

Evidence that cAMP-dependent Protein Kinase and a Protein Factor Are Involved in Reactivation of Triton X-100 Models of Sea Urchin and Starfish Spermatozoa

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ABSTRACT A fraction obtained from detergent-extract of sea urchin or starfish spermatozoa using DEAE-cellulose chromatography reactivated Triton X-100 models of the spermatozoa in a cAMP-dependent manner. The DEAE fraction contained cAMP-dependent protein kinase with a high level of specific activity. Rabbit muscle inhibitor protein highly specific for cAMP-dependent protein kinases inhibited the ability of the DEAE fraction to induce reactivation of Triton X-100 models. This inhibition paralleled inhibition of cAMP-dependent protein kinase activity of the DEAE fraction, suggesting participation of the enzyme in the cAMP-dependent reactivation of Triton X-100 models. However, cAMP-dependent protein kinase further purified from the DEAE fraction was incapable of reactivating these models by itself. A protein factor which was separated from the protein kinase in the course of purification of the enzyme was found to also be necessary for the reactivation. When cAMP-dependent protein kinase was pretreated with protein kinase inhibitor before addition of the protein factor, the reactivation of Triton X-100 models was no longer detected. However, after the protein factor had been incubated with cAMP and cAMP-dependent protein kinase, protein kinase inhibitor did not repress reactivation of Triton X-100 models. We propose that the reactivation needs phosphorylation of the protein factor by cAMP-dependent protein kinase.

Triton X-100 models (TXM) of sea urchin spermatozoa developed by Gibbons and Gibbons (1) favor studies of the flagellar movement on a physiological as well as a biochemical basis. Treatment of TXM with anti-Fragment 1A serum (2) or extraction of the outer arms (3) causes a decrease in the beat frequency of models. The cyclic attachment and detachment of dynein arms to and from the adjacent B tubule have been considered the molecular basis of the flagellar movement producing beating forces. These analyses have been focused on the interactions of doublet microtubules with dynein arms.

On the other hand, cyclic nucleotide has frequently been proposed as a regulator of mammalian flagellar motility. Hamster cauda epididymal spermatozoa become progressively motile when the epididymal semen is diluted into a medium containing calcium ions. The initiation of a beat is accompanied by accumulation of cyclic AMP (cAMP) within the spermatozoa (4). Hoskins' work (5) indicates that an inhibitor of cyclic nucleotide phosphodiesterase or exogenous cAMP increases or maintains mammalian sperm motility as well as

respiration and fructolytic rates. In addition, spermatozoa from various sources contain high levels of activity of cAMP-dependent protein kinase (6-9), adenylate cyclase (4, 10-12), cyclic nucleotide phosphodiesterase (4, 11, 12), phosphoprotein phosphatase (13), and several kinds of proteins phosphorylated in a cAMP-dependent manner (14). Although there has been no observation that cAMP stimulates the movement of TXM prepared from sea urchin spermatozoa (1), cAMP does promote motility of mammalian sperm models free from plasma membranes and mitochondrial components (15, 16). These results may indicate that cAMP acts on the motile apparatus itself. However, there is a gap between the action of cAMP and the actual participation of cAMP-dependent protein kinase in the flagellar movement. Our interest has focused on the regulation of flagellar movement, and we found recently that a detergent-soluble fraction of sea urchin spermatozoa gives cAMP-sensitivity to TXM. We here show the evidence that reactivation of TXM needs cAMP-dependent protein kinase and a protein factor.

MATERIALS AND METHODS

Spermatozoa of sea urchins (*Anthocidaris crassispina*, *Pseudocentrotus depressus*, and *Hemicentrotus pulcherrimus*) and those of starfish (*Asterias amurensis*) were used as experimental materials. The sea urchin spermatozoa were collected by injecting 0.5 M KCl into the coelom of the animal, and were stored as dry sperm until use. The starfish spermatozoa were obtained according to the method described by Mabuchi et al. (17).

