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AN ADENOSINE 3′:5′ MONOPHOSPHATE DEPENDENT PROTEIN KINASE FROM SEA URCHIN SPERMATOZOA *

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Summary

A cyclic AMP dependent protein kinase (EC 2.7.1.37) from sea urchin sperm was purified to near homogeneity and characterized. A 68-fold purification of the enzyme was obtained. This preparation had a specific activity of 389 000 units/mg protein with protamine as the substrate. On the basis of the purification required, it may be calculated that the protein kinase constitutes as much as 1.5% of the soluble protein in sperm. There appeared to be a single form of the enzyme in sea urchin sperm, based on the behavior of the enzyme during DEAE-cellulose and Sephadex G-200 column chromatography. Magnesium ion was required for enzyme activity. The rate of phosphorylation of protamine was stimulated 2.5-fold by an optimal concentration of 0.9 M NaCl. The K_m for ATP (minus cyclic AMP) was 0.119 ± 0.013 (S.D.) and 0.055 mM ± 0.009 (S.D.) in the presence of cyclic AMP. The specificity of the enzyme toward protein acceptors, in decreasing order of phosphorylation, was found to be histone f1 protamine, histone f2b, histone f3 and histone f2a; casein and phosvitin were not phosphorylated. The holoenzyme was found to have an apparent molecular weight of 230 000 by Sephadex G-200 chromatography. In the presence of $5 \cdot 10^{-6}$ M cyclic AMP, the holoenzyme was dissociated on Sephadex G-200 to a regulatory subunit of molecular weight 165 000 and a catalytic subunit of M_r 73 000. The dissociation could also be demonstrated by disc gel electrophoresis in the presence and absence of cyclic AMP.

Introduction

Adenosine 3′ : 5′ monophosphate-dependent protein kinases (EC 2.7.1.37) which catalyze the phosphorylation from ATP of casein, protamine and histone

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Abbreviation: EGTA, ethyleneglycol-bis-(β-aminoethylether)-*N,N′*-tetraacetic acid.

have been found in different eucaryotic and procaryotic cells [1—4]. Protein kinase activity is comparatively high in the trout testis [5]. More recently, Garbers et al. [6] and Hoskins et al. [7] have both reported cyclic AMP-dependent protein kinase in bovine epididymal spermatozoa. In our laboratory, we have found that the activity of protein kinase in the sperm of the sea urchin *Lytechinus variegatus* was 30-fold higher than in oocytes or gastrulae embryos [8]. The relatively high levels of protein kinase in the sea urchin sperm suggest that the enzyme may play an important role in gametogenesis, regulation of sperm motility or the fertilization process itself. We have therefore characterized the sea urchin sperm protein kinase as a part of a continuing study.

Materials

All chemicals used in this study were reagent grade; cyclic AMP, cyclic GMP, salmon protamine and calf thymus histone mixtures were obtained from Sigma Chemical Company. Histone fractions, f1, f2a, f2b, f3 were obtained from Worthington Biochemical Company. Calcium phosphate gels were prepared by the method of Colowick [9].

Cyclic [^{3}H]AMP (24.4 Ci/mmol) was purchased from New England Nuclear Company. [γ-^{32}P]ATP (100—700 cpm/pmol) was prepared as reported by Soderling et al. [10].

The gametes from local sea urchins were obtained by injecting 1 ml of isotonic KCl (0.56 M) into the perivisceral cavity. The male sea urchin was then placed aboral surface down to shed into a Syracuse watch glass [11]. The pooled sperm were washed twice after suspension in Millipore-filtered sea water by centrifugation at 4000 × *g* for 5 min. The pelleted sperm were stored at —20°C until used.

Methods

Extracts of the sperm were prepared by suspending 2 vols. of cells in 1 vol. of buffer (50 mM Tris · HCl, 5 mM magnesium chloride, 1 mM dithiothreitol, pH 7.4) followed by sonication for 2 min. The homogenates were centrifuged at 20 000 × *g* for 30 min. An aliquot of the supernatant was reserved for analysis and the remainder was centrifuged at 200 000 × *g* for a preliminary purification of the enzyme.

