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PURIFICATION AND CHARACTERIZATION OF A PROTEIN KINASE IN *TETRAHYMENA* CILIA

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SUMMARY

A protein kinase was detected in *Tetrahymena* cilia and partially purified by hydroxyapatite column chromatography and Sephadex G-200 gel filtration. This enzyme preferred casein to histone and protamine as substrate protein. The apparent K_m for casein was about 0.6 mg/ml and that for ATP was about 25 μM when casein was used as substrate protein. The pH optimum for casein phosphorylation was about 8.5. 10 mM Mg^{2+} was required for its maximal activity. In the absence of Mg^{2+}, other divalent cations such as Mn^{2+} and Co^{2+} could partially substitute for Mg^{2+}. The molecular weight of the enzyme was estimated to be about 53 000.

The ciliary protein kinase was found to catalyze the phosphorylation of tubulin prepared from ciliary axonemes. Furthermore, adenosine 3′,5′-monophosphate showed little effect on the phosphorylation of casein, histone, protamine and tubulin.

INTRODUCTION

It has been demonstrated recently that a mammalian brain tubulin fraction prepared by the method of Weisenberg *et al.*[1] shows protein kinase activity. Goodman *et al.*[2] have shown that the brain tubulin fraction contains a protein kinase which phosphorylates tubulin and that this activity is enhanced by the addition of adenosine 3′,5′-monophosphate (cyclic AMP). This was confirmed later by Lagnado *et al.*[3]. In addition, evidence has been presented that a protein kinase, which was purified after Miyamoto *et al.*[4], catalyzes the phosphorylation of tubulin[2,5]. On the other hand, Soifer *et al.*[6] and Soifer[7] have observed in a brain tubulin fraction another type of protein kinase which catalyzes the phosphorylation of casein and lysine-rich histone but cannot phosphorylate tubulin.

In addition to the data obtained from *in vitro* studies, there is evidence that tubulin is actually phosphorylated *in vivo*. According to Eipper[8], brain tubulin contained 0.8 mole phosphate per mole of tubulin dimer, and incubation of brain slices with ortho[^{32}P]phosphate resulted in phosphorylation of only the β-subunit of the tubulin dimer.

Besides brain kinases, cyclic AMP-dependent protein kinases have been partially purified from the sonicates of spermatozoa of the ox[9,10] and the sea urchin[11]. As the sonication caused tail disintegration on all spermatozoa with their heads remaining intact, those authors proposed a possibility that the protein kinases take part in the flagellar motile system.

The present results, described below, demonstrate that a protein kinase can be extracted from the axoneme fraction of *Tetrahymena* cilia, and does catalyze phosphorylation of ciliary tubulin *in vitro*.

MATERIALS AND METHODS

Tetrahymena pyriformis W was grown in a culture medium containing 2% (w/v) polypeptone, 0.5% (w/v) yeast extract and 0.85% (w/v) glucose with constant aeration. Cells in the stationary phase were used as experimental materials. Cilia were isolated by the method of Gibbons[12] with some modifications as follows; the $CaCl_2$ concentration in the ethanol–calcium procedure was increased to 20 mM and a slight contamination due to the cell bodies was removed by passing the solution through a glass filter after the routine procedure in which the cilia were separated from the cell bodies by a brief centrifugation. Axonemes were prepared by the method of Stephens and Levine[13].

[γ-^{32}P]ATP was prepared following the method described by Glynn and Chappell[14]. Ortho[^{32}P]phosphate was purchased from The Radiochemical Centre, Great Britain, ATP from Kojin, Japan, and cyclic AMP from Daiichi, Japan. Proteins were obtained from commercial sources, as follows; casein, Merck; calf thymus histone (type II-A), herring protamine (grade III), soybean trypsin inhibitor and ovalbumin from Sigma; and bovine serum albumin and bovine γ-globulin from Armour. Hydroxyapatite was prepared according to the method of Tiselius *et al.*[15].

