Biochimica et Biophysica Acta, 370 (1974) 130–139

BBA 67348

PROTEIN KINASES IN *TETRAHYMENA* CILIA

II. PARTIAL PURIFICATION AND CHARACTERIZATION OF ADENOSINE 3′,5′-MONOPHOSPHATE-DEPENDENT AND GUANOSINE 3′,5′-MONOPHOSPHATE-DEPENDENT PROTEIN KINASES

HIROMU MUROFUSHI

Department of Biophysics and Biochemistry, Faculty of Science, University of Tokyo, Hongo, Bunkyo-ku, Tokyo (Japan)

(Received May 27th, 1974)

SUMMARY

Adenosine 3′,5′-monophosphate (cyclic AMP)- and guanosine 3′,5′-monophosphate (cyclic GMP)-dependent protein kinase (EC 2.7.1.37) activities were detected in the Triton extract of *Tetrahymena* cilia. When the Triton extract was chromatographed on a DEAE-cellulose column, one peak of cyclic AMP-dependent protein kinase activity and three peaks of cyclic GMP-dependent protein kinase activity were detected, one of which was further purified with CM-Sephadex column chromatography.

Two of the cyclic GMP-dependent protein kinases had quite similar properties, prefering histone and casein for substrate, and were specifically activated by a low concentration of cyclic GMP. The other one, cyclic GMP-dependent protein kinase, which preferentially phosphorylated histone and protamine, was less susceptible to cyclic GMP.

The activity of cyclic AMP-dependent protein kinase was highly stimulated by a low concentration of cyclic AMP and showed preference for protamine or histone as substrate protein.

The apparent molecular weights of all the cyclic GMP-dependent protein kinases were about $1 \cdot 10^5$, that of the cyclic AMP-dependent kinase being about $6 \cdot 10^4$.

INTRODUCTION

Recently, several reports have been published on the regulatory effects on cyclic nucleotides on the flagellar and ciliary motile system. Morton et al. [1] have shown that quiescent hamster sperm is activated by either calcium, cyclic AMP, cyclic GMP or cyclic UMP. Cyclic nucleotides and phosphodiesterase inhibitors activate the respiration of bovine epididymal spermatozoa [2,3]. In the case of *Chlamydomonas*, increase in cyclic AMP levels caused by methylxanthine or aminophylline inhibits flagellar movement and flagellar regeneration [4]. Similar results have been obtained on *Tetrahymena* cilia [5].

In the studies of enzymes related to cyclic nucleotides, adenyl cyclases were detected in the sperm of man, the dog, the hamster and the sea urchin [6, 7]. Furthermore, it has been reported that the sea urchin spermatozoon contains a high level of guanyl cyclase activity [8, 9]. Cyclic AMP-dependent protein kinases have been partially purified from the sonicates of sperm from the ox [10, 11] and the sea urchin [12, 13].

The author has already reported that a cyclic AMP-independant protein kinase is localized in the axoneme of *Tetrahymena* cilia and that the enzyme can phosphorylate tubulin [14]. In the present paper, identification, partial purification and some characterization of cyclic AMP- and cyclic GMP-dependent protein kinases in the Triton extract of *Tetrahymena* cilia are described.

MATERIALS AND METHODS

Biological materials

The isolation method of *Tetrahymena* cilia was described previously [14]. From a 5-l culture, about 3 ml of wet-packed cilia were routinely obtained. Triton extract was prepared by the method of Stephens and Levine [15] with a modification that the cilia was exposed to 10 vol. of 1% Triton X-100 containing 30 mM Tris–HCl buffer (pH 8.2), 3 mM $MgCl_2$ and 50 mM KCl. After the axonemes were precipitated, the supernatant was clarified with a further centrifugation at $10^5 \times g$ for 1 h followed by dialysis against 20 mM Tris–HCl buffer (pH 8.0) containing 0.1 mM dithiothreitol to yield the Triton extract fraction.

Protein kinase assay

The assay method was essentially the same as that of the previous paper [14]. The standard assay mixture contained 5 μmoles of potassium phosphate buffer (pH 6.4), 1 μmole of $MgCl_2$, 0.1 μmole of dithiothreitol, 0.1 mg of whole histone, 0.01 μmole of [^{32}P]ATP ($5 \cdot 10^5$–$2 \cdot 10^6$ cpm) and enzyme in a total volume of 0.1 ml. If indicated, 3 μM cyclic AMP or cyclic GMP was added to the assay mixture. 1 unit of enzyme activity was defined as the amount of enzyme which catalyzed the transfer of 1 nmole of ^{32}P from ATP to histone per 1 min in the standard assay system containing 3 μM cyclic AMP or cyclic GMP.