DEAE-Cellulose chromatography. Pre-swollen DE-52 was obtained from Whatman Company. The 200 000 × *g* supernatant, 41 ml at a concentration of 1 mg of protein per ml, was applied onto a column (1.6 cm × 11 cm) of DE-52 previously equilibrated with 0.05 M Tris · HCl, 5 mM $MgCl_2$, and 1 mM dithiothreitol (dithiothreitol buffer, pH 7.4). The column was washed with dithiothreitol buffer and eluted with a linear gradient of 0—0.4 M NaCl in a total volume of 200 ml (Fig. 1).

Ammonium sulfate concentration. The fractions 42—48 (Fig. 1) of the DEAE-cellulose column containing the enzyme activity were then combined. The material was concentrated by the addition of ammonium sulfate to 70% saturation. After centrifugation at 20 000 × *g* for 30 min, the precipitate was

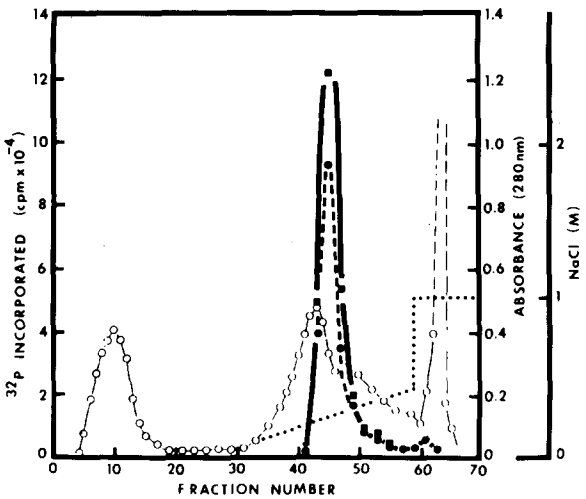

Fig. 1. DEAE-Cellulose chromatography of sea urchin sperm protein kinase. About 40 mg of the ultracentrifuged protein was applied onto the column as indicated in the Material and Methods. ○———○ absorbance 280 nm; ■———■, in the presence of $5 \cdot 10^{-6}$ M cyclic AMP; ●- - - -● in the presence of $4 \cdot 10^{-4}$ M cyclic GMP; . . . . . NaCl concentration.

suspended in 2 ml of dithiothreitol buffer and dialyzed overnight against the same buffer.

Sephadex G-200 gel filtration. The dialyzed material (2 ml) was applied to a Sephadex G-200 column (2.5 cm × 32 cm) equilibrated with dithiothreitol buffer and 0.1 M KCl. The active fractions were pooled and concentrated by ultrafiltration. The enzyme was eluted as a single peak of activity, as shown in the results of an earlier experiment (Fig. 2).

Calcium phosphate gel fractionation. After ultracentrifugation, the enzyme was absorbed onto calcium phosphate gel and fractionated according to the

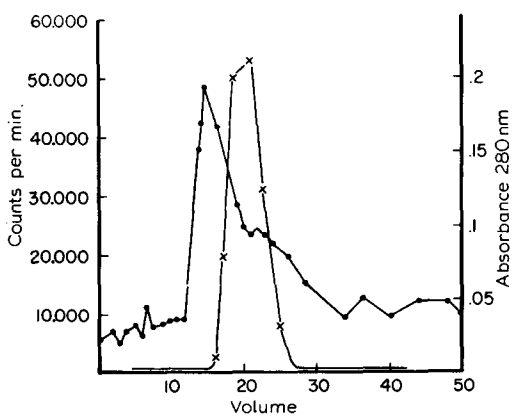

Fig. 2. Sephadex G-200 chromatography of sea urchin sperm protein kinase. 2 ml of the $(NH_4)_2SO_4$ concentrated and redissolved enzyme was applied to the column. ●———● absorbance 280 nm; X———X protein kinase activity in the presence of $5 \cdot 10^{-6}$ M cyclic AMP.

method of Kuo et al. [12]. The amount of gel added was about 5 mg of dry gel per absorbance unit (280 nm) of the enzyme. The cyclic GMP-dependent protein kinase activity was eluted with two washings of 50 mM sodium phosphate buffer containing 2 mM EDTA pH 7.0 (eluate 1), and the cyclic AMP-dependent enzyme was eluted with two washings of 200 mM sodium phosphate containing 2 mM EDTA, pH 7.0 buffer (eluate 2).