Protein kinase assay

A standard assay mixture, unless otherwise indicated, contained 5 μmoles of Tris–HCl (pH 8.5), 1 μmole of $MgCl_2$, 0.1 μmole of dithiothreitol, 0.2 μg of casein, 0.01 μmole of [^{32}P]ATP ($4 \cdot 10^5$–$20 \cdot 10^5$ cpm) and enzyme, in a total volume of 0.1 ml. The reaction was started by the addition of enzyme and allowed to proceed for 5 min at 25 °C. It was found that the reaction was linear for at least 10 min. After incubation, 0.04 ml of the reaction mixtures was pipetted onto Whatman No. 1 paper discs and washed once with 10% trichloroacetic acid and three times with 5% trichloroacetic acid. After washing finally with ethanol and ether, the radioactivities were counted in a toluene-based scintillator containing PPO (4 g/l) and dimethyl-POPOP (0.2 g/l) with an Aloka liquid scintillation system (Model LSC-651). When protamine was used as substrate protein, 0.6 mg of bovine serum albumin was added to the assay mixture before pipetting onto paper discs and washing was performed four times with 10% trichloroacetic acid. In the case of histone, 18% trichloroacetic acid was applied. All the trichloroacetic acid solutions mentioned above contained 1% sodium pyrophosphate. One unit of enzyme activity was defined as the amount of enzyme which catalyzes the transfer of 1 nmole of ^{32}P from ATP to casein per 1 min in the standard assay system at 25 °C.

Polyacrylamide gel electrophoresis

Sodium dodecylsulfate polyacrylamide gel electrophoresis was performed according to the method of Weber and Osborn[16] with some modifications[17]. The gels (0.6 cm × 6.5 cm) were made of 7.5% acrylamide–0.2% methylenebisacrylamide.

In order to identify protein components which incorporated radioactive phosphate, gels were sliced into 37 equal pieces with a hand-made gel slicer and digested in 0.3 ml of 30% H_2O_2 (ref. 18) in counting bials. To the solubilized gels, were added 0.3 ml of 1 M HCl and 10 ml of toluene-based scintillator containing 2.8 g/l of PPO, 0.14 g/l of dimethyl-POPOP and 30% (v/v) of Nonions NS-210 (nonylphenolpolyethoxyether, Nippon Oil and Fats Co. Ltd)[19] and the radioactivities were measured as described above.

Protein determination

The amount of protein was determined by the method of Lowry *et al.*[20] using bovine serum albumin as a standard.

RESULTS

(1) Enzyme purification

Solubilization of enzyme. About 5 ml of packed wet cilia was routinely obtained from a 9-l culture, from which axonemes were prepared using 1% (v/v) Triton X-100 (ref. 13).

To the purified axonemes, about 20 ml of 0.6 M KCl containing 0.5 mM EDTA, 0.1 mM ATP, 0.1 mM dithiothreitol and 1 mM Tris–HCl (pH 8.2) were added and homogenized vigorously with a teflon homogenizer. The homogenate was dialyzed against 500 ml of the same buffer for 5 h and centrifuged at 50 000 × *g* for 20 min. The pellet was re-extracted with about 20 ml of the same KCl solution. Both the supernatants were combined. Most of the protein kinase activity (more than 95%) was detected in the supernatant by this extaction procedure.

Hydroxyapatite column chromatography. When the KCl extract was chromatographed on hydroxyapatite, three peaks of protein kinase activity (Peaks A, B and C) were consistently eluted as shown in Fig. 1. The fractions of the main peak, C (tubes 53–65) were combined.

$(NH_4)_2SO_4$ fractionation. Solid $(NH_4)_2SO_4$ (0.35 g/ml) was added to the hydroxyapatite fraction. The precipitates formed were collected by centrifugation, dissolved and dialyzed against 10 mM Tris–HCl (pH 8.4) containing 0.6 M KCl, 0.1 mM ATP and 0.1 mM dithiothreitol. This fraction was called the "$(NH_4)_2SO_4$ fraction".

