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STUDIES ON FLAGELLAR ATPase FROM SEA URCHIN SPERMATOZOA

II. EFFECT OF TRYPSIN DIGESTION ON THE ENZYME

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SUMMARY

The flagellar ATPase (ATP phosphohydrolase, EC 3.6.1.3), dynein, from sea urchin spermatozoa was digested with trypsin (EC 3.4.4.4), leading to production of an enzymatically active protein. The protein was partially purified by column chromatography on Sepharose 4B and designated as Fragment A. Fragment A was still contaminated with a few minor proteins as shown by disc electrophoresis. Disc electrophoresis on polyacrylamide gel and molecular-sieve chromatography suggested that the molecular size of Fragment A is smaller than that of the parent ATPase although in sucrose density gradient centrifugation both enzymes have the same sedimentation coefficient.

In contrast to the parent flagellar ATPase, Fragment A could not recombine with the EDTA-treated flagella, and the ATPase activity of Fragment A was enhanced by the addition of a microtubule fraction.

These properties of Fragment A are discussed in connection with interaction of the intact flagellar ATPase (arms) with the outer doublet microtubules.

INTRODUCTION

In a recent paper[1], we reported a method for purifying the flagellar ATPase (ATP phosphohydrolase, EC 3.6.1.3) from the sea urchin spermatozoa by column chromatography on Sepharose 4B and hydroxylapatite, and it was also demonstrated that both crude and purified enzymes can recombine with EDTA-treated flagella or outer doublet microtubules in the presence of divalent cations. Moreover, some properties of the purified enzyme were found to be basically similar to those of dynein from *Tetrahymena* cilia described by Gibbons[2]. Therefore, it was suggested that the flagellar ATPase corresponds to the projections (arms)[3,4] attached to the A-tubulus of the outer doublet microtubules. In this connection it seemed desirable to clarify the possibility that the ATPase could be composed of two parts, one having ATPase activity and the other having recombination activity.

During the course of studies along these lines, a protein fragment designated

Fragment A, which possessed enzyme activity but not recombination activity, was obtained from the flagellar ATPase of the sea urchin spermatozoa by means of trypsin (EC 3.4.4.4) digestion. The present paper describes a method for preparing Fragment A and presents some properties of the protein.

MATERIALS AND METHODS

Materials

The sea urchin *Hemicentrotus pulcherrimus* was used throughout the work. Glycerinated sperm suspension was prepared as described previously[1].

Preparation of purified flagellar ATPase

Starting from glycerinated sperm suspension, flagellar ATPase was purified by column chromatography on Sepharose 4B and hydroxylapatite as described in the previous paper[1]. Since it had been demonstrated that the multiple peaks of ATPase activity on the hydroxylapatite column originated from a single enzyme, in the present work the active fractions on the column were combined and used as the purified flagellar ATPase. The enzyme thus prepared and partially purified ATPase are referred to as "intact ATPase" or "intact enzyme" in the present paper.

Preparation of EDTA-treated flagella, B-tubulin and A-tubule

EDTA-treated flagella and outer doublet microtubules were prepared by the methods given in the previous report[1]. B-tubulin and A-tubule were obtained by thermal fractionation of outer microtubules according to the method of Stephens[5].

Digestion of flagellar ATPase with trypsin

When crude flagellar ATPase was digested with trypsin, a protein fragment having ATPase activity was produced. This protein fragment was partially purified and designated "Fragment A". The detailed preparation procedures will be described in Results.