Disc gel electrophoresis. The gel buffer system used was based on that described by Davis et al. [13] using a Buchler disc gel electrophoresis apparatus. Polyacrylamide gels (7%) were cast in a buffer containing 30 mM HCl adjusted to pH 7.9 with 2 M Tris base. They were then soaked in 5 mM dithiothreitol in the same buffer overnight. Samples to be run were dialyzed for 2 h against the same buffer consisting of 0.25 M HCl adjusted to pH 5.9, with 2 M Tris base and 20% glycerol. Bromophenol blue was used as the tracking dye. Duplicate 100 μl samples were loaded onto the gels. The electrophoresis was carried out at 4°C for 1 h at a current of 2 mA per tube.

One of the gels were stained with Coomassie blue and the other cut into 2 mm thick slices. Protein kinase was eluted from the slices by placing them in 150 μl of 0.05 M Tris · HCl, 5 mM $MgCl_2$, and 1 mM dithiothreitol buffer (pH 7.5) overnight at 0°C. 20 μl were used for protein kinase assays and 100 μl were used for the assay of cyclic AMP binding. Each gel slice was individually analyzed.

Protein concentration. This was determined by the method of Lowry et al. [14] with bovine serum albumin as a standard.

Protein kinase assays. These were done as previously described [15].

Cyclic [^{3}H]AMP binding assays. The assay of cyclic AMP binding activity was done according to the method of Tao et al. [16]. The binding mixture, contained in a final volume of 0.11 ml, was 2 μmol of Tris · HCl pH 7.5, 1 μmol $MgCl_2$, 20 pmol of cyclic [^{3}H]AMP (1600 cpm/pmol) and various amounts of protein kinase. The mixture was incubated at 37°C for 3 min and all subsequent procedures were carried out at 0°C. The binding mixture, after dilution with cold 0.02 M Tris · HCl, 10 mM $MgCl_2$ pH 7.5, was passed through Millipore filters (HA 0.45-μ pore size) which had been pre-equilibrated with 10 ml of the cold Tris · HCl, $MgCl_2$ buffer. The filters were then washed with 20 ml of the cold buffer and placed in a vial containing 10 ml of Aquasol for counting.

Results

Purification

Sperm was obtained from male sea urchin *L. variegatus* and sonicated to prepare a crude extract. All procedures were carried out at 0—4°C. As a freeze-thaw cycle of the pelleted sperm gave an extract of a higher specific activity (about 2.5 times) than extracts obtained from freshly collected unfrozen sperm [17], it was included in the procedure. The 20 000 × *g* supernatant was centrifuged for 90 min at 200 000 × *g*; this usually resulted in a small increase in total activity, probably due to the removal of ATPase or a kinase inhibitor.

Purification was carried out as described in the methods. The overall purification was 68-fold with a specific activity of 389 000 units/mg protein and the

TABLE 1

PURIFICATION OF SEA URCHIN SPERM PROTEIN KINASE

Purification step	Protein (mg)	Activity * (units)	Specific activity (units/mg)	Purification (fold)	Yield (%)
20 000 × *g* supernatant after freeze-thaw cycle of pelleted sperm	53.3	729 000	14 800	2.5 **	
200 000 × *g* supernatant	39.7	902 000	22 700	3.9	100
DEAE-cellulose	5.0	458 000	90 900	15.8	50
Ammonium sulfate	4.7	412 000	80 300	15.2	45
Sephadex G-200	0.6	232 000	389 000	67.8	26

* 1 unit = 1 pmol ^{32}P incorporated/min.

** Extract prepared from unfrozen sperm gave a specific activity of 5700 units/mg.

recovery was 26% (Table I). One unit of protein kinase activity was defined as that amount of enzyme which transfers one pmol of [^{32}P] from [γ-^{32}P]ATP to protein substrate per min.

Following the Sephadex G-200 filtration, the enzyme migrated as one major band with a faint, fast moving band after 7% polyacrylamide disc gel electrophoresis (Fig. 3). The enzyme activity was associated with the major electrophoresis band. The minor band may represent partially dissociated protein kinase.

Fig. 3. Disc gel electrophoresis of sea urchin sperm protein kinase. The enzyme was purified to the Sephadex G-200 step, concentrated, and 5 μg of enzyme in 100 μl were put onto the gel.

