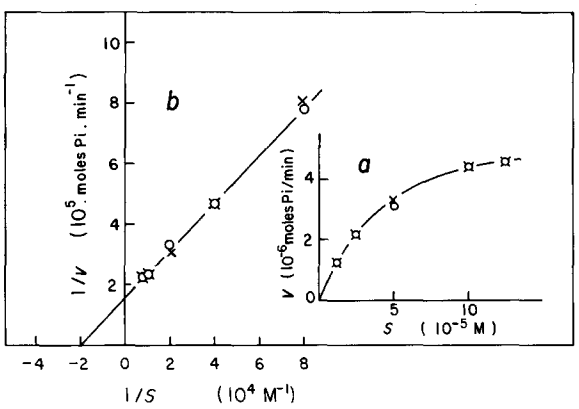

Fig. 10. Enzyme activity as a function of ATP concentration. The assay solution contained 125 mM Tris–HCl buffer (pH 8.3), 20 mM $MgCl_2$ and different concentrations of ATP. The concentrations of the enzymes, HA-1 (×) and HA-2 (○) fractions, were 10 and 7 μg protein per ml, respectively. b. Double reciprocal plot of the data presented in Fig. 10a.

TABLE IV

RECOMBINATION BETWEEN SOLUBILIZED ATPase (CRUDE EXTRACT) AND EDTA-TREATED FLAGELLA

The amounts of ATPase and EDTA-treated flagella were 2 and 4 mg protein per ml, respectively.

Dialyzing solution	*Sample*	*ATPase activity (total units)*		*Recombination** (%)
		In supernatant	*In precipitate*	
Tris–Ca	A	2.06	0.27	
	B	0.00	0.00	
	C	0.55	1.75	73
	C − (A + B)	−1.51	+1.48	
Tris–Mg	A	2.84	0.17	
	B	0.00	0.00	
	C	1.92	1.24	32
	C − (A + B)	−0.92	+1.07	
Tris–Ca, Mg	A	2.46	0.14	
	B	0.00	0.00	
	C	1.18	1.60	52
	C − (A + B)	−1.28	+1.46	
Tris–EDTA	A	2.97	0.00	
	B	0.14	0.00	
	C	3.37	0.00	−9
	C − (A + B)	+0.26	0.00	

* Recombination percent = $[(A + B) - C]/A \times 100$, where A, B and C represent the ATPase activities found in the supernatants obtained from samples A, B and C, respectively.

TABLE V

RECOMBINATION BETWEEN PURIFIED ATPase (HA-3) AND EDTA-TREATED FLAGELLA

The concentrations of ATPase and EDTA-treated flagella were 0.45 and 3.8 mg protein per ml, respectively.

Dialyzing solution	*Sample*	*ATPase activity (total units)*		*Recombination (%)*
		In supernatant	*In precipitate*	
Tris–Ca	A	0.46	0.10	
	B	0.00	0.32	100
	C	0.00	0.96	
	C−(A+B)	−0.46	+0.54	
Tris–Mg	A	0.63	0.05	
	B	0.01	0.32	57
	C	0.28	0.79	
	C−(A+B)	−0.36	+0.42	
Tris–EDTA	A	0.84	0.00	
	B	0.16	0.18	−11
	C	1.05	0.20	
	C−(A+B)	+0.05	+0.02	

treated flagellar fractions, respectively, were then prepared. Each sample was dialyzed for 2 days against 1 l of 10 mM Tris–HCl buffer (pH 8.3) containing 0.1 % β-mercaptoethanol, and 10 mM $CaCl_2$, 10 mM $MgCl_2$, 5 mM $CaCl_2$ *plus* 5 mM $MgCl_2$ or 1 mM EDTA. After dialysis each sample was centrifuged at 70000 × *g* for 60 min. The precipitate was suspended in each buffer solution for dialysis. The supernatant and precipitate thus obtained were assayed for ATPase activity. Table IV shows the results of a recombination experiment between the solubilized ATPase and EDTA-treated flagella. The activity lost from the supernatant was recovered quantitatively from the precipitate. The recombination percentages in the case of adding Mg^{2+}, Ca^{2+}, and Mg^{2+} *plus* Ca^{2+} as divalent cations were 32, 73, and 52 %, respectively. On the other hand, recombination was not seen when EDTA was added to the dialyzing solution.