Molecular weight estimation

The molecular weights of the enzymes were estimated with Sephadex G-200 gel filtration. Two types of column (1.7 cm $\times$ 54 cm) were prepared; one was equilibrated with 20 mM Tris–HCl buffer (pH 8.0) containing 0.1 mM dithiothreitol and the other with 20 mM Tris–HCl buffer (pH 8.0) containing 0.1 mM dithiothreitol and 0.6 M KCl. Details on gel filtration were shown in the previous paper [14].

Protein determination

The amount of protein was determined according to the method of Lowry et al. [16]. using bovine serum albumin as a standard.

All the chemicals and proteins were the same as those used in the previous report [14]. Cyclic GMP was purchased from Boehringer.

RESULTS

(I) Enzyme purification

DEAE-cellulose column chromatography. When the Triton extract was chromatographed on a DEAE-cellulose column, four peaks of protein kinase activity were consistently eluted. As was shown in Fig. 1, three of them were cyclic GMP-dependent

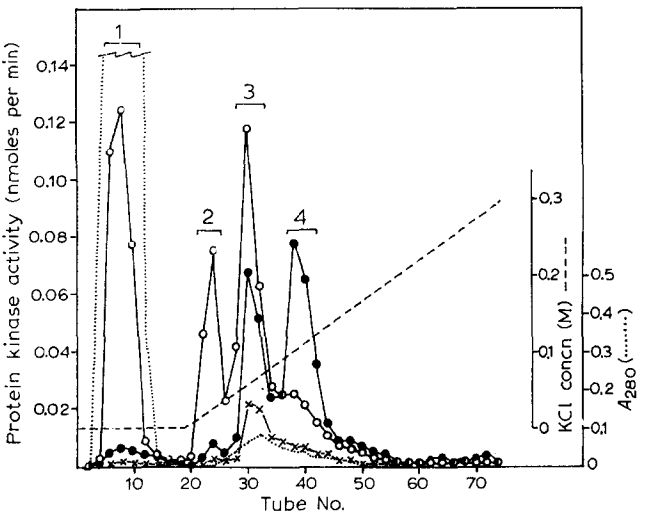

Fig. 1. DEAE-cellulose column chromatography of the Triton extract. 32.5 ml of Triton extract were loaded on a DEAE-cellulose column (1.5 cm × 16 cm) equilibrated with 20 mM Tris–HCl buffer (pH 8.0) containing 0.1 mM dithiothreitol. After the column was washed with the same buffer (about 50 ml), a linear gradient of KCl was started (from 0 to 0.35 M, total 400 ml), collecting fractions of 6 ml. Aliquots of 50 μl from each fraction were assayed using the standard assay system. Protein kinase activity: ●—●, in the presence of 3 μM cyclic AMP; ○—○, in the presence of 3 μM cyclic GMP; ×—×, in the absence of cyclic nucleotide; · · · · · , $A_{280\ nm}$. 1, cyclic GMP-dependent protein kinase 1; 2, cyclic GMP-dependent protein kinase 2; 3, cyclic GMP-dependent protein kinase 3; 4, cyclic AMP-dependent protein kinase.

and one was preferentially stimulated by cyclic AMP. They are called here cyclic GMP-dependent protein kinase 1, cyclic GMP-dependent protein kinase 2, cyclic GMP-dependent protein kinase 3 and cyclic AMP-dependent protein kinase. Almost all of Triton X-100 appeared in the unadsorbed fraction coincident with cyclic GMP-dependent protein kinase 1. The high absorbance at 280 nm in this peak was mostly due to Triton X-100. The peak fractions of each cyclic GMP-dependent protein kinase 2 (Tubes 21–25), cyclic GMP-dependent protein kinase 3 (Tubes 28–33) and cyclic AMP-dependent protein kinase (Tubes 37–42) were combined, concentrated through ultrafiltration (Diaflo, Amicon, membrane UM-2) and dialyzed against 20 mM Tris–HCl buffer (pH 8.0) containing 0.1 mM dithiothreitol. The cyclic GMP-dependent protein kinase 1 fraction (Tubes 6–10) was dialyzed against 20 mM potassium phosphate buffer (pH 7.0) containing 0.1 mM dithiothreitol.