TABLE II

SPECIFIC ACTIVITIES OF PURIFIED PROTEIN KINASES

1 unit = 1 pmol [^{32}P] transferred/min.

Source	Specific activity (units/mg protein)	Purification (fold)	Reference
Beef heart	807 000	1220	Rubin et al. 1972 (27)
Bovine brain	271 449	155	Miyamoto et al. 1973 (34)
Sea urchin sperm	389 000	68	Lee and Iverson 1972 (8)
Bovine sperm	36 000	19	Hoskins et al. 1972 (7)
Bovine sperm (3 forms)	20 000—40 000	30	Garbers et al. 1973 (6)

The Sephadex G-200 eluate, if kept at 4°C in 0.5 mg bovine serum albumin/ml, had only 30% of the initial activity after 6 days, 15% after 2 weeks and 8% after 3 weeks.

The 68-fold purification required for the isolation of sea urchin sperm protein kinase indicates that the enzyme comprises as much as 1.5% of the total soluble protein. By comparison, enzyme data from beef heart muscle (Table II) suggests that protein kinase constitutes less than 0.1% of the soluble protein in mammalian tissue. That protein kinase from sea urchin sperm constitutes an appreciable fraction of the soluble protein, confirms our earlier observations of high levels of protein kinase activity in the sperm [18]. It may be that sperm will be the source of choice to investigators who wish to study the enzymology of protein kinase, for the enzyme is relatively easy to isolate from this source.

We have also assayed bovine sperm extracts obtained from frozen bull semen. As noted earlier, freezing of sea urchin sperm resulted in a large increase in specific activity. Therefore, data from frozen and unfrozen sea urchin sperm is included in Table III. Protein kinase levels for sea urchin sperm and bovine sperm, using our assay technique, gave results which are much higher than those found in nonreproductive tissues by others, ranging from one to three orders of magnitude higher [19,20,21]. Sperm or testicular extracts are clearly rich in protein kinase activity. Our own observations and the recent studies of the bovine sperm enzyme [6,7], suggest that high levels of protein kinase may be a general phenomenon extending to sperm of other organisms.

TABLE III

PROTEIN KINASE ACTIVITY IN CRUDE EXTRACTS OF TESTIS AND SPERM

Assays were carried out on 200 000 × *g* supernatants prepared as described in Methods.

Source of tissue	Protein kinase activity (units/mg Protein)
Rat testis	8 200
Bovine sperm from semen	33 000 *
Sea urchin sperm	20 000 *
	6 790

* Sperm were subjected to a freeze-thaw cycle prior to sonication.

Hoskins et al. [7] and Garbers et al. [6] reported a 19 and 30-fold purification of protein kinase from bovine epididymal spermatozoa (Table II). Garbers et al. [6] suggested that the enzyme consists of as much as 10% of the soluble protein. This estimate may be high for the homogeneity of the preparations was not established. The results are also complicated by multiple forms of protein kinase, Hoskins et al. [7] found two forms and Garbers et al. [6] three forms in bovine sperm as opposed to a single form in the sea urchin sperm (Figs. 1 and 2). The multiple forms may be due to proteolysis, as opposed to their real nature in vivo.

Assay

A summary of some of the properties important to the assay of protein kinase in the sea urchin sperm is shown in Table IV. The enzyme required 1 mM dithiothreitol and 0.6 mM EGTA for maximal activity. It was optimally stimulated by 0.9 M NaCl. This effect was only seen when protamine was used as the substrate and not when histone was used; thus, it appeared to be a substrate-directed effort. This salt effect is similar to that observed with trout testis protamine kinase [5]. Na_2SO_4 (0.1 M) could replace the 0.9 M NaCl (Table IV), showing that the enzyme was affected by high ionic strength. Mg^{2+} was required for enzyme activity, which was optimal at 20 mM. This seems somewhat high, since the ATP concentration used was only 0.2 mM; however, it may be that the enzyme has a requirement for Mg^{2+} in addition to that required for the formation of the MgATP complex [22]. Other divalent cations such as Mn^{2+}, Ca^{2+} and Cu^{2+} were ineffective.