Disc electrophoresis on polyacrylamide gel

Disc electrophoresis on polyacrylamide gel was carried out at 4 °C after the method of Davis[6]. Polyacrylamide gel preparations, 7.5% at pH 8.9 and 2.5% at pH 6.7, were used for small-pore separation gel and large-pore spacer gel, respectively. A sample solution containing about 10–500 μg protein was applied to the spacer gel and then a small amount of Sephadex G-25 powder was added to the sample. An electric current was supplied at 1 mA per tube (gel column size: 5 mm × 70 mm), until bromphenol blue migrated to the end of the tube at the anode side. Protein on the gel was stained with 0.1% amido black in 7% acetic acid for about 30 min, followed by electrolytic destaining of the background. For the detection of ATPase, the gel was incubated with the reaction mixture for assay of ATPase (see below) at 30 °C for about 30 min. The excess reaction mixture was then removed and P_i liberated was detected by immersing the gel in the color-developing reagents for P_i as directed by Lohmann and Jendrassik[7]. Within 10–20 min at 37 °C a blue band appeared against an almost colorless background, indicating the position of the enzyme. After several hours, however, the entire background became blue.

Sucrose density gradient centrifugation

The linear sucrose gradient was prepared from a mixing chamber containing 2.4 ml of 20% sucrose (w/v) in 10 mM Tris–HCl buffer (pH 8.3), 0.2 mM EDTA and 0.1% β-mercaptoethanol, with an adjacent reservoir containing 2.6 ml of 5% sucrose in the same buffer mixture. A sample solution (0.1 ml), dissolved in the above buffer mixture, was layered on the top of the gradient. Then the gradient was centrifuged for 14 h at 35 000 rev./min in the RPS 40 rotor of a Hitachi 65 P ultracentrifuge. Then, the bottom of the tube was punctured, and 8-drop fractions were collected. The above procedures were carried out at 3 °C. Each fraction was analyzed with respect to protein concentration or ATPase activity. The sucrose concentration of each fraction was determined with an Abbe refractometer (Erma).

ATPase assay

The standard assay for ATPase activity of Fragment A was carried out by incubating at 30 °C for 5 min in a test tube containing 150 μmoles Tris–HCl buffer (pH 8.3), 3 μmoles $MgCl_2$, 2.5 μmoles ATP and the enzyme solution in a final volume of 1 ml. One unit of enzyme activity was defined as the amount causing liberation of 1 μmole P_i per min under the above conditions.

The assay mixture for the intact ATPase and analytical methods for the determination of P_i were the same as described previously[1].

Determination of protein concentration

Protein was determined by the method of Lowry *et al.*[8], using bovine serum albumin as the standard, or by measuring absorbance at 280 nm.

Reagents

Trypsin (2 times crystallized, salt-free, lyophilized) was purchased from Worthington. Catalase (EC 1.11.1.6) (from cow liver, 2 times crystallized) was a generous gift from Dr T. Ohoka of Tokyo Metropolitan University. Other chemicals were obtained from commercial sources.

RESULTS

Preparation of Fragment A

Starting from 400 ml of glycerinated sperm suspension, the crude flagellar ATPase was prepared as described previously[1]. The crude extract (about 87 ml) was concentrated to 8.8 ml with the use of a Diaflo membrane filter and dialyzed against 10 mM Tris–HCl buffer (pH 8.3) containing 0.2 mM EDTA and 0.1% β-mercaptoethanol for 18 h. The dialyzed solution was then mixed with 1 ml of trypsin solution (10 mg/ml of 1 mM HCl) and incubated at 20 °C for 3 h. In the reaction mixture the ratio of total protein to trypsin was 5:1. The tryptic digest was applied to a Sepharose 4B column (2.8 cm × 95 cm), equilibrated with 10 mM Tris–HCl buffer (pH 8.3) containing 0.2 mM EDTA and 0.1% β-mercaptoethanol. Protein was eluted with the same buffer mixture at a flow rate of 15 ml/h with a fraction size of 5 ml. As shown in Fig. 1, a single peak of ATPase activity appeared. The active fractions (No. 52–60) were pooled and subjected to further purification.