Sephadex G-200 gel filtration. For further purification, the $(NH_4)_2SO_4$ fraction was gel filtered through a Sephadex G-200 column. The protein kinase activity was eluted as a single peak as is shown in Fig. 2. The active fractions (tubes 36–46) were combined.

From the results summarized in Table I, the apparent specific activity of the Sephadex G-200 fraction was found to be about 60-fold higher than that of the KCl extract of the axoneme. The enzyme in the Sephadex G-200 fraction was fairly unstable, about 50% of the activity was lost in one week at 0 °C. The enzyme involved in the $(NH_4)_2SO_4$ fraction was more stable, little loss of the activity was detected when

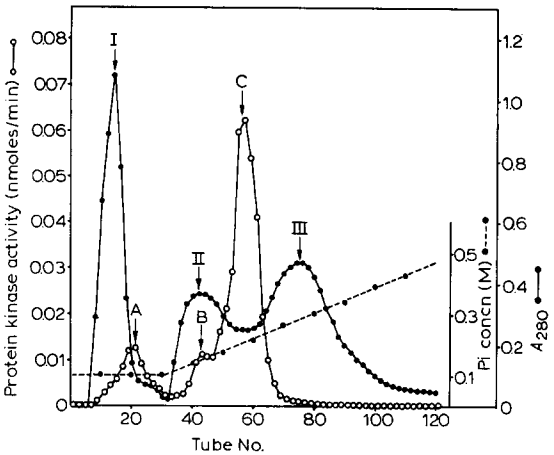

Fig. 1. Hydroxyapatite column chromatography of the KCl extract. 40 ml of the KCl extract were loaded on the hydroxyapatite column (2 cm × 12.5 cm) equilibrated with 0.1 M potassium phosphate buffer (pH 7.5) containing 0.1 mM ATP and 0.1 mM dithiothreitol. After the column was washed with about 60 ml of the same solution, adsorbed materials were eluted with a linear gradient of phosphate buffer containing 0.1 mM ATP and 0.1 mM dithiothreitol (from 0.1 – 0.6 M, total 500 ml), collecting fractions of 4 ml. Aliquots of 50 μl from each fraction were assayed using the standard assay mixture.

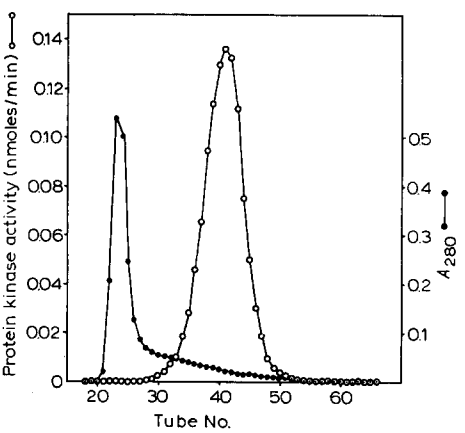

Fig. 2. Gel filtration of the $(NH_4)_2SO_4$ fraction on Sephadex G-200. The $(NH_4)_2SO_4$ fraction was loaded on Sephadex G-200 column (2 cm × 47 cm) equilibrated with 10 mM Tris–HCl (pH 8.4) containing 0.6 M KCl, 0.1 mM ATP and 0.1 mM dithiothreitol, and eluted with the same buffer solution, collecting fractions of 2.5 ml. Th enzyme activity of each fraction was assayed with aliquot of 50 μl using the standard assay system.

stored for a month at 0 °C. To determine the characteristics of this ciliary protein kinase, the $(NH_4)_2SO_4$ fraction was used as enzyme source.

(II) General properties of ciliary protein kinase

The pH optimum of the enzyme activity was about 8.5. A second peak was detected at around pH 7 (Fig. 3). The apparent K_m for ATP was about 25 μM.