Preparation of Sperm TXM and Definition of the Index of Motility

Dry sperm were diluted with 20 vol of Ca-free sea water at 25°C to make a stock sperm suspension, which was used within 4 h. A 30- μ l portion of this suspension was added to 0.5 ml of an extraction medium (0.04% Triton X-100 and 1 mM DTT in 10 mM Tris-HCl, 2 mM MgSO₄, 150 mM KCl, 0.1 mM EDTA, pH 8.1 [TMKE]) at room temperature and gently swirled for ~30 s. A 30- μ l portion of the extracted sperm suspension was diluted with 3 ml of an ATP-containing medium (1 mM ATP and 1 mM DTT in TMKE), immediately followed by observation of TXM in 30 μ l of the above ATP-containing medium without a cover slip. Dark-field illumination was used for the measurement of motility, an index of which was determined as % motility, defined as % motility = (the number of motile TXM/total TXM) \times 100. When the effects of some fractions on the motility of TXM were studied, those dialyzed against TMKE containing 0.1 mM DTT were added to 2 vol of TXM suspension in the ATP-containing medium, and % motility was calculated. % Motility in the absence of cAMP ([-cAMP]) was a value at 5 min after each fraction was added, and % motility in the presence of cAMP ([+cAMP]) was that at 3 min after the final addition of cAMP, unless otherwise indicated.

Extraction of Soluble Materials from Spermatozoa and Preparation of DEAE-adsorbed Fraction

Unless otherwise mentioned, preparations were performed at 0–4°C. Spermatozoa were washed twice with Ca-free sea water. Packed wet spermatozoa were then homogenized with eight strokes in 2 vol of a solution containing 0.2% Nonidet P-40, 0.1 mM ATP, 0.1 mM DTT, 1 mM EGTA, and TMKE, followed by centrifugation at 12,000 g for 40 min. The supernate was stored at –80°C until use. The thawed supernate was dialyzed against two changes of a buffer solution (40 vol) consisting of 0.1 mM DTT and 20 mM Tris-HCl (pH 8.1), and then centrifuged at 100,000 g for 1 h to remove aggregates. The 100,000 g supernate called sperm extract was charged on a DEAE-cellulose column pre-equilibrated with the same buffer solution. After the column was thoroughly washed with the same solution to remove Nonidet P-40, the adsorbed materials were eluted with 0.5 M KCl containing 0.1 mM DTT and 20 mM Tris-HCl (pH 8.1) to obtain the DEAE-adsorbed fraction.

Protein Kinase Assay

Protein kinase activity was assayed by the method reported by Murofushi (18) using calf thymus whole histones as substrate. 1 U of the activity was defined as the amount of enzyme which catalyzes transfer of 1 pmol ³²P/min from ATP to histone in the standard assay system at 25°C.

Partial Purification of cAMP-dependent Protein Kinase

The sperm extract of *Hemicentrotus* (250 mg protein) was chromatographed on a DEAE-cellulose column (2.3 \times 41 cm) equilibrated with 20 mM Tris-HCl (pH 8.1) containing 0.1 mM DTT. By elution with a linear concentration gradient of KCl from 0 to 0.5 M, a single peak of the activity for cAMP-dependent protein kinase was detected at a position of 0.15 M. This fraction was dialyzed against 20 mM potassium phosphate buffer (pH 7.0) containing 0.1 mM DTT, and further chromatographed on a hydroxyapatite column (1.5 \times 26 cm) equilibrated with the same solution. Adsorbed materials were eluted with a linear gradient of phosphate buffer from 20 mM to 0.2 M. The enzyme was eluted with 120 mM phosphate buffer. For further purification, the fraction was condensed by ultrafiltration and with Aquacide, and subjected to gel filtration using an apparatus for high-speed liquid chromatography (HLC-803 system equipped with two G 3000 SW columns in series (each 7.5 \times 600 mm), Toyo Soda Manufacturing Co.). The column was pre-equilibrated with TMKE containing 0.1 mM DTT. The enzyme emerged just after the void volume fraction and was

used as a partially purified cAMP-dependent protein kinase. The purification steps are summarized in Table I.

Partial Purification of Protein Kinase Inhibitor

Protein kinase inhibitor was partially purified from rabbit skeletal muscle by the method of Walsh et al. (19), except that the final gel filtration step was omitted. 1 U of inhibitor activity was defined as the amount which inhibits 2 U of the protein kinase activity by half. The specific activity of the inhibitor finally obtained was 36,000 U/mg protein.