The above active fractions (46 ml) were concentrated to 4.5 ml by ultra-

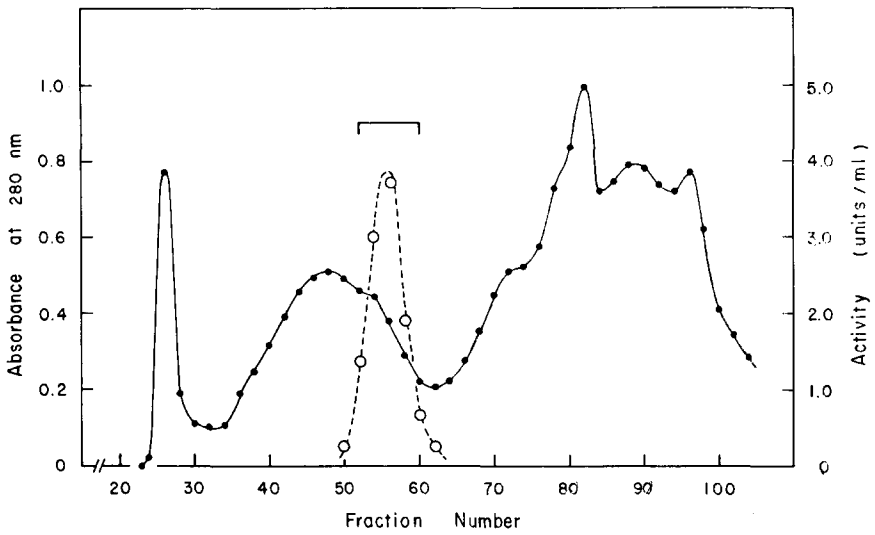

Fig. 1. Column chromatography of the first trypsin digest of the crude extract on Sepharose 4B Conditions are described in the text. Fraction 26 appears to be the void volume. ●—●, $A_{280\ nm}$ ○---○, ATPase activity.

filtration and 0.2 ml trypsin solution (10 mg/ml of 1 mM HCl) was added to the concentrate. In the mixture the ratio of total protein to trypsin was 10:1. After incubation at 20 °C for 1 h, the mixture was rechromatographed on a Sepharose 4B column under the same conditions as described above. The elution profile is presented in Fig. 2. The active fractions (No. 52–60) were combined and designated as Fragment A.

Typical data for the preparation of Fragment A are summarized in Table I.

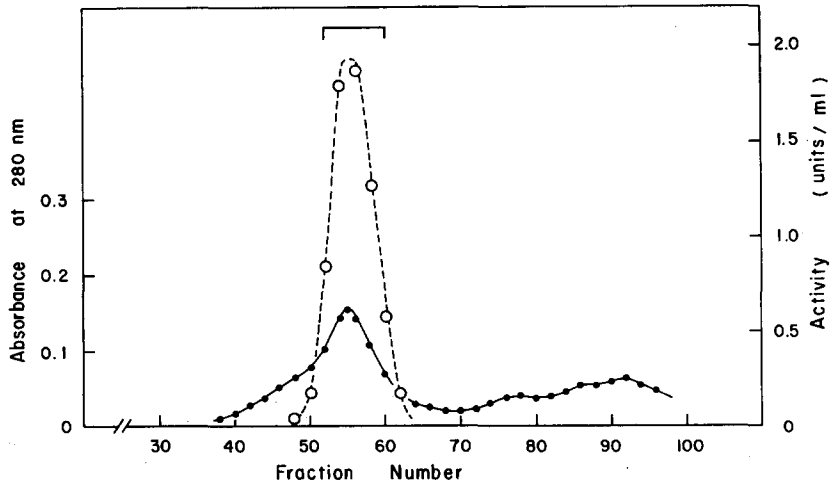

Fig. 2. Column chromatography of the second trypsin digest of the active effluent from the first Sepharose 4B column. Detailed conditions are the same as in Fig. 1. ●—●, $A_{280\ nm}$; ○---○, ATPase activity.