Time course

The assays were normally carried out at pH 7.7 in the absence or presence of $5 \cdot 10^{-6}$ M cyclic AMP using protamine as the protein substrate. These conditions had previously been shown to be optimal for the assay of sea urchin protein kinase [15]. The phosphorylation of protamine by crude extracts became rapidly non-linear with respect to time, whereas after ultracentrifugation it remained linear for a longer period. A possible cause for lengthening the time of linearity of phosphorylation is the removal of an ATPase during ultracentrifugation. Phosphorylation of protein by the purified enzyme was linear at least

TABLE IV

ASSAY SYSTEM FOR PROTEIN KINASE

	Relative activity
Complete	100
— Dithiothreitol (1 mM)	93
— EGTA (0.6 mM)	33
— NaCl (0.9 M)	43
— NaCl + Na_2SO_4 (0.1 M)	84
— Mg^{2+} (18 mM) + EGTA (6 mM)	9
— Mg^{2+} + Ca^{2+} or Mn^{2+}	0
— Protamine	0
+ NaF (5 mM)	69
+ Theophylline	74

10 min and was stimulated 4-fold by cyclic AMP. When different concentrations (1—6 μg) of enzyme were used in the presence or absence of cyclic AMP, the reaction was linear with increasing enzyme concentration. The fact that activity was found in the absence of cyclic AMP suggests a partially dissociated enzyme. However, it has been shown that protamines and histones can cause dissociation and activation of protein kinase [23,24].

Effect of varying concentrations of ATP

The effect of varying concentrations of ATP on protein kinase activity in the absence and presence of cyclic AMP is shown in Fig. 4. Values obtained in the presence of cyclic AMP were significantly lower than in its absence. The K_m for ATP in the absence of cyclic AMP was 0.119 mM ± 0.013 (S.D.), and in the presence of cyclic AMP and 0.055 mM ± 0.009 (S.D.) The significantly different K_m values were obtained from fitted curves using the computer program of Hanson et al. [25] as adapted and revised by Dr. J.F. Woessner (University of Miami). One would expect identical K_m values in the presence or absence of cyclic AMP (Fig. 4). It may be that cyclic AMP has a separate allosteric effect on the catalytic subunit as well as causing dissociation of the holoenzyme.

Specificity of cyclic nucleotide activation

We have previously shown that extracts of sea urchin sperm contained a protein kinase activated by both cyclic AMP and cyclic GMP. Maximal stimulation was observed with 10^{-6} M cyclic AMP, whereas a cyclic GMP concentration of 10^{-4} M was required for maximal stimulation [15]. The question arose as to whether one or two protein kinases were present in our enzyme preparation. Examination of the cyclic GMP dependent activity during the DEAE-cellulose chromatography (Fig. 1) showed that it co-migrated with cyclic AMP-dependent activity. In addition, determination of the ratio of cyclic GMP to cyclic AMP dependent activities throughout the purification procedure showed that this remained constant (Table V). Further, the two cyclic nucleotide activities were found to exhibit similar heat-inactivation curves (not shown).

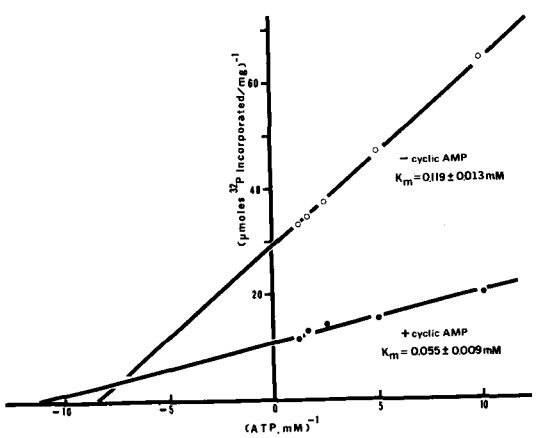

Fig. 4. Double reciprocal plot showing the effect of ATP concentration on protein kinase activity in the absence or presence of cyclic AMP. ○———○ in the absence of cyclic AMP; ●———● in the presence of $5 \cdot 10^{-6}$ M cyclic AMP.

TABLE V

RATIO OF CYCLIC GMP TO CYCLIC AMP-DEPENDENT PROTEIN KINASE ACTIVITIES

The results were expressed as counts per minute. 2—16 μg of enzyme were used.