Others

The amount of protein was determined by the method of Lowry et al. (20) using bovine serum albumin as a standard. Hydroxyapatite was prepared following the method of Mains et al. (21). [γ -³²P]ATP was prepared according to the method described by Glynn and Chappell (22). DEAE-cellulose column chromatography was performed using DE-52 from Whatman. A Diaflo membrane PM-10 from Amicon Corp. (Scientific Sys. Div., Lexington, Mass.) was used for ultrafiltration. ATP and cAMP were obtained from Kyowa Hakko Co. (Tokyo). 5'-AMP and cGMP were from Kohjin Co. (Tokyo) and Boehringer Mannheim Biochemicals (Indianapolis, Ind.), respectively. Trypsin, soybean trypsin inhibitor and calf thymus whole histones were purchased from Sigma Chemical Co. (St. Louis, Mo.). Aquacide was from Calbiochem-Behring Corp. (American Hoescht Corp., La Jolla, Calif.).

RESULTS

Cyclic AMP-dependent Reactivation of TXM

TXM prepared by the present procedure from *Pseudocentrotus* spermatozoa gradually ceased to undulate (Fig. 1). Cyclic AMP alone did not show any effect on motility of models. TXM quickly stopped beating when the DEAE-adsorbed fraction of the sperm extract was added, but began to beat after

TABLE I
Purification of cAMP-dependent Protein Kinase from Sea Urchin Spermatozoa

Fraction	Total	Total	Specific	Yield	Purification (x-fold)
	protein	activity	activity		
	mg	U	U/mg	%	
Sperm extract	250	40,000	160	100	1
DEAE-cellulose fraction	13	28,000	2,200	70	14
Hydroxyapatite fraction	3.4	11,000	3,200	28	20
High-speed liquid chromatography fraction	0.31	2,900	9,400	7	59

Protein kinase activity was measured using the standard assay mixture.

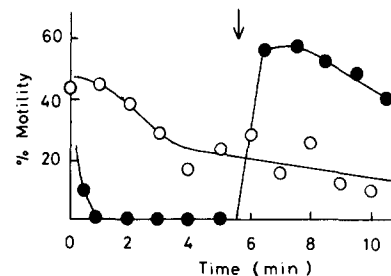


FIGURE 1 Time-course of changes in % motility of TXM in the presence or absence of DEAE-adsorbed fraction and motility reactivation by cAMP. O, control, without addition of DEAE-adsorbed fraction. ●, 20 μ g of DEAE-adsorbed fraction was contained in 30 μ l of TXM suspension. Cyclic AMP (10 μ M) was added at the point indicated by the arrow.

further addition of cAMP at a final concentration of 10 μM . When TXM were added to the mixture of the DEAE-adsorbed fraction and cAMP, a high level of % motility was maintained.

Cyclic AMP-induced reactivation was also observed in TXM of *Anthocidaris*, *Hemicentrotus*, and *Asterias* spermatozoa. The DEAE-adsorbed fraction from *Pseudocentrotus* or *Asterias* spermatozoa could cross-react with TXM of *Hemicentrotus* spermatozoa, suggesting that the factors involved in the DEAE-adsorbed fraction are not species-specific. The DEAE-adsorbed fraction used in the experiments described below was from *Pseudocentrotus* spermatozoa, and the TXM mainly from *Hemicentrotus* spermatozoa.

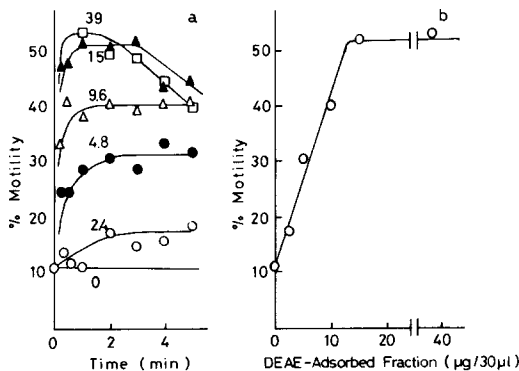


FIGURE 2 Effect of the concentration of DEAE-adsorbed fraction on % motility in the presence of 10 μM cAMP. (a) Time-course of % motility after cAMP addition. Figures on each curve indicate the amount of protein in DEAE-adsorbed fraction added in $\mu\text{g}/30\ \mu\text{l}$. (b) Relationship between the plateau levels of % motility and concentrations of DEAE-adsorbed fraction. The plateau levels of % motility vs. concentrations of DEAE-adsorbed fraction were plotted from the data shown in a.