CM-Sephadex column chromatography. The dialyzed cyclic GMP-dependent protein kinase 1 fraction was further chromatographed on a CM-Sephadex column.

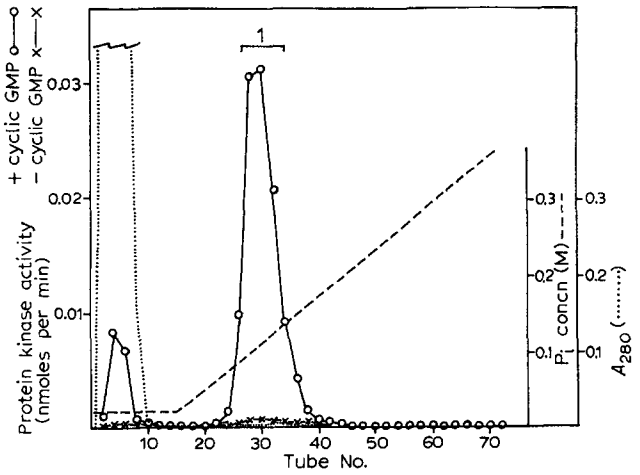

Fig. 2. CM-Sephadex column chromatography of cyclic GMP-dependent protein kinase 1 (DEAE-cellulose fraction). A dialyzed DEAE-cellulose fraction of cyclic GMP-dependent protein kinase 1 (27 ml) was applied to a CM-Sephadex (C-50) column (1.5 cm × 17 cm) equilibrated with 20 mM potassium phosphate buffer (pH 7.0) containing 0.1 mM dithiothreitol. After the column was washed with 45 ml of the same buffer, adsorbed materials were eluted with a linear gradient of phosphate buffer containing 0.1 mM dithiothreitol (from 0.02 to 0.4 M, total 400 ml). Each fraction was 6.5 ml, the enzyme activity of which was measured with an aliquot of 50 μl using the standard assay mixture in the absence or presence of 3 μM cyclic GMP. 1, cyclic GMP-dependent protein kinase 1.

TABLE I

PURIFICATION STEPS OF CYCLIC NUCLEOTIDE-DEPENDENT PROTEIN KINASES

The activity was measured with the standard assay system in the absence or presence of 3 μM cyclic AMP or cyclic GMP.

Fraction	Protein (mg)	Spec. act. (units per mg)			Total activity (units)	
		– cyclic nucleotide	+ cyclic AMP	+ cyclic GMP	+ cyclic AMP	+ cyclic GMP
Triton extract	51.0	0.42	6.03	6.10	307	311
DEAE-cellulose fraction						
Cyclic GMP-dependent protein kinase 1	12.7	0.07	–	6.71	–	85.2
Cyclic GMP-dependent protein kinase 2	0.37	2.41	–	74.2	–	27.4
Cyclic GMP-dependent protein kinase 3	1.54	4.80	–	29.7	–	45.6
Cyclic AMP-dependent protein kinase	1.52	1.04	140	–	212	–
CM-Sephadex fraction						
Cyclic GMP-dependent protein kinase 1	0.49	1.72	–	25.9	–	12.7

Cyclic GMP-dependent protein kinase activity emerged in a major peak at 0.1 M potassium phosphate buffer with a minor peak at the unadsorbed fraction (Fig. 2). Tubes 26–34 were combined, condensed with ultrafiltration followed by dialysis against 20 mM Tris–HCl buffer (pH 8.0) containing 0.1 mM dithiothreitol to yield a purer cyclic GMP-dependent protein kinase 1 which was essentially free from Triton X-100. The purification steps were summarized in Table I.

To determine the characteristics of the enzymes, the CM-Sephadex fraction was used as an enzyme source of cyclic GMP-dependent protein kinase 1 and DEAE-cellulose fractions as cyclic GMP-dependent protein kinase 2, 3 and cyclic AMP-dependent protein kinase.

(II) Properties of the protein kinases

Specificities for substrate protein. The enzyme activities were measured using whole histone, protamine, casein and brain tubulin as substrate in the absence or presence of 3 μM cyclic GMP for cyclic GMP-dependent protein kinase 1, 2 and 3 and 3 μM cyclic AMP for cyclic AMP-dependent protein kinase. As was shown in Table II, cyclic GMP-dependent protein kinase 1 and 2 preferred casein and histone. On the other hand, cyclic GMP-dependent protein kinase 3 and cyclic AMP-dependent protein kinase showed a higher reaction rate when protamine or histone was used as substrate protein. Tubulin could hardly be phosphorylated by all the four species of enzymes.