TABLE I

SUMMARY OF PREPARATION OF FRAGMENT A

Step	*Total volume (ml)*	*Total protein (mg)*	*Total enzyme activity (units)*	*Specific activity (units/mg)*	*Yield (%)*
Crude extract	87	345	176*	0.51	100
Digest of crude extract	9.8	570**	312	0.55	178
First Sepharose 4B	46	20.6	92	4.46	52
Second Sepharose 4B	47	7.1	54	7.55	31

* The assay system for Fragment A was used.

** This value was considerably higher than that expected from the value in the crude extract. Increment of total activity was also observed in this step. Reasonable explanations for these phenomena remained obscure.

Fragment A prepared by the above procedures showed a specific activity of 7.55 μmoles P_i per min per mg protein.

Changes of disc electrophoretic patterns of flagellar ATPase during preparation of Fragment A

In order to follow the digestion of the ATPase with trypsin, samples obtained from each step of the above preparation procedures were analyzed by disc electrophoresis on polyacrylamide gel. Results are presented in Figs 3 and 4. In Fig. 3, the right columns (a, b and c) and left columns (A, B and C) show bands obtained after staining for enzyme activity and proteins, respectively. As shown in Column a of Fig. 3, the activity band is located between the spacer gel and the top of the sepa-

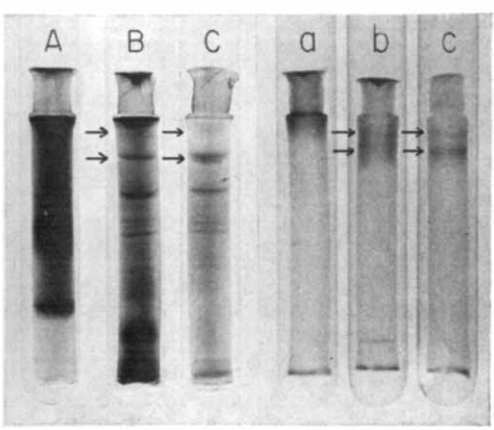

Fig. 3. Disc electrophoresis of intact and trypsin-treated flagellar ATPase on polyacrylamide gel. Right columns (a, b and c): protein bands revealed by the enzyme activity. Left columns (A, B and C): protein bands stained with amido black. Arrows in right figures indicate positions of enzymatically revealed protein bands. Arrows in left figures indicate positions of corresponding protein bands. In the right columns, the end of the gel was stained blue, when the gel was immersed into the color-developing reagents for P_i. This color reaction is not due to the enzyme activity, because it is irrespective of preincubation with ATP. A and a: crude extract (417 μg protein). B and b: first trypsin digest of the crude extract (490 μg). C and c: the active effluent from the first column chromatography on Sepharose 4B (109 μg).

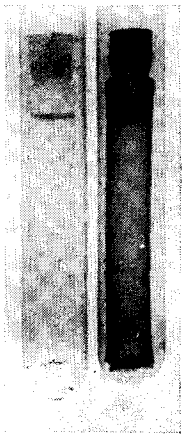

Fig. 4. Disc electrophoresis of Fragment A on polyacrylamide gel. About 15 μg protein per column was applied. Right column: protein band revealed by the enzyme activity. Left column: protein bands stained with amido black.

ration gel. It thus appears that the intact enzyme cannot penetrate into the separation gel composed of 7.5% polyacrylamide. In the case of the crude enzyme digested with trypsin, however, two bands of ATPase activity appeared in the separation gel (Fig. 3, Column b). The positions of the activity bands corresponded exactly to those of the protein bands (Column B). Columns C and c in Fig. 3 represent the electrophoretic pattern of the sample partially purified through the first column chromatography on Sepharose 4B. The profile, especially the appearance of two activity bands, was essentially the same as that found in the case of the digested crude enzyme.

Fig. 4 shows the electrophoretic patterns of Fragment A. When the gel was stained with amido black after electrophoresis, one major and a few minor protein bands appeared on the column. However, a single activity band, which corresponded to the major protein band, was obtained. Since the major band in Fig. 4 corresponds to the faster activity band on the Column c in Fig. 3, the slower activity band on the same column seems to be an intermediate formed from the crude enzyme during trypsin digestion.