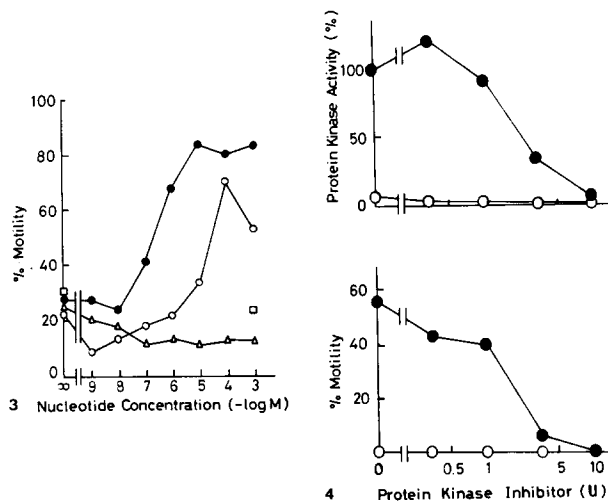


FIGURE 3 Nucleotide specificity for the reactivation of TXM. % motility was determined in the presence of 39 μg DEAE-adsorbed fraction/ $30\ \mu\text{l}$ at 3 min after the addition of various concentrations of the following nucleotide: cAMP (●); cGMP (○); 5'-AMP (△); and ATP (□).

FIGURE 4 Effect of protein kinase inhibitor on protein kinase activity in DEAE-adsorbed fraction (a) and on % motility (b). Protein 1 329kinase activity was measured in the same condition as that used for motility determination, except for the addition of histones as substrate and the omission of TXM. Protein kinase inhibitor activity represents the values in $30\ \mu\text{l}$ of the assay system. ○, (-) cAMP; ●, (+) cAMP.

It should be mentioned here that TXM of sea urchin sperm flagella without the head and middle piece were also reactivated to undulate by cAMP in the presence of the DEAE-adsorbed fraction.

The plateau level of % motility after cAMP addition was found to be dependent on the amount of the DEAE-adsorbed fraction added to the models (Fig. 2a). When the plateau levels were plotted vs. the amounts of the DEAE-adsorbed fraction, a linearity was observed up to 15 μg of the DEAE-adsorbed fraction (Fig. 2b), although the full activation level changed from one batch of spermatozoa to another. The data shown in Fig. 2 indicate that the mode of the reactivation caused by the DEAE-adsorbed fraction with cAMP was stoichiometric rather than catalytic. Fig. 2a also shows that the addition of $>15\ \mu\text{g}$ of the fraction causes decline of % motility after reaching maximal levels.

Cyclic AMP at low concentration preferentially elevated % motility (Fig. 3). Half-maximal reactivation occurred at 0.3 μM . Cyclic guanosine monophosphate (cGMP) increased % motility only at higher concentrations, revealing the concentration for the half-maximal reactivation to be on the order of 10 μM . 5'-AMP was inactive. It is evident that the reactivation is specific for cAMP.

When the movement of TXM was repressed in the presence of the DEAE-adsorbed fraction, the addition of 1 mM ATP never initiated the beating. The occurrence of the motionless states of TXM was not due to exhaustion of ATP in the