Effect of pH. Fig. 3 illustrates the effect of pH on the activities of the cyclic nucleotide-dependent protein kinases. In the case in which histone was used as the substrate protein, all the enzymes showed similar pH curves revealing their optima at

TABLE II

SUBSTRATE SPECIFICITY OF CYCLIC NUCLEOTIDE-DEPENDENT PROTEIN KINASES

The enzyme activity was assayed with the standard assay mixture except for the substrate proteins (1 mg/ml). If indicated, the reaction mixture contained either 3 μM cyclic GMP for cyclic GMP-dependent protein kinase 1, 2 and 3 or 3 μM cyclic AMP for cyclic AMP-dependent protein kinase, respectively. Porcine brain tubulin was prepared with the method of Shelanski et al. [17] followed by further purification with Sephadex G-200 gel filtration (Kuriyama, unpublished). 0.010, 0.019, 0.024 and 0.029 unit of enzymes were used per assay for cyclic GMP-dependent protein kinase 1, 2, 3 and cyclic AMP-dependent protein kinase, respectively.

Substrate	Relative activity (%)							
	Cyclic GMP-dependent protein kinase						Cyclic AMP-dependent protein kinase	
	1		2		3			
cyclic nucleotide present	−	+	−	+	−	+	−	+
Histone	4	100	4	100	10	100	4	100
Protamine	4	22	2	39	20	104	19	127
Casein	22	111	5	123	5	67	1	47
Tubulin	7	12	3	4	6	1	1	13

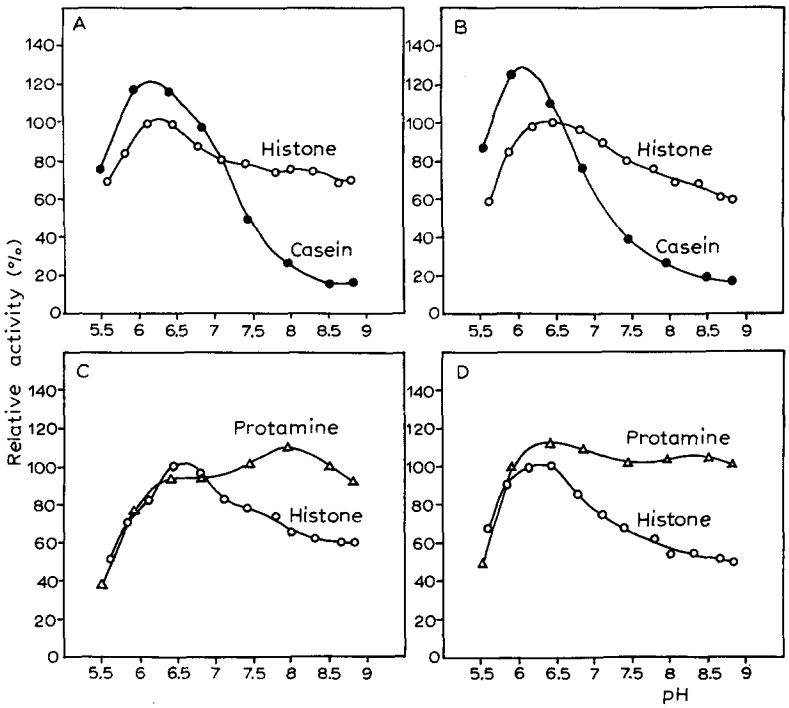

Fig. 3. Effect of pH on the enzyme activity. The enzyme activity was measured in the presence of 3 μM cyclic AMP for cyclic AMP-dependent protein kinase (D) or 3 μM cyclic GMP for cyclic GMP-dependent protein kinase 1 (A) 2 (B) and 3 (C), respectively, with the standard assay mixture except for the variation in pH of the potassium phosphate buffer (50 mM) and substrate protein (1 mg/ml) as indicated. 0.012, 0.017, 0.033 and 0.032 unit of enzymes were used for each assay of cyclic GMP-dependent protein kinase 1, 2, 3 and cyclic AMP-dependent protein kinase, respectively.

around pH 6.4. Casein was phosphorylated more rapidly than histone at lower pH and acted as a poorer substrate at higher pH. The pH optima for casein phosphorylation by cyclic GMP-dependent protein kinase 1 and 2 were equal to or slightly lower than that of the histone phosphorylation (Fig. 3A and B). On the other hand, cyclic GMP-dependent protein kinase 3 and cyclic AMP-dependent protein kinase phosphorylated protamine more than histone at a pH range between 7 and 8.5. At lower pH, the reaction rates of histone and protamine phosphorylation by the two enzymes were nearly equal (Figs. 3C and D).