	Cyclic AMP ($5 \cdot 10^{-6}$ M)	Cyclic GMP ($4 \cdot 10^{-4}$ M)	G/A
Crude extract	2473	2125	0.85
Ultracentrifuged	5751	4865	0.85
DEAE-Cellulose	1863	1558	0.84
Sephadex G-200	2752	2325	0.84

Kuo et al. [12] described the separation of two types of protein kinase from Arthropoda. Both were stimulated by cyclic AMP and cyclic GMP, but one had an optimal response to low concentrations of cyclic AMP, and the other had optimal response to low concentrations of cyclic GMP. We used their calcium phosphate gel procedure to examine crude extracts of sea urchin sperm to confirm that the activity we studied was of the cyclic AMP dependent type (Table VI). According to Kuo et al. [12], the cyclic AMP dependent protein kinase should appear in eluate 2. Over 99% of the total activity in the crude extract did appear in this eluate and had the same response to the cyclic nucleotides as the purified enzyme (Table VI). However, eluate 1 did contain a small amount of activity (0.17% relative to eluate 2), which behaved like the cyclic GMP dependent protein kinase of Kuo et al. [12]. Maximal response to cyclic GMP was seen at 10^{-6} M, while 100-fold higher levels of cyclic AMP were required for maximal stimulation. Thus, there appeared to be a small, but identifiable amount of a cyclic GMP dependent protein kinase in the sea urchin sperm.

Specificity of protein acceptors

Table VII shows the ability of protein kinase to phosphorylate different substrates. The activity with protamine, routinely used as the substrate, was employed as the standard rate (100%) of phosphorylation. 100 μg of substrate per 100 μl of incubation mixture were used in each case. The specificity towards a series of calf thymus histones as classified by the terminology of Johns [26] were used. The lysine rich histones (f1, f2b) are the best substrates, while

TABLE VI

CALCIUM PHOSPHATE GEL SEPARATION OF CYCLIC GMP AND CYCLIC AMP DEPENDENT PROTEIN KINASES FROM SEA URCHIN SPERM

Fraction	Total protein kinase activities						
	No addition	Cyclic GMP			Cyclic AMP		
		(A) 10^{-6} M	(B) 10^{-4} M	Ratio (A)/(B)	(A) 10^{-6} M	(B) 10^{-4} M	Ratio (A)/(B)
Eluate 1	12	162	142	1.1	38	185	0.21
Eluate 2	4810	23 400	80 000	0.29	91 600	71 410	1.28

TABLE VII

SPECIFICITY TOWARD PROTEIN ACCEPTORS

100 μg of each substrate were used per 100 μl of incubation mixture.

Substrate	Relative activity
Protamine sulfate	100
Calf thymus histones:	
mixture	7.9
f1 (very lysine rich)	114
f2b (slightly lysine rich)	65
f3 (arginine rich)	8.3
f2a (arginine rich)	7.8
Whole histone, sea urchin gastrulae	15.7
Sea urchin sperm, histone f2b	224
Casein	0
Phosvitin	0

the arginine rich and unfractionated histones (f3, f2a) are poor substrates. Whole histones extracted from isolated sea urchin gastrula chromatin, and purified f2b histone from the sperm of *Strongylocentrotus purpuratus* (gifts from Dr. M.E. McClure, University of Texas) were tested as substrates. The sea urchin derived substrates are better than those from the mammalian source. The acidic proteins, casein and phosvitin, were not phosphorylated.

Physical properties and mechanism of response to cyclic AMP

Sephadex G-200 chromatography. 2 ml of the ultracentrifuged sample was passed through a Sephadex G-200 column (2.5 cm × 32 cm). The protein