Fig. 4 illustrates the divalent cation requirement for ciliary protein kinase. Mg^{2+} was most effective among all the cations investigated, and revealed the optimum

TABLE I

PURIFICATION STEPS OF A PROTEIN KINASE FROM *Tetrahymena* CILIA

The activity was determined with the standard assay system.

Fraction	*Protein (mg)*	*Total activity (units)*	*Specific activity (units/mg)*
Cilia	654	65.8	0.10
Axoneme	338	144	0.43
KCl extract	109	201	1.85
Hydroxyapatite fraction	9.6	122	12.7
$(NH_4)_2SO_4$ fraction	8.0	122	15.3
Sephadex G-200 fraction	0.60	64.1	107

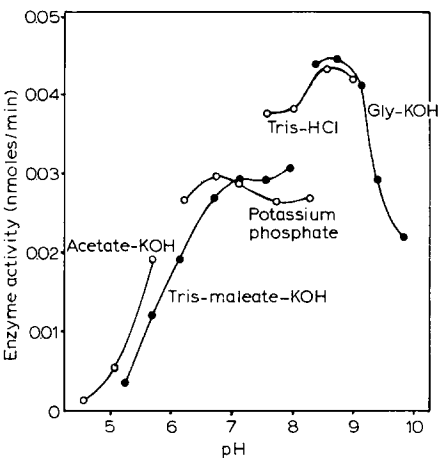

Fig. 3. Effect of pH on the rate of casein phosphorylation. The reaction rate was determined with the standard assay system except for the buffers indicated. The concentration of each buffer was 50 mM. 0.043 unit of the enzyme ($(NH_4)_2SO_4$ fraction) was used per incubation.

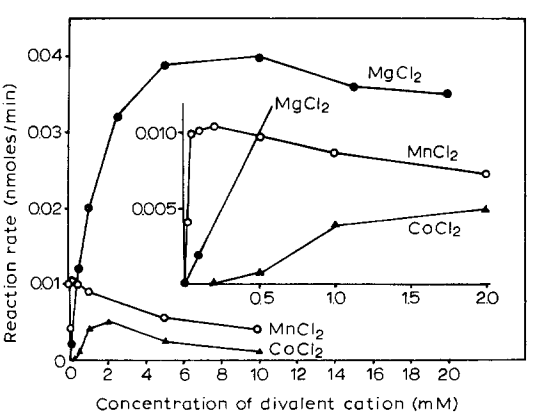

Fig. 4. Divalent cation requirement. The reaction rate was measured with the standard assay mixture except that $MgCl_2$ was substituted for various concentrations of divalent cations indicated. 0.040 unit of the enzyme ($(NH_4)_2SO_4$ fraction) was used per incubation.

at around 10 mM. Mn^{2+} and Co^{2+} were less effective with their optima at 0.2 and 2 mM, respectively. Each 2, 5 and 10 mM of Ca^{2+}, Sr^{2+}, Ni^{2+}, Zn^{2+} and Cd^{2+} were completely ineffective.

Among the proteins tested for substrate specificity, casein was the best substrate for the enzyme in the present assay condition. The apparent K_m for casein was about 0.6 mg/ml. Protamine and histone (8 mg/ml at the maximum) were poorer substrates than casein, revealing one-fifth and one-fourth of the rate of casein phosphorylation, respectively.

10^{-8}–10^{-4} M cyclic AMP showed little effect on the phosphorylation of casein, histone and protamine.

The enzyme activity was depressed as the concentration of KCl and NaCl increased. The effects of both salts were quite similar. About half of the enzyme activity was depressed by the addition of 0.2 M KCl or NaCl.

It has been established by Miyamoto *et al.*[4] and Guthrow *et al.*[21] that Ca^{2+} inhibits cyclic AMP-dependent protein kinases in the presence of Mg^{2+}. The same inhibitory effect of Ca^{2+} was true with the present enzyme. $CaCl_2$ reduced the enzyme activity to half the original level, however, by increasing the concentration up to 10 mM, the phosphorylation was largely diminished.