Therefore, the pH curve of cyclic GMP-dependent protein kinase 1 resembled that of cyclic GMP-dependent protein kinase 2, and that of cyclic GMP-dependent protein kinase 3, cyclic AMP-dependent protein kinase.

Effects of cyclic AMP and cyclic GMP. The effects of cyclic AMP and cyclic GMP on the activities of the protein kinases were examined using histone as substrate protein. As mentioned later, it was revealed that cyclic GMP-dependent protein kinase 3 (DEAE-cellulose fraction) was contaminated with cyclic AMP-dependent protein kinase (about one fourth of the amount of the cyclic GMP-dependent protein kinase). To eliminate the contamination, cyclic GMP-dependent protein kinase 3 was

further purified with Sephadex G-200 gel filtration under lower ionic strength. The flow through fraction was condensed and used as the enzyme source of cyclic GMP-dependent protein kinase 3. As for the other three enzymes, the CM-Sephadex fraction for cyclic GMP-dependent protein kinase 1 and the DEAE-cellulose fractions for cyclic GMP-dependent protein kinase 2 and cyclic AMP-dependent protein kinase were used in this experiment.

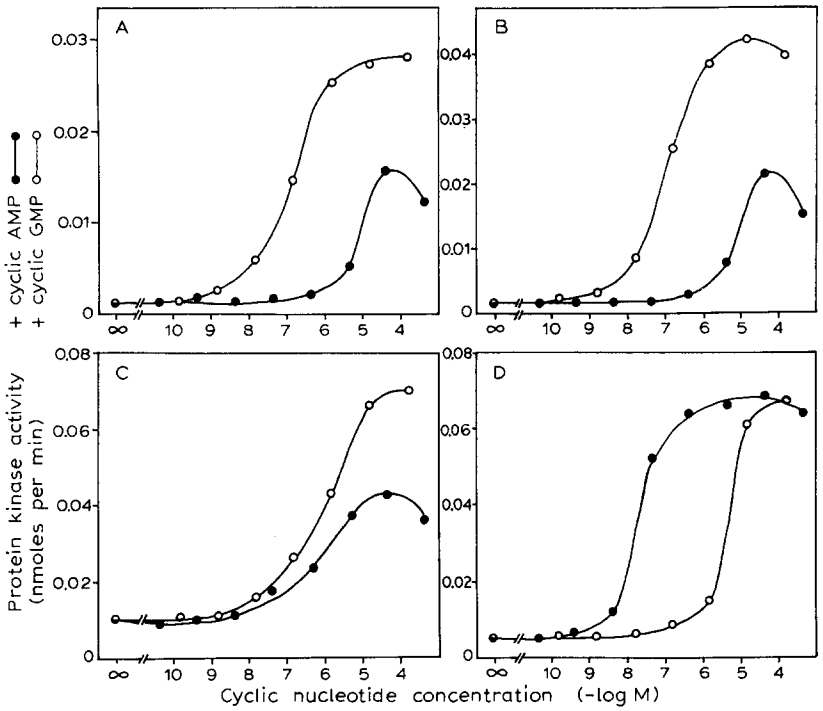

Fig. 4. Effect of various concentrations of cyclic AMP and cyclic GMP on the reaction rate. The enzyme activity was measured with the standard assay mixture including various concentrations of cyclic AMP or cyclic GMP as indicated. As enzyme sources, the CM-Sephadex fraction (cyclic GMP-dependent protein kinase 1, A) and the DEAE-cellulose fraction (cyclic GMP-dependent protein kinase 2 (B) and cyclic AMP-dependent (D)) were used. As for the assay of cyclic GMP-dependent protein kinase 3 (C) the enzyme fraction free from cyclic AMP-dependent protein kinase was prepared in the following manner (see Text); cyclic GMP-dependent protein kinase 3 (DEAE-cellulose fraction) was gel filtered on a Sephadex G-200 column equilibrated with 20 mM Tris–HCl buffer (pH 8.0) containing 0.1 mM dithiothreitol followed by condensation of the flow through fraction to yield the cyclic GMP-dependent protein kinase fraction essentially free from contamination of cyclic AMP-dependent protein kinase.