Comparison of elution patterns from Sepharose 4B column between the intact enzymes and Fragment A

Curves a, b and c in Fig. 5 represent the elution profiles of the crude ATPase, partially purified ATPase and Fragment A, respectively. The first sample showed two activity peaks as already reported[1], with the main peak at Fraction 44. The partially purified enzyme had a single peak at Fraction 44. In contrast to these intact enzymes, Fragment A showed a single peak at Fraction 55. Catalase, used for comparison, was eluted with peak Fraction 80 under the same conditions (Curve d).

In Sephadex column chromatography it is well known that the elution position of the protein is correlated with its Stokes radius[9]. Recently, Gerlich[10] showed that this correlation also holds for Sepharose column chromatography. From the above results it seems very likely that the Stokes radius of flagellar ATPase is decreased by treatment with trypsin.

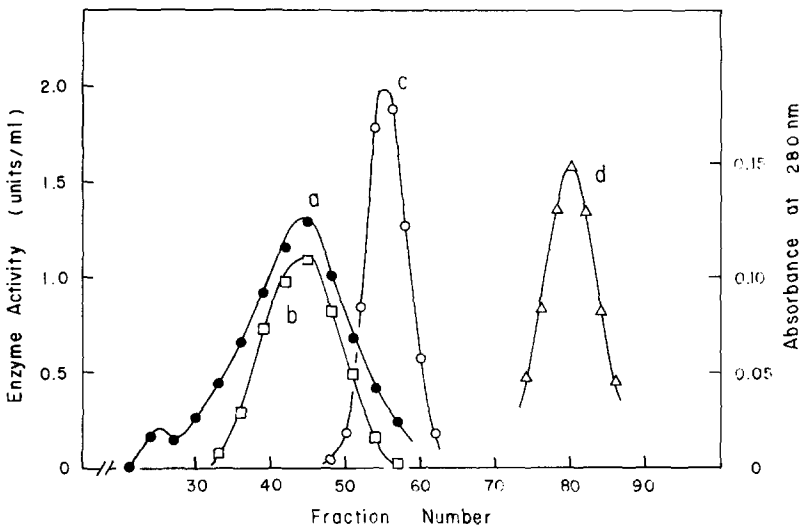

Fig. 5. Comparison of elution patterns from Sepharose 4B column between the intact enzymes and Fragment A. Curves a, b and c (expressed as ATPase activity) show elution patterns of the crude enzyme, partially purified enzyme and Fragment A, respectively. Curve d (expressed as protein concentration) indicates elution profile of catalase for comparison. The crude enzyme was prepared from glycerinated sperm suspension. The partially purified enzyme corresponded to the active effluent (No. 32–53) from the first Sepharose 4B column chromatography of the crude enzyme. In both cases, each 15 ml of the concentrated sample was applied to the column. The chromatographic conditions were the same as in Fig. 2. The elution pattern of Fragment A was cited from Fig. 2.

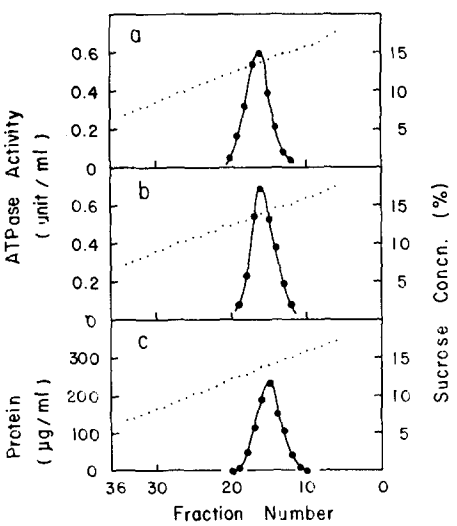

Fig. 6. Sucrose density gradient centrifugation of flagellar ATPase. Sucrose gradients were placed in each of three buckets of the RPS 40 rotor. Purified intact enzyme (170 μg protein in 100 μl) was layered on Gradient 1, Fragment A (160 μg protein in 100 μl) on Gradient 2 and catalase (100 μg protein in 100 μl) on Gradient 3, respectively. After the rotor was run at 3 °C for 14 h at 35 000 rev./min, the gradient was fractionated and analyzed for ATPase activity (intact enzyme and Fragment A) or protein concentration (catalase) as described in Materials and Methods. Dotted lines in the figures indicate the sucrose concentration. a, purified intact enzyme; b, Fragment A; c, catalase.