The molecular weight of ciliary protein kinase was estimated with Sephadex G-200 gel filtration (Fig. 5). The apparent molecular weight was estimated to be about 53 000, which was close to that of tubulin.

(III) Tubulin phosphorylation catalyzed by ciliary protein kinase

Identification of tubulin as the major component of Peak I fraction from hydroxyapatite chromatography. Tubulin is a main component of the axoneme and it is known

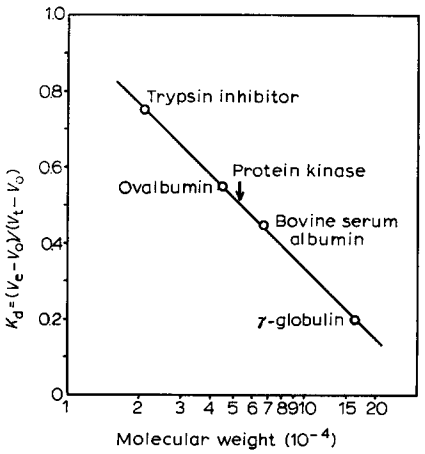

Fig. 5. Molecular weight estimation of ciliary protein kinase with Sephadex G-200 gel filtration. As protein markers, soybean trypsin inhibitors, ovalbumin, bovine serum albumin and γ-globulin were solubilized separately in 1 ml of 10 mM Tris–HCl (pH 8.4)–0.6 M KCl containing Blue Dextran and sucrose. As source, 1 ml of the $(NH_4)_2SO_4$ fraction was mixed with Blue Dextran as well. Each samples was run separately. The Sephadex G-200 column (2 cm × 47 cm) was equilibrated with Tris–HCl and the elution was performed with the same buffer. 2 ml of fractions were collected. Marker proteins were detected by the measurement of $A_{280\ nm}$. The void and column volumes were measured by the aid of Blue Dextran and sucrose, respectively. The assay condition of protein kinase was the same as shown in Fig. 2.

that a considerable amount of ciliary tubulin can be extracted with 0.6 M KCl[22]. In the present work, the KCl extract of the axoneme was separated into three peaks of Protein I, II and III (see Fig. 1). Fig. 6A represents the electrophoretic patterns of the protein components in Peaks I, II and III. The run-off fraction (Peak I) exhibited the presence of a major component. From its mobility, it was supposed that the protein might be tubulin. Therefore, the major component of Peak I was further analyzed by electrophoresis on sodium dodecylsulfate polyacrylamide gels (Fig. 6B) with a marker of ciliary tubulin derived from outer fibers[23]. Since the outer fiber consists mainly of tubulin, the major band of solubilized outer fiber (Fig. 6B-c) was identified as tubulin. The R_F value of the main band of Peak I (Fig. 6B-a) was coincident with that of the major band of solubilized outer fiber (Fig. 6B-c), and the molecular weight was estimated to be 54 000, which was close to that obtained by Renaud *et al.*[22]. Moreover, the R_F value of the main band was identical with that of porcine brain tubulin prepared by the method of Weisenberg *et al.*[1]. These facts support the idea that the major component of Peak I was ciliary tubulin.