Figs 4A and B shows that cyclic GMP-dependent protein kinase 1 and 2 were preferentially activated by cyclic GMP. Half-maximal activation occurred in the order of 10^{-7} M and the responses of the two enzymes towards cyclic GMP and cyclic AMP were quite similar. On the other hand, cyclic GMP-dependent protein kinase 3 was less sensitive to cyclic GMP revealing half-maximal activation in a range of 10^{-6} M

(Fig. 4C). Cyclic AMP-dependent protein kinase was stimulated specifically by cyclic AMP at a lower concentration. Half-maximal activation was observed in the order of 10^{-8} M (Fig. 4D).

Molecular weight estimation. The molecular weight of cyclic AMP-dependent protein kinase was estimated with a Sephadex G-200 column equilibrated with 20 mM Tris–HCl buffer (pH 8.0) containing 0.1 mM dithiothreitol. The apparent molecular weight was about $6 \cdot 10^4$ (Fig. 5A).

When cyclic GMP-dependent protein kinase 1, 2 and 3 were gel filtered through a Sephadex G-200 column at a low ionic strength, all of the cyclic GMP-dependent

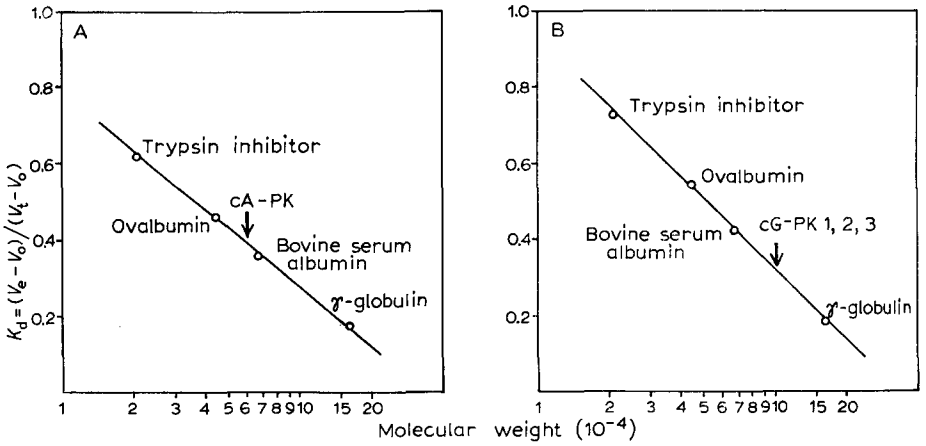

Fig. 5. Molecular weight estimation of the enzymes. The cyclic AMP-dependent protein kinase (DEAE-cellulose fraction, 0.16 mg) was gel filtered through a Sephadex G-200 column (1.7 cm × 54 cm) equilibrated with 20 mM Tris–HCl buffer (pH 8.0) containing 0.1 mM dithiothreitol (A). Cyclic GMP-dependent protein kinase 1 (CM-Sephadex fraction, 0.053 mg), cyclic GMP-dependent protein kinase 2 (DEAE-cellulose fraction, 0.087 mg) and cyclic GMP-dependent protein kinase 3 (DEAE-cellulose fraction, 0.21 mg) were dialyzed against 20 mM Tris–HCl buffer (pH 8.0) containing 0.6 M KCl and 0.1 mM dithiothreitol and loaded separately on a column of Sephadex G-200 column (1.7 cm × 54 cm) equilibrated with the same buffer (B). For each species of enzyme, the reaction rate was determined with a series of the standard assay mixtures containing 3 μM cyclic AMP, 3 μM cyclic GMP or none of the cyclic nucleotide in order to confirm whether each enzyme maintained its sensitivity to cyclic AMP or cyclic GMP. The details were described in the previous paper [14]. cA-PK, cyclic AMP-dependent protein kinase; cG-PK1, 2 and 3, cyclic GMP-dependent protein kinases 1, 2 and 3.

protein kinase activities were detected in the void volume. In the case of the gel filtration of cyclic GMP-dependent protein kinase 3, a minor peak of cyclic AMP-dependent protein kinase activity emerged at the position of molecular weight of about $6 \cdot 10^4$. These data suggested that the cyclic GMP-dependent protein kinases existed in the form of complexes or aggregates under low ionic strength. Therefore, gel filtrations were performed in the presence of 0.6 M KCl. In this case, the enzyme activities which had been detected at the void volume disappeared completely. The estimated molecular weights of cyclic GMP-dependent protein kinase 1, 2 and 3 were all about $1 \cdot 10^5$ (Fig. 5B).