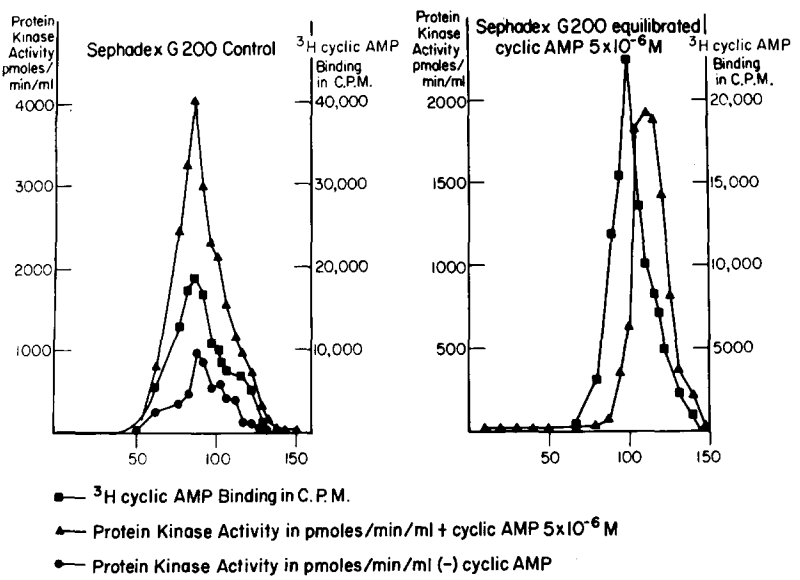

Fig. 5. Behavior of protein kinase on Sephadex G-200 in the absence and presence of cyclic AMP. Details are described in the text.

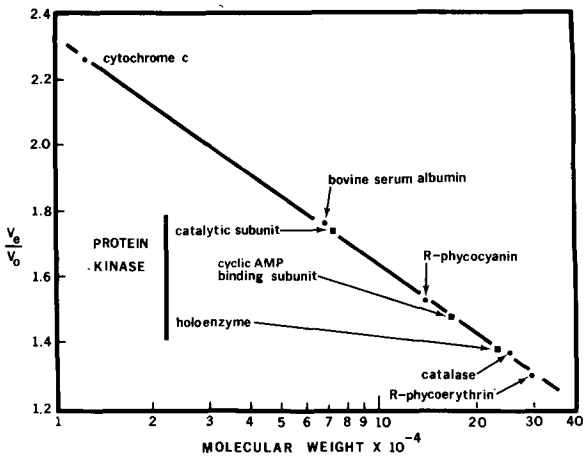

Fig. 6. Molecular weight determination of protein kinase and its subunits by Sephadex G-200 column chromatography.

kinase activity and the cyclic AMP binding activity were eluted in the same peak (Fig. 5). The same Sephadex G-200 column was then equilibrated with $5 \cdot 10^{-6}$ M cyclic AMP. To 2 ml of the ultracentrifuged sample cyclic AMP was added ($1 \cdot 10^{-6}$ M final concentration) and preincubated at 0°C for 20 min. When this sample was passed through the column, it was found that the protein kinase activity was dissociated from the cyclic AMP binding activity (Fig. 5).

The column was calibrated with proteins of known molecular weights (Fig. 6): phycoerythrin, 290 000; catalase, 250 000; R-phycocyanin, 138 000; bovine serum albumin, 67 000; and cytochrome *c*, 12 400. V_e (elution volume) was determined by reading the maximum absorption peak of the standards. Blue dextran was used to determine the V_0 (void volume). The molecular weight of sperm protein kinase holoenzyme was estimated to be 230 000. In the presence of cyclic AMP, the molecular weight of the regulatory subunit was found to be 165 000 and the catalytic subunit was 73 000. This suggests a 1 : 1 stoichiometry between the two types of subunits.

The stoichiometry of the regulatory (R) and catalytic (C) subunits have been clearly elucidated from the beef heart protein kinase [27]. The enzyme was earlier found to have a M_r of 240 000, by Sephadex chromatography, and could be dissociated into a C subunit of 30 000 and a R subunit of 170 000 [27]. These data are similar to ours. In a more recent study, Rosen et al. [28] reported a M_r of 170 000. The R subunit is a dimer of 98 000, while the C subunit is a dimer of 76 000. The subunit composition was 2C2R. The inconsistency of these data with gel filtration data was explained on the basis that the R subunit is very asymmetrical, with an axial ratio of about 12 [28]. Molecular weights determined by gel filtration are based on the assumption that the proteins are globular but are actually related directly to Stokes radii. An asymmetric protein will, therefore, give a higher apparent molecular weight when globular proteins are used as standards. Further studies will be needed to establish the exact subunit structure of the sea urchin enzyme.