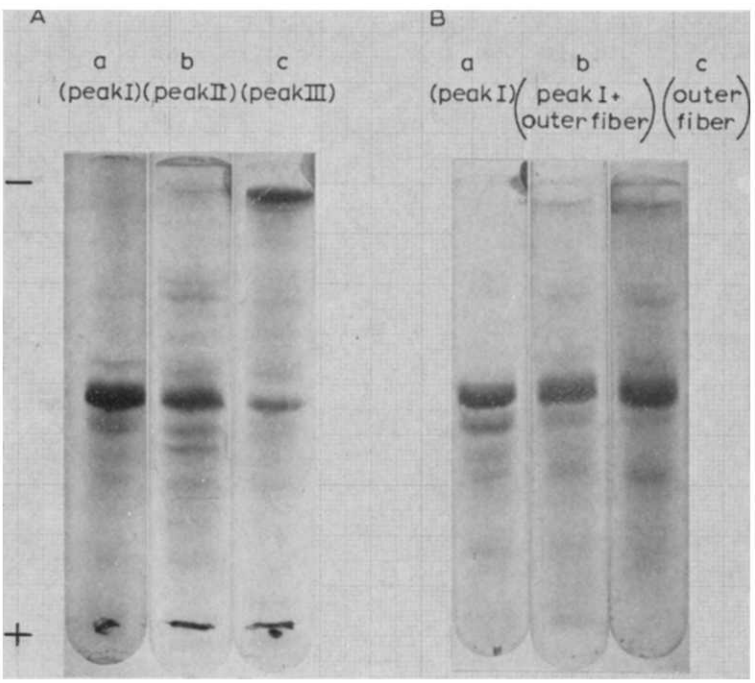

Fig. 6. (A) Sodium dodecylsulfate polyacrylamide gel electrophoresis of components in Peak I, II and III. Several main fractions of each Peak I, II and III were combined and concentrated. After treated with 8 M urea–1% sodium dodecylsulfate–1% 2-mercaptoethanol–5 mM EDTA–20 mM Tris–HCl (pH 8.5), about 50 μg of each protein fraction was applied on sodium dodecylsulfate polyacrylamide gels. a, Peak I; b, Peak II, and c, Peak III. (B) Sodium dodecylsulfate polyacrylamide gel electrophoresis of components in Peak I and solubilized outer fibers as a marker of tubulin. Several main fractions of Peak I of hydroxyapatite column chromatography were combined and dialyzed against the urea-sodium dodecylsulfate–2-mercaptoethanol–EDTA–Tris buffer. Outer fibers were directly solubilized in the same solution and dialyzed as well. About 50 μg of Peak I protein and solubilized outer fibers were applied to Gels a and c, respectively. About 25 μg of both fractions were mixed and applied, b.

Time course of tubulin phosphorylation catalyzed by ciliary protein kinase. The ability of this as a substrate of ciliary tubulin was further investigated. In order to obtain a concentrated tubulin fraction for assay, the Peak I fraction was further fractionated by $(NH_4)_2SO_4$ precipitation. This fraction is, hereafter, called the "axonemal tubulin fraction".

Although most of protein kinase was separated from ciliary tubulin by the hydroxyapatite column chromatography, the Peak I fraction still contained a detectable amount of protein kinase (see Fig. 1). After it was concentrated by $(NH_4)_2SO_4$ fractionation to obtain the axonemal tubulin fraction as substrate, a fairly high contaminating protein kinase activity was detected without addition of exogenous enzyme. When purified protein kinase was added, a higher rate of protein phosphorylation was demonstrated (Fig. 7A).