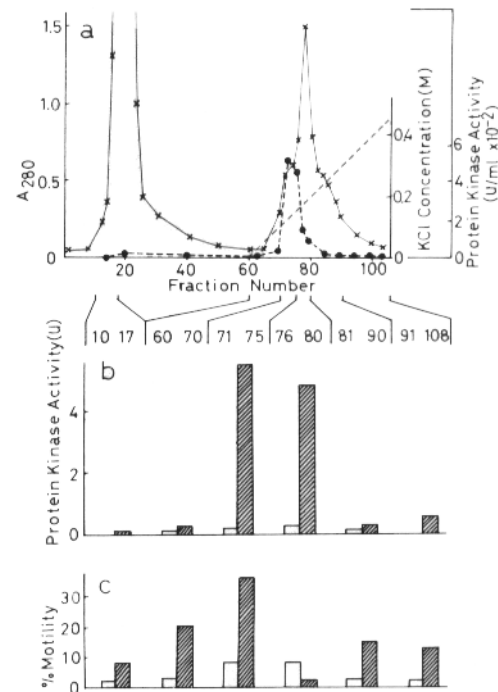


FIGURE 5 DEAE-cellulose column chromatography of sperm extract. Sperm extract of *Hemicentrotus* (508 mg protein) was applied on a DEAE-cellulose column ($3.4 \times 30\text{ cm}$) equilibrated with 20 mM Tris-HCl (pH 8.1) containing 0.1 mM DTT. After unadsorbed materials were washed out with $\sim 400\text{ ml}$ of the same solution, elution was carried out by a linear concentration gradient of KCl from 0 to 0.5 M in 20 mM Tris-HCl (pH 8.1) and 0.1 mM DTT, total 500 ml, collecting fractions of 9.5 ml. (a) Elution profile. Protein kinase activity was assayed using the standard assay mixture. A_{280} (x—x). KCl concentration (---). Protein kinase activity (●—●). (b) Protein kinase activity in $30\ \mu\text{l}$ of assay solution for TXM reactivation system. (c) Activity of each fraction in enhancing % motility. □, (-) cAMP; ▨, (+) cAMP.

reactivation system. This suggests that cAMP affects the motile apparatus directly, not via an ATP-generating system.

Participation of cAMP-dependent Protein Kinase in Reactivation of TXM

The action of cAMP is generally mediated through cAMP-dependent protein kinase (23). This suggested to us that the cAMP-dependent reactivation of TXM was also the case. In fact, the DEAE-adsorbed fraction contained this enzyme with a high level of specific activity (177 U/mg), and the medium condition for TXM reactivation strongly supported the enzyme activity. In addition, the reactivation profiles obtained by changing concentration of cAMP and cGMP (Fig. 3) closely resembled those obtained for cAMP-dependent protein kinases from sperm flagella of sea urchin (9) and from *Tetrahymena* cilia (18).

To confirm that this cAMP-dependent reactivation was driven by cAMP-dependent protein kinase, we employed an inhibitor protein which is highly specific for protein kinases from a variety of sources (19). When the inhibitor protein was added to the DEAE-adsorbed fraction, % motility of TXM did decrease in parallel with inhibition of the cAMP-dependent protein kinase activity in the DEAE-adsorbed fraction (Fig. 4). The parallel relation strongly indicates that the enzyme actually participates in the reactivation. It should be noted here that protein kinase inhibitor by itself did not affect the motility of TXM at all.

We partially purified cAMP-dependent protein kinase from the sperm extract of *Hemicentrotus* (Materials and Methods) to obtain further evidence on the role of this enzyme in TXM reactivation. When 17 U of the kinase (high-speed liquid chromatography fraction) was added to TXM suspension in a total volume of 30 μ l, % motility was 12% for (-) cAMP, and 10% for (+) cAMP. This indicated clearly that the partially purified enzyme fraction alone was not capable of reactivating TXM.

Participation of Regulatory Factor in Reactivation of TXM

Since the partially purified enzyme was incapable of reactivating the models, it was reasonable to assume that some factor

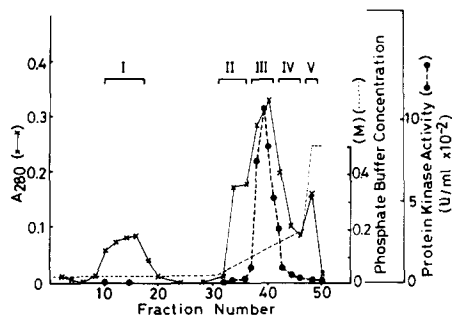


FIGURE 6 Hydroxyapatite column chromatography of fractions 71-75 obtained by DEAE-cellulose column chromatography. Fractions 71-75 (40 mg) were loaded on a hydroxyapatite column (1.5 \times 26 cm) equilibrated with 20 mM potassium phosphate buffer (pH 7.0) containing 0.1 mM DTT. After the column was washed with 75 ml of the same buffer solution, adsorbed materials were eluted with a linear concentration gradient of phosphate buffer (from 20 mM to 0.2 M, total 60 ml) and then with 0.5 M phosphate buffer. Each fraction was 5 ml. The roman numerals in the figure indicate fractions combined. A_{280} (x—x). Phosphate buffer concentration (---). Protein kinase activity (●—●).