DISCUSSION

It was shown in this paper that *Tetrahymena* cilia contained multiple species of cyclic nucleotide-dependent protein kinase; two of which (cyclic GMP-dependent protein kinase 1 and 2) were specifically activated by cyclic GMP, one (cyclic GMP-dependent protein kinase 3) was less specific to cyclic GMP than cyclic GMP-dependent protein kinase 1 and 2 and the last one (cyclic AMP-dependent protein kinase) was highly activated by a low concentration of cyclic AMP. Cyclic GMP-dependent protein kinase 1 and 2 were quite similar with respect to the pH curve, preference to substrate proteins, response to cyclic GMP and cyclic AMP and molecular weight, except for the chromatographic behavior. These two enzymes might be essentially the same species. On the other hand, cyclic GMP-dependent protein kinase 3 resembled cyclic AMP-dependent protein kinase in the specificity for substrate proteins and response to variation of pH. The former was more susceptible to cyclic GMP than cyclic AMP and the latter was highly specific for cyclic AMP. These data suggest the presence of a subtle control system of protein phosphorylation via a wide variation of cyclic AMP and cyclic GMP concentration in cilia.

The localization of these enzymes was considered to be in the soluble fraction or the membrane of cilia. When histone was used as substrate protein, more than 80% of cyclic AMP and cyclic GMP-dependent protein kinase activities could be extracted with 1% Triton leaving little cyclic nucleotide-dependent protein kinase activity in the axoneme. The physiological substrate of the enzymes are quite unclear at the present time. Further investigation must be performed on the precise characterization of the kinases and on their biological significance in the ciliary motile system.

ACKNOWLEDGEMENT

The author wishes to express his thanks to Professor H. Sakai for his valuable suggestions and discussions. And the author was greatly indebted to Mr M. Hoshino for his kind gift of the Triton extract of *Tetrahymena* cilia.

REFERENCES

1 Morton, B., Harrigan-Lum, J., Albagli, L. and Jooss, T. (1974) Biochem. Biophys. Res. Commun. 56, 372–379
2 Garbers, D. L., Lust, W. D., First, N. L. and Lardy, H. A. (1971) Biochemistry 10, 1825–1831
3 Hoskins, D. D. (1973) J. Biol. Chem. 248, 1135–1140
4 Rubin, R. W. and Filner, P. (1973) J. Cell Biol. 56, 628–635
5 Wolfe, J. (1973) J. Cell. Physiol. 82, 39–48
6 Gray, J. P., Hardman, J. G., Hammer, J. L., Hoos, R. T. and Sutherland, E. W. (1971) Fed. Proc. 30, 1267Abstr.
7 Morton, B. and Albagli, L. (1973) Biochem. Biophys. Res. Commun. 50, 697–703
8 Gray, J. P., Hardman, J. G., Bibring, T. and Sutherland, E. W. (1970) Fed. Proc. 29, 608 Abstr.
9 Hardman, J. G., Beavo, J. A., Gray, J. P., Chrisman, T. D., Patterson, W. D. and Sutherland, E. W. (1971) Ann. N.Y. Acad. Sci. 185, 27–35
10 Hoskins, D. D., Casillas, E. R. and Stephens, D. T. (1972) Biochem. Biophys. Res. Commun. 48. 1331–1338
11 Garbers, D. L., First, N. L. and Lardy, H. A. (1973) J. Biol. Chem. 248, 875–879
12 Lee, M. Y. W. and Iverson, R. M. (1972) Exp. Cell Res. 75, 300–305

13 Lee, M. Y. W. and Iverson, R. M. (1973) J. Cell Biol. 55, 150a
14 Murofushi, H. (1973) Biochim. Biophys. Acta 327, 354–364
15 Stephens, R. E. and Levine, E. E. (1970) J. Cell Biol. 46, 416–421
16 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
17 Shelanski, M. L., Gaskin, F. and Cantor, C. R. (1973) Proc. Natl. Acad. Sci. U.S. 70, 765–768