Sucrose density gradient centrifugation

Fig. 6 shows patterns obtained after centrifugation of purified intact ATPase (a), Fragment A (b) and catalase (c) through a sucrose density gradient. The distance travelled from the meniscus by Fragment A was equal to that of the intact enzyme. According to the method of Martin and Ames[11], $s^{0,725}_{20,w}$ values of both ATPase preparations were calculated to be 10.8, using the value of 11.3 for catalase as a marker protein. In contrast with the results of the disc electrophoresis and molecular-sieve chromatography, experimental results obtained from the sucrose density gradient centrifugation method appear to indicate that the intact enzyme would not be attacked by trypsin. This discrepancy will be discussed in a later section.

Loss of recombination ability of Fragment A with EDTA-treated flagella

The recombination of Fragment A with the flagellar fraction was examined by a method similar to the one described in the previous paper[1]. Samples A, B, and C, containing 1 ml of Fragment A, 1 ml of EDTA-treated flagellar fraction, and 1 ml of Fragment A *plus* 1 ml of EDTA-treated flagellar fraction, respectively, were prepared. Each sample was dialyzed for 18 h against 500 ml of 10 mM Tris-HCl buffer (pH 8.3) containing 0.1% β-mercaptoethanol and 10 mM $CaCl_2$, 10 mM $MgCl_2$ or 1 mM EDTA. After dialysis, each sample was centrifuged at 70 000 $\times$ *g* for 60 min. The precipitates were suspended in the buffer solution used for dialysis and homogenized with a glass homogenizer. The supernatant and precipitate thus obtained were assayed for ATPase activity. The recombination ability was expressed as a recombination percentage which was defined as $(A + B) - C/A \cdot 100$[1], where *A*, *B*, and *C* represented the ATPase activities found in the supernatants obtained from Samples A, B, and C, respectively. As shown in Table II, the recombination percentages obtained after dialysis against Tris–Ca^{2+}, Tris–Mg^{2+} and Tris–EDTA solutions are −48, −66 and −40%, respectively. Recombination was not observed even

TABLE II

FAILURE OF RECOMBINATION BETWEEN FRAGMENT A AND EDTA-TREATED FLAGELLA

Fragment A (152 μg protein per ml) and EDTA-treated flagella (6.1 mg protein per ml) fractions had initial total activities of 1.14 units and 0.10 unit, respectively. The recombination percentage was calculated as $(A + B) - C/A \cdot 100$ (*A*, *B*, and *C* as supernatant values)[1].

Dialyzing solution	*Sample*	*ATPase activity (total units)*		*Recombination (%)*
		In supernatant	*In precipitate*	
Tris–Ca^{2+}	A	0.67	0.00	
	B	0.00	0.02	−48
	C	0.99	0.13	
	(A+B)−C	−0.32	−0.13	
Tris–Mg^{2+}	A	0.77	0.00	
	B	0.02	0.03	−66
	C	1.30	0.11	
	(A+B)−C	−0.51	−0.08	
Tris–EDTA	A	0.96	0.00	
	B	0.09	0.04	−40
	C	1.43	0.11	
	(A+B)−C	−0.38	−0.07	

when the concentration of Ca^{2+} or Mg^{2+} was lowered to 2.5 mM. On the other hand, the intact crude ATPase could recombine with the EDTA-treated flagella of the same batch, and the recombination percentages obtained after dialysis against Tris–Ca^{2+}, Tris–Mg^{2+} and Tris–EDTA solutions were 67, 38 and 0%, respectively. These values agreed well with those observed in our previous experiments (*cf.* Table IV in Ogawa and Mohri[1]). From these results it is concluded that Fragment A has no recombination activity.