TABLE II
Effect of Hydroxyapatite Fractions (I to V) on the Motility of the Triton Models

	Hydroxyapatite fraction				
	I	II	III	IV	V
	U				
Protein kinase activity*					
(-)cAMP	0.01	0.03	0.17	0.06	0.01
(+)cAMP	0.09	0.03	3.76	0.88	0.09
Activity for inducing reactivation (% motility)					
(-)cAMP	12	13	14	14	10
(+)cAMP	8	6	19	16	12
Activity for inducing reactivation by supplementing fraction III‡ (% motility)					
(-)cAMP	15	12	35	24	19
(+)cAMP	24	23	35	39	66

* Values in 30 μ l of assay system for motility.

‡ Contained 3.7 U of protein kinase activity.

TABLE III
Sensitivity of the Factor in Fraction V to Proteolytic or Heat Treatment *

Treatment	% Motility	
	(-)cAMP	(+)cAMP
None	14	49
Trypsin‡	18	18
Heat§	16	15

* Fraction III (0.94 μ g containing 3.7 U of protein kinase activity) was mixed with 1.2 μ g of treated fraction V in a total volume of 30 μ l.

‡ Trypsin was added to fraction V in a ratio of trypsin to fraction V (wt/wt) of about 1:7. 60 min after incubation at 25°C, the digestion was stopped by the addition of an excess soybean trypsin inhibitor.

§ Fraction V was heated at 65°C for 5 min and then centrifuged to clarify.

other than cAMP-dependent protein kinase was further necessary for the reactivation and that it was removed in the course of purification of the enzyme. This possibility was also suggested by the data indicating that the mode of action was not catalytic but stoichiometric (Fig. 2). Upon measuring the ability of all the enzyme fractions obtained at each purification step to enhance the motility of TXM in a cAMP-dependent manner, it became clear that the enzyme fraction obtained at the hydroxyapatite column chromatography step no longer reactivated TXM in the presence of cAMP. Therefore, we focused on the fractions obtained by DEAE-cellulose chromatography to find some regulatory factor for reactivation of TXM. Fig. 5 indicates the data at the step of DEAE-cellulose column chromatography. The activity of cAMP-dependent protein kinase emerged from fraction 71 to fraction 80 as a single peak, which was divided into left and right halves (fractions 71-75 and fractions 76-80). The former half, showing the highest activity of the enzyme, reactivated TXM with cAMP. But the latter half, showing the second highest enzyme activity, did not induce the cAMP-dependent reactivation. This fact indicates that a factor necessary for the TXM reactivation is separable from the enzyme.

When fractions 71-75 were chromatographed on a hydroxyapatite column, five fractions (from I to V), including the

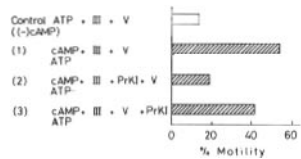


FIGURE 7 Blockage of motility activation by protein kinase inhibitor. After mixing of ATP, cAMP and fraction III, fraction V, and protein kinase inhibitor were added at intervals of 30 s in the order indicated at the left of the figure. % Motility was assayed 30 s after the final addition of TXM suspension. ATP and cAMP concentrations in the preincubations were 1 mM and 10 μ M, respectively. Abbreviations used in this figure are: III, fraction III (2.6 U of protein kinase activity); V, fraction V (1.2 μ g of protein); PrKI, protein kinase inhibitor (30.7 U). These are values in 30 μ l of the assay system for motility.

enzyme fraction, were obtained (Fig. 6). However, none of these fractions had the ability to reactivate Triton models (Table II). This was consistent with the above result, that the enzyme purified by hydroxyapatite chromatography did not reactivate the movement of TXM. We concluded from this result that the kinase (fraction III) was separated from the factor at this step. Recombination experiments revealed that it was fraction V which showed the most pronounced reactivation of TXM in the presence of fraction III (Table II). In addition, fraction V was sensitive to both proteolytic and heat treatments (Table III). These results showed that reactivation required two factors present in fractions from the DEAE-cellulose column: a cAMP-dependent protein kinase in fraction III and a protein factor in fraction V.