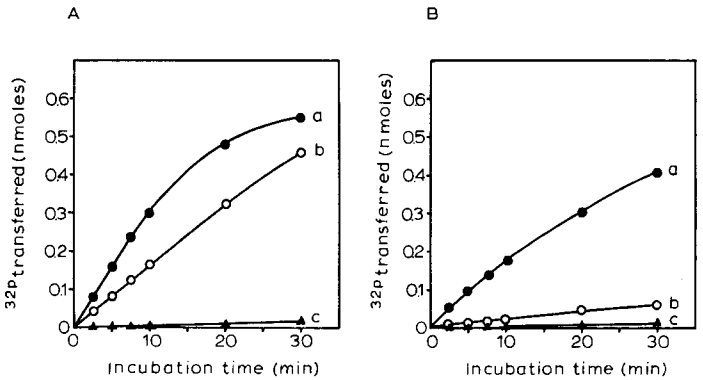

Fig. 7. Time-cource of phosphorylation of the axonemal tubulin fraction (A), and of the urea-treated tubulin fraction (B), with and without exogenous protein kinase. The Peak I fraction of hydroxyapatite chromatography (Fig. 1, tubus 7–18) were combined and proteins were precipitated with $(NH_4)_2SO_4$ (0.31 g/ml), dissolved in 30 mM Tris–HCl (pH 8.3) and dialyzed against the same buffer to obtain the axonemal tubulin fraction. As for the preparation of the urea-treated tubulin fraction, the axonemal tubulin fraction was dialyzed against 5 M urea containing 30 mM Tris–HCl (pH 8.3), 20 mM 2-mercaptoethanol and 0.5 mM EDTA for 1 h at 0 °C. Urea was then removed by dialysis against 30 mM Tris–HCl (pH 8.3). 0.28 mg of the axonemal tubulin fraction or the urea-treated tubulin fraction was used per incubation as substrate. The time-course of the reactions were measured with the standard assay mixture except for the substrate proteins. 0.030 unit of exogenous protein kinase ($(NH_4)_2SO_4$ fraction) was added when indicated.
(A) a, (+)axonemal tubulin fraction, (+)exogenous enzyme;
b, (+)axonemal tubulin fraction, (−)exogenous enzyme;
c, (−)axonemal tubulin fraction, (+)exogenous enzyme.
(B) a, (+)urea-treated tubulin fraction, (+)exogenous enzyme;
b, (+)urea-treated tubulin fraction, (−)exogenous enzyme;
c, (−)urea-treated tubulin fraction, (+)exogenous enzyme.

It was difficult to separate the contaminating protein kinase from the tubulin fraction. Therefore, an attempt was made to reduce the enzyme activity in the axonemal tubulin fraction. When the fraction was treated with 5 M urea (see legend of Fig. 7), the enzyme activity decreased to less than 15% of the original level. This fraction was designated as "urea-treated tubulin fracton".

As shown in Fig. 7B, the urea-treated tubulin fraction served as a substrate for ciliary protein kinase. The rate of phosphorylation in this fraction was about half of that of casein at the same protein concentration. From these data, it was strongly

suggested that the axonemal tubulin molecule could actually serve as a phosphate acceptor for this enzyme. In order to obtain evidence an experiment was designed to analyze phosphorylated proteins by electrophoresis.

Sodium dodecylsulfate polyacrylamide gel electrophoresis of phosphorylated proteins. Phosphorylated proteins in the urea-treated tubulin fraction were electrophoresed and the radioactivity of the sliced gels was counted (Fig. 8). A peak of the radioactivity emerged coincident with the major band of protein. It was concluded, therefore, that tubulin actually served as a substrate for ciliary protein kinase.