As mentioned above, the recombination percentages are not 0% but have negative values in the case of Fragment A. This may be explained in terms of the following considerations. In the first place, the total activity of Fragment A used for the above experiments decreased from the initial value of 1.14 units (or 1.14 units/ml) to 0.67, 0.77 or 0.96 unit, as seen in supernatants of Sample A (Table II), presumably due to denaturation of Fragment A during dialysis. In the second place, theoretically, the total activity of the supernatant of Sample C must be 1.24 units (1.14 units of Fragment A *plus* 0.10 unit of EDTA-treated flagella). However, an apparent increment in the total activity of Sample C was observed in the case of Tris–Mg^{2+} (1.30 units) or Tris–EDTA (1.43 units) solution. As described below, the enzyme activity of Fragment A was enhanced by the addition of the supernatant of EDTA-treated flagella which had been subjected to prolonged dialysis against Tris–EDTA solution. Since the supernatant of Sample C contained the Fragment A *plus* the supernatant of Sample B (dialyzed EDTA-treated flagella), the increment of the activity could be ascribed to the activating effect of some material(s) derived from the EDTA-treated flagella. At any rate, since the recombination percentage is defined as $(A + B) - C/A \cdot 100$, the recombination percentages would become negative as a result of a decrease in the A value and an increment in the C value in the present experiments.

Activation of ATPase activity of Fragment A by microtubule fractions

As shown in Curve a of Fig. 7, the enzyme activity of Fragment A is enhanced

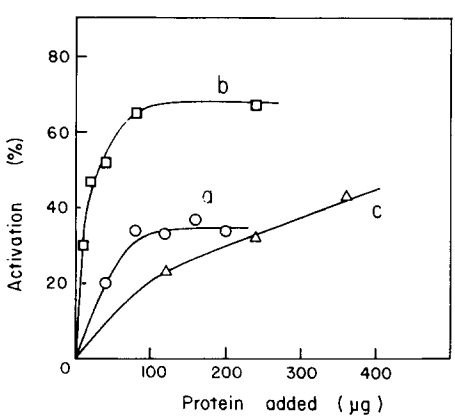

Fig. 7. Effect of microtubule fractions on the ATPase activity of Fragment A. Microtubule fractions of varying amounts of protein were added to the assay mixture. The protein amount of Fragment A was 15 μg. The supernatant of EDTA-treated flagella was prepared by centrifugation after dialysis against Tris–EDTA solution as described in the text. Curve a: supernatant of EDTA-treated flagella; b, B-tubulin fraction; c, A-tubule fraction.

by 35% in the presence of the supernatant of EDTA-treated flagella. It is known that microtubule fractions, especially B-tubulin, are gradually released from EDTA-treated flagella or outer doublet microtubules during prolonged dialysis or standing[5]. Hence, the activating effect of B-tubulin and A-tubule fractions on the enzyme activity of Fragment A was tested. As seen in Fig. 7 (Curves b and c), the enzyme is activated by both fractions. The B-tubulin fraction appears to have a stronger activating effect. Since these microtubule fractions have not yet been purified, it is uncertain at present whether the activation of the enzyme by the A-tubule fraction is caused by A-tubule itself or by B-tubulin contained in the A-tubule fraction. There is also another possibility that some unknown factor(s) might be involved in the activation. It is noteworthy that the ATPase activity of the intact enzyme, crude or purified one, cannot be increased by microtubule fractions.