Furthermore, it was suggested that cAMP-dependent phosphorylation of the protein factor supported the reactivation of the TXM. The following experiment (Fig. 7) was carried out to study this possibility. Fraction III was first mixed with ATP, cAMP, and Mg^{2+} , then mixed with fraction V or kinase inhibitor and incubated for a period of 30 s; the inhibitor or fraction V, respectively, was then added. A TXM suspension was finally added to this mixture. Three combinations of the experiments are shown in Fig. 7 in comparison with a control ([–] cAMP). (1) When TXM were added to the mixture of fraction III and V without addition of the kinase inhibitor, a pronounced reactivation was consistently observed. (2) Addition of the kinase inhibitor to fraction III before mixing in fraction V suppressed to a great extent the reactivation of TXM successively added. (3) In contrast, when the addition of kinase inhibitor was preceded by mixing fraction V with fraction III, significant reactivation was detected—that is, the inhibitor no longer greatly decreased the beating of TXM. We can assume that phosphorylation of the protein factor in fraction V was maintained in the first case; that, in contrast, phosphorylation of the protein factor was kept inhibited in the second case; and that phosphorylation of the protein factor had occurred before the kinase inhibitor was added in the third case. This also suggested that cAMP-dependent phosphorylation of some components in the TXM itself was not needed for the reactivation.

DISCUSSION

Here we report motionless TXM of sea urchin and starfish spermatozoa which showed cAMP-dependent reactivation. We point out that our TXM are essentially the same as the quiescent ones reported by Gibbons and Gibbons (24): (a) its low % motility was not due to exhaustion of ATP; (b) the micro-

scopic image of the motionless models before reactivation by cAMP resembled the quiescent ones reported by Gibbons and Gibbons; and (c) potentially asymmetric TXM prepared by their method also showed cAMP-dependent reactivation in the presence of the DEAE-adsorbed fraction (data not shown).

The cAMP-dependent reactivation of TXM seems to be mediated by cAMP-dependent protein kinase, because (a) the DEAE-adsorbed fraction contained this enzyme with a specific activity of 177 U/mg, (b) the model reactivation was highly specific for cAMP, (c) the medium condition for the reactivation supported the enzyme activity, and (d) % motility of TXM was depressed in parallel with inhibition of the cAMP-dependent protein kinase activity by protein kinase inhibitor.

Moreover, the results indicated below support the conclusion that a factor other than the enzyme is also necessary for the reactivation: (a) The mode of action of the DEAE-adsorbed fraction seems to be not catalytic but stoichiometric. (b) Partially purified enzyme fraction alone is not capable of reactivating TXM. (c) When mixed back with the protein factor fraction free from cAMP-dependent protein kinase activity, the enzyme fraction regained the ability to reactivate TXM in a cAMP-dependent manner. The experiments using the protein kinase inhibitor present the hypothesis that phosphorylation of the regulatory protein factor in fraction V results in reactivation of TXM. Purification and identification of the protein factor and the mode of its action remain to be solved.

We have noticed that cAMP sometimes does not affect TXM prepared from very fresh spermatozoa. This is consistent with the results obtained by Gibbons and Gibbons (1), who reported that addition of cAMP had no effect on the motility of sea urchin sperm TXM. However, it is suggested that preincubation of spermatozoa at room temperature made their TXM susceptible to cAMP-dependent reactivation for beating. This might be due to dephosphorylation of the protein factor to some extent during preparation of TXM, which leads to quiescent state of the models.

Studies of ciliary and flagellar movement have hitherto focused only on the axoneme, especially on the interactions between microtubules and dynein accompanying sliding among groups of outer fibers. In contrast, this paper gives evidence that detergent solubilizes factors necessary for the regulation of flagellar movement.

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