Table V shows the results of recombination experiments between the purified ATPase and EDTA-treated flagella. Samples A, B, and C contained 2 ml of the purified ATPase (HA-3) fraction, 2 ml of EDTA-treated flagellar fraction, and 2 ml of ATPase *plus* 2 ml of the EDTA-treated flagellar fractions, respectively. The dialysis was performed for 18 h. The recombination percentages in the case of adding Mg^{2+} and Ca^{2+}, were 57 and 100 % respectively, and there was again no recombination in the presence of EDTA.

Similar results were obtained when the EDTA-treated flagella, which seemed to consist mainly of outer doublet microtubules, were replaced by a more purified outer doublet fraction prepared by treating EDTA-treated flagella with 1 % Triton X-100 to remove the remaining flagellar membranes.

DISCUSSION

GIBBONS[6] reported that the purified ATPase from Tetrahymena cilia is still contaminated with other proteins. The sucrose-density gradient centrifugation method

adopted by him to Tetrahymena cilia appeared to be unadequate for purification of the flagellar ATPase from sea urchin spermatozoa because of a rather large volume of the crude extract. It might be desired, therefore, to develop other improved devices. Several trials including $(NH_4)_2SO_4$ fractionation, column chromatography on DEAE-, ECTEOLA- or CM-cellulose gave no satisfactory results. As described in the present paper, with the use of Sepharose 4B and hydroxylapatite column systems, we could obtain the purified ATPase having a specific activity of 3.55 μmoles P_i/min per mg protein starting from a large volume of the crude extract. The method was highly reproducible and the best preparation had a higher specific activity than ciliary ATPase (2.70 μmoles P_i/min per mg protein)[6]. Furthermore, the purified enzyme was stable in the presence of β-mercaptoethanol.

A characteristic feature of flagellar ATPase revealed by the present study is that the enzyme resolves into multiple activity peaks on hydroxylapatite column chromatography (see Figs. 2 and 4). Similar phenomenon was also observed when tropocollagen was applied to the hydroxylapatite column, and was interpreted as due to microheterogeneity of tropocollagen molecules[22]. The present results, that re-chromatography of an ATPase fraction (HA-1), once obtained as a single peak by hydroxylapatite column, again resulted in a resolution of the activity into several peaks (Fig. 4), might not be explained in the terms of microheterogeneity of ATPase molecules. The results rather suggest that the multiple peaks are derived from a single form of the enzyme. It is not certain at present moment whether the difference in the extent of aggregation of ATPase is reflected in the occurrence of these multiple peaks on the column.

The properties of the purified ATPase resemble those so far reported by several workers[2–14] in either flagella or cilia, and might not be affected through processes for purifying ATPase. For instance, we can find similar pH activity curves between ciliary ATPase[6,10] and flagellar ATPase (see Fig. 6). A similar situation is found concerning the effect of divalent cations on ATPase activity, but the ratio of Mg^{2+}-activation to Ca^{2+}-activation in flagellar ATPase is higher than that in ciliary ATPase.

However, present experiments also indicate that there are some differences between flagellar and ciliary ATPase. In sea urchin, when the Mg^{2+}-ATPase activity was measured with varying amounts of KCl, a maximum of 20% activation over the level in the absence of K^+, was observed around 0.3 M (*cf.* STEPHENS AND LEVINE[10]). On the other hand, in Tetrahymena when the ATPase activity of 14 S dynein was assayed at low KCl concentration, the specific activity of 14 S dynein was 2–3 times that of 30 S dynein. Higher KCl concentrations increased the activity of 14 S dynein and decreased the activity of 30 S dynein, with a cross-over point around 0.15 M KCl.

It was suggested that minor and major peaks of ATPase activity appearing on the Sepharose 4B column (see Fig. 1) represent the aggregated and non-aggregated types of ATPase. We also showed there are no difference of enzymatic properties between aggregated and non-aggregated types of ATPase, while GIBBONS[6] reported that the degree of polymerization affects the properties of ciliary ATPase.

Furthermore, according to GIBBONS[2,4], another difference between 30 S and 14 S dynein is that only the former can recombine with the outer doublet microtubules from which the ATPase is released. The present results, however, revealed that even the purified preparation of the flagellar ATPase can recombine in high percentages with the microtubules if dialyzed against a solution containing Ca^{2+} or

Mg^{2+}, the former being more effective (Tables IV and V). When the purified preparation (HA-3) was rechromatographed on Sepharose 4B column, most activity appeared on the same position of the major fractions in Fig. 1. (K. Ogawa and H. Mohri, unpublished observation.)

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