DISCUSSION

In the previous paper[1], we reported that crude or purified flagellar ATPase (dynein) has a pronounced property of recombining with outer microtubules. In the present work, an ATPase preparation named "Fragment A", lacking recombination activity, was obtained from the crude flagellar ATPase by means of trypsin digestion. An enzyme having analogous properties can also be prepared from the purified ATPase by a similar method and formation of Fragment A from the intact enzyme is inhibited by soy bean inhibitor for trypsin (Ogawa, K., unpublished). These results provide support for the view that the parent flagellar ATPase might be composed at least of two parts, one having ATPase activity and the other having recombination activity, although the protein fraction, presumably responsible for the latter activity, has not yet been obtained.

Although experiments were conducted with aims different from those of the present work, two papers describing the effect of trypsin on flagellar ATPase have already been published. Mohri *et al.*[12] described that when "Fraction I" (dynein *plus* microtubules) from the sea urchin spermatozoa is analyzed by the sedimentation method before and after treatment with trypsin, the same sedimentation coefficients are obtained. Summers and Gibbons[13] also reported that axonemes isolated from the sperm of the sea urchin retained their original structure, when "briefly" digested with trypsin. Both groups of authors concluded that the flagellar ATPase (or arms) is rather resistant to trypsin digestion. However, the concentration of trypsin adopted by the above authors was much lower than that used in the present experiment.

In the present work, centrifugation through a sucrose density gradient showed that the sedimentation coefficients of the intact ATPase and Fragment A are the same and calculated to be 10.8 S. In contrast, the results obtained from disc electrophoresis (Figs 3 and 4) and molecular-sieve chromatography (Fig. 5) revealed that the molecular size of Fragment A would be smaller than that of the intact enzyme. Several lines of experiments might be required in order to elucidate these contradictory results. It is noticeable, however, that in the case of fibrous protein the sedimentation coefficient does not entirely depend on its molecular size. For example, it is well known that the sedimentation coefficient of myosin, a fibrous protein, is 6.4 S and smaller than that of heavy meromyosin (7.2 S), a fragment produced from myosin with trypsin digestion[14]. Concerning the shape of dynein, Gibbons and Rowe[15]

proposed from observation on electron micrographs that 14-S dynein from *Tetrahymena* cilia is a globular type. The molecular weight was determined to be 600 000 from ultracentrifugation data. Mohri *et al.*[12] also reported that the flagellar ATPase from the sea urchin spermatozoa is a globular protein with a sedimentation coefficient of 10–12 S and a molecular weight of 350 000–370 000 as calculated by measuring the target size for high-energy electrons. If the ATPase is a globular protein, however, each apparent molecular weight calculated from the sedimentation coefficient might be expected to be considerably smaller than the values described above. This consideration together with the fact (Fig. 5, Curve d) that catalase (11.3 S) is eluted from the Sepharose 4B column later than the ATPase (10.8 S) leads to a supposition that the flagellar ATPase would not be a simple globular protein.

It is believed that in cilia or flagella one end of the arms is attached to A-tubule and the other end is facing the adjacent B-tubule of outer microtubules[3,4,13,16], and the flagellar ATPase corresponds to the arms[3,12,13]. The present result may imply that the distal portion of the arms, facing the adjacent B-tubule, involves the active site required for the enzyme activity. Concerning the mechanism underlying the flagellar movement, Summers and Gibbons[13] discussed the possibility that the interaction between microtubules occurs through the arms. In this connection, it seems worthwhile to mention that the ATPase activity of Fragment A is increased by microtubule fractions, especially by B-tubulin fraction. However, a gap is usually observed in electron micrographs between the arms and the adjacent B-tubule[13,16]. Elucidation of interactions between flagellar ATPase and microtubules must await further study.

Besides activation of Fragment A by microtubule fraction, one more remarkable difference in enzyme activity between the intact enzyme and Fragment A is the susceptibility of the latter to the concentration of ATP or Mg^{2+}. At a constant concentration of Mg^{2+}, high concentration of ATP induces substrate inhibition, whereas high concentration of Mg^{2+} also inhibits the enzyme activity at a fixed concentration of ATP. These and related observations will be detailed elsewhere.

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