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Novel Adenosine 3′,5′-Cyclic Monophosphate Dependent Protein Kinases in a Marine Diatom†

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Received December 27, 1988; Revised Manuscript Received April 7, 1989

ABSTRACT: Two novel adenosine 3′,5′-cyclic monophosphate (cAMP) dependent protein kinases have been isolated from the diatom *Cylindrotheca fusiformis*. The kinases, designated I and II, are eluted from DEAE-Sephacel at 0.10 and 0.15 M NaCl. They have a high affinity for cAMP and are activated by micromolar cAMP. They exhibit maximal activity at 5 mM Mg^{2+} and pH 8 with the preferred phosphate donor ATP (K_m = 0.1 mM) and phosphate acceptor histone H1 (K_m = 0.2 μM). They phosphorylate sea urchin sperm histone H1 on a single serine site in the sequence Arg-Lys-Gly-Ser(^{32}P)-Ser-Asn-Ala-Arg and have an apparent M_r of 75 000 as determined by gel filtration and sucrose density sedimentation. In the kinase I preparation a single protein band with an apparent M_r of about 78 000 is photolabeled with 8-azido[^{32}P]cAMP and is also phosphorylated with [γ-^{32}P]ATP in a cAMP-dependent manner, after autoradiography following sodium dodecyl sulfate gel electrophoresis. The rate of phosphorylation of the 78 000-dalton band is independent of the enzyme concentration. The photoaffinity labeling and cAMP-dependent phosphorylation also occur on a 78 000-dalton protein in the kinase II preparation. The catalytic activities of the two kinases cannot be freed by cAMP affinity column chromatography. The apparent size of the kinases remains unchanged upon preincubation with 0.5 mM cAMP followed by sucrose density sedimentation in the presence of the nucleotide. The results indicate that (i) these diatom cAMP-dependent protein kinases are monomeric proteins, possessing both the cAMP-binding regulatory and catalytic domains on the same polypeptide chain, (ii) the enzymes do not dissociate into smaller species upon activation by binding cAMP, and (iii) self-phosphorylation of the enzymes by an intrapeptide reaction is cAMP dependent. The two diatom cAMP kinases are refractory to the heat-stable protein kinase modulator from rabbit muscle, but they respond differently to proteolytic degradation and to inhibition by arachidonic acid and several microbial alkaloids.

There is mounting evidence that the physiological action of adenosine 3′,5′-cyclic monophosphate (cAMP)[1] and cGMP in eukaryotic cells is mediated through the activation of cAMP-dependent and cGMP-dependent protein kinases (cAMP and cGMP kinases) (Walsh et al., 1968; Kuo & Greengard, 1969). These enzymes have similar substrate specificity, amino acid composition, self-phosphorylation, and mechanism of activation (Rosen et al., 1977; Edelman et al., 1987). They also share a conserved catalytic core with many other proteins such as tyrosine kinase, protein kinase C, myosin light chain kinase, the epidermal growth factor receptor, and the transforming protein $p60^{v\text{-}src}$ (Gill & McCune, 1979; Taylor, 1987). These characteristics have led many investi-

† This work was supported by the National Science Foundation (Grant DCB-8417815).
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[1] Abbreviations: cAMP, adenosine 3′,5′-cyclic monophosphate; cGMP, guanosine 3′,5′-cyclic monophosphate; cAMP kinase, cAMP-dependent protein kinase; cGMP kinase, cGMP-dependent protein kinase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HPLC, high-performance liquid chromatography; PMSF, phenylmethanesulfonyl fluoride; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; BSA, bovine serum albumin; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; TCA, trichloroacetic acid; kDa, kilodalton(s); K-252a, (8*R**,9*S**,11*S**)-(−)-9-hydroxy-9-(methoxycarbonyl)-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1*H*,8*H*,11*H*-2,7b,11a-triazadibenzo[*a*,*g*]cycloocta[*cde*]trinden-1-one.

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gators to propose that the cAMP and cGMP kinases belong to a related family of proteins that have evolved from a common ancestor (Gill & McCune, 1979; Lincoln & Corbin, 1983; Taylor, 1987). Although the cAMP and cGMP kinases are closely related, they are distinguished from each other by a number of structural and functional features. The cAMP kinases generally contain two cAMP-binding regulatory (R) subunits and two catalytic (C) subunits as tetrameric holoenzymes (Rosen et al., 1977; Edelman et al., 1987). The M_r values of several isoenzymic forms of the R subunit are about 45000, and that of the C subunit is about 40000. Activation by binding cAMP involves the reversible dissociation of the holoenzyme to yield the active C subunit (Rosen et al., 1977; Edelman et al., 1987) as expressed in eq 1.

$$R_2C_2 + 4cAMP \rightleftharpoons (R\ 2cAMP)_2 + 2C \quad (1)$$

$$(RC)_2 + 4cGMP \rightleftharpoons (2cGMP\ RC)_2 \quad (2)$$

In contrast, native mammalian cGMP kinases are homodimeric proteins containing the R and C domains on the same polypeptide chain; the holoenzymes have M_r of about 150000, and the M_r 75000 monomers are partially held together by intermolecular disulfide bonds (Gill et al., 1976, 1977; Takai et al., 1976). The enzyme is activated merely by cGMP binding (eq 2). Both the cAMP and cGMP kinases can catalyze maximal self-phosphorylation without activation by cAMP or cGMP (Rosen et al., 1977; Lincoln & Corbin, 1983; Edelman et al., 1987). Three unusual cyclic nucleotide dependent protein kinases have been reported in the literature. Like the cGMP kinase, an enzyme isolated from grasshoppers behaves as a dimeric protein, but has high affinity for both cAMP and cGMP (Vardanis, 1980). A second enzyme, from a nematode, has a high affinity for cAMP, but it apparently does not dissociate into subunits upon chromatography by a cAMP affinity column (Vardanis, 1984). The structural nature of the latter enzyme is unclear. Recently, Miglietta and Nelson (1988) have isolated a third enzyme, a cGMP kinase from the cilia of *Paramecium tetraurelia*, which appears to be a monomer with an apparent M_r of 77000 and with the cGMP-binding and catalytic activities on the same polypeptide. We have been investigating the role of cAMP kinase in the silicon-regulated cell cycle of the diatom *Cylindrotheca fusiformis*. During the course of this study we have discovered two cAMP kinases which differ from any previously described cyclic nucleotide dependent protein kinases.

Experimental Procedures

Materials. [2,8-^{3}H]cAMP was purchased from Amersham. [γ-^{32}P]ATP, 8-azido[α-^{32}P]ATP, and 8-azido[^{32}P]cAMP were from ICN. Sephadex G-150 and DEAE-Sephacel were from Pharmacia. Purified histone H1 and the other histone subfractions from sea urchin sperm were the generous gift of Dr. Donald C. Porter (this institution). The protein kinase inhibitor and the catalytic subunit of cAMP-dependent protein kinase were purified from rabbit muscle and were the generous gift of Dr. James Woodgett (Salk Institute). Staurosporine and the K-252 compounds were the generous gift of Kyowa Hakko Kogyo Co., Ltd. All other materials and chemicals were from Sigma, including cAMP–agarose and calmodulin.

Cell Culture and Extract. Cultures of *C. fusiformis* were grown autotrophically in a synthetic seawater medium, and exponential cells were collected by low-speed centrifugation as described previously (Aline et al., 1984). Cells were washed once with 0.5 M NaCl at 4 °C and resuspended to about 2 × 10^9 cells/mL in the ice-cold buffer containing 20 mM Tris-HCl, pH 8, 0.5 mM EDTA, 5 mM $MgCl_2$, 0.5 mM PMSF, 2.5 mM DTT, and 10% glycerol. Cells were immediately sonicated at 0–4 °C with four 5-s bursts in a Branson sonicator (microtip, setting 2). After centrifugation at 10000*g* for 20 min at 4 °C, the supernatant was used for enzyme purification.

Enzyme Purification. All enzyme purification was performed at 0–4 °C. The 1000*g* supernatant obtained from two 1.5-L cultures (4 × 10^{10} cells) was chromatographed on Sephadex G-150. The Sephadex column (2.5 × 90 cm) was equilibrated and eluted in 7.4-mL fractions at 10 mL/h with the buffer containing 20 mM Tris-HCl at pH 8, 5 mM $MgCl_2$, 2.5 mM DTT, 0.5 mM PMSF, and 10% glycerol. Aliquots of 50 μL and of 0.2 mL were used to determine cAMP kinase activity and cAMP-binding activity, respectively. Gel filtration fractions containing the kinase activity were pooled and loaded onto a DEAE-Sephacel column (1.5 × 11 cm) equilibrated with the buffer. After loading, the column was washed with 50 mL of the buffer and subsequently developed, at 15 mL/h in 6-mL fractions, with 200 mL of a linear salt gradient in the buffer. Attempts were made to further purify the two cAMP kinase fractions, eluted from DEAE-Sephacel, by affinity column chromatography. Fractions containing either of