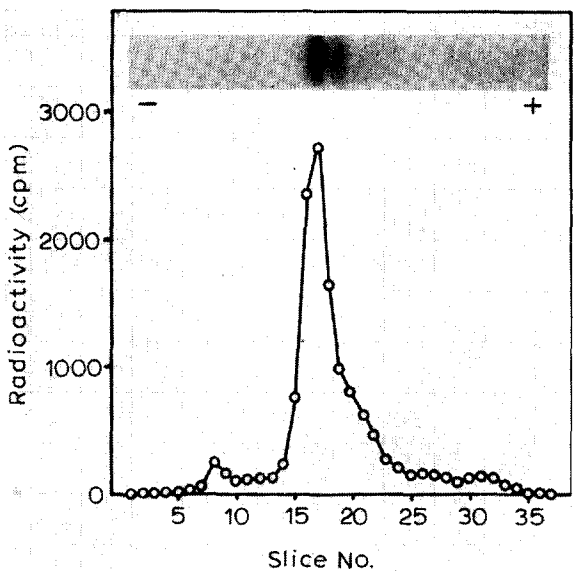

Fig. 8. Identification as tubulin of phosphorylated protein by sodium sodium dodecylsulfate polyacrylamide gel electrophoresis. 1 ml of an assay mixture containing 2.8 mg of urea-treated tubulin fraction was incubated at 25 °C for 1 h. The concentration of the reagents were the same as those in the standard assay mixture. 0.30 unit of the enzyme ($(NH_4)_2SO_4$ fraction) was used. Equal volume of 20% trichloroacetic acid containing 2% sodium pyrophosphate was added to stop the reaction. The precipitates were then washed five times with 5% trichloroacetic acid–1% sodium pyrophosphate and were dissolved in 0.5 ml of the urea–sodium dodecylsulfate–2-mercaptoethanol–EDTA–Tris buffer (see legend of Fig. 6), followed by dialysis against the same solution. About 50 μg of the protein was electrophoresed. After staining, the gels were sliced and their radioactivities were counted as in Materials and Methods.

Effect of cyclic AMP on the phosphorylation of tubulin. As described in Section II, cyclic AMP did not significantly affect the phosphorylation of casein, histone and protamine. The same held true with the effect of varying the concentration of cyclic AMP (10^{-10}-10^{-4} M) in tubulin phosphorylation. In addition, the phosphorylation of tubulin in the axonemal tubulin fraction catalyzed by the contaminating enzyme was not influenced by cyclic AMP.

DISCUSSION

It has been established that *Tetrahymena* cilia contains a protein kinase which catalyzes the phosphorylation of ciliary tubulin. In the present paper, the extraction

of the enzyme was usually carried out with the axoneme. When the outer-fiber fraction was obtained according to Gibbons[23], most of the protein kinase activity (about 70%) remained in the fraction, from which the same enzyme fraction was extracted and purified by hydroxyapatite column chromatography. This indicates that the protein kinase is tightly bound to the outer fiber.

Even when the outer-fiber suspension was incubated with [^{32}P]ATP, a considerable amount of the radioactivity was detected in the acid precipitate. This may confirm that the true substrate of the protein kinase is tubulin. The phosphorylation in the outer-fiber fraction was not also affected by cyclic AMP.

It has been reported so far that tubulin phosphorylation is cyclic AMP dependent *in vitro*[2,3]. It is noteworthy, in the case of the present ciliary kinase, that the Triton X-100 treatment and the KCl extraction induced a three times increase in the total activity of protein kinase (see Table I). Therefore, a possibility was considered that the R-subunit[23–25] might be removed during these steps. However, the possibility may be ruled out, because the protein kinase activity of the whole axoneme fraction was hardly affected by 10^{-8}–10^{-4} M cyclic AMP. Furthermore, the incorporation of ^{32}P from ATP into the outer-fiber fraction was also cyclic AMP independent. A possibility still remained, however, that the R-subunit might be removed by Triton X-100 used in the procedure of the axoneme preparation. But it is reasonable to decide that ciliary kinase contains no R-subunit *in situ*, judging from the fact that brain protein kinases which are cyclic AMP dependent could be prepared after Triton treatment[26]. Further experiments are required to demonstrate the absence of cyclic AMP-binding protein in cilia.

As inhibitor other than R-subunit, on the other hand, might be removed with the KCl extraction, or ciliary protein kinase might be partially masked in the axonemal structure and be activated in this procedure.

In the treatment of tubulin with urea to reduce the contaminating protein kinase activity, care should be taken due to the following possibility: Proteins which have little substrate activity might become good substrates after urea treatment. It is true that the urea treatment slightly enhanced the activities of ovalbumin, bovine serum albumin and γ-globulin, as substrate, however, these activities were 10-fold lower than that of the urea-treated tubulin fraction in the present work. The activity of urea-treated bovine serum albumin was maximally only about one-seventh of that of the same concentration of the urea-treated tubulin fraction. Furthermore, the activity of casein was not affected by the urea treatment. For further analysis of tubulin phosphorylation *in vitro*, it is necessary to prepare pure native tubulin which is free of protein kinase activity.

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