Disc gel electrophoresis. Further confirmation for the dissociation of the

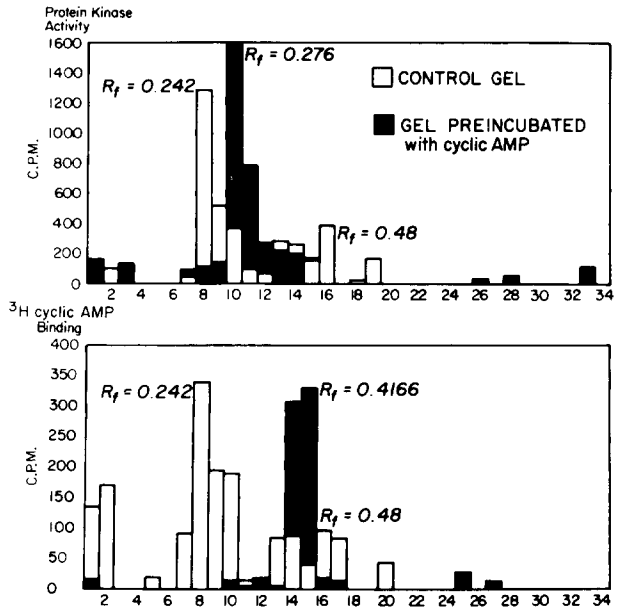

Fig. 7. Disc gel electrophoresis of the purified enzyme in the absence or presence of cyclic AMP. The upper diagram is protein kinase activity. The lower diagram is cyclic [^{3}H] AMP-binding activity.

catalytic and regulatory subunits by cyclic AMP was shown by electrophoresis on 7% disc gels. An enzyme preparation purified to the Sephadex G-200 step was used. After the gels were run, 34 slices were cut and each slice assayed for both protein kinase and cyclic AMP binding activity. In the control gel, the protein kinase activity and the cyclic AMP binding activity migrated at the same time (R_m 0.24). When the enzyme had been preincubated with cyclic AMP, the kinase activity migrated at a slightly faster rate (R_m 0.28) whereas the regulatory subunit had a R_m of 0.42 (Fig. 7). These data confirmed that cyclic AMP leads to dissociation of the holoenzyme into cyclic AMP binding and catalytic subunits.

Discussion

These studies have confirmed our original observations that sea urchin sperm contained high levels of protein kinase activity; the degree of purification required to bring the enzyme to near-homogeneity indicates that protein kinase does in fact constitute as much as 1.5% of the soluble protein in sea urchin sperm. The properties of the purified enzyme appear to be similar to those described for protein kinases from other sources. The major question which remains to be resolved is the specific function(s) of this enzyme in the sperm. It seems unlikely that such a specialized cell would retain such high levels of an enzyme unless it served some special function. It may be noted that this work, as well as that of others [6,29] indicates that high levels of protein kinase are present in the sperm of higher organisms as well. It has been suggested that cyclic nucleotides may control the onset of sperm motility [6]. In this regard,

it may be speculated that protein phosphorylation may be responsible for the activation of energy metabolism, such as has been described for glycogen and lipid metabolism [3]. Since Mann [30] had indicated that there are no detectable glycogen reserves in sperm it is of interest that Hoskins et al. [29] have recently reported that cyclic AMP increases fructolysis in bovine sperm. Alternatively, it may be speculated that protein kinase may affect or control the contractile elements of the sperm, i.e. tubulin [21]. Relevant to this possibility is the fact that tubulin from rat brain has been shown to be a phosphoprotein [32]. Recent studies have also shown that elements of the mammalian muscle contractile system can be phosphorylated in vitro by protein kinases [33], although the role of phosphorylation in the control of the contractile process remains unknown.

Preliminary work in this laboratory also showed that spermidine and spermine isolated from the sea urchin *Strongylocentratus purpuratus* (gifts from Dr. Diane H. Russell, National Cancer Institute, Baltimore) at the concentration of $1 \cdot 10^{-5}$ M inhibited the enzyme activity by 50 and 23% respectively. To our knowledge, this is the first observation that the polyamines, spermine and spermidine inhibit protein kinase activity. It is not possible at this point to postulate the physiological role of the polyamines in the sea urchin with relation to protein kinase activity. Further studies are needed to clarify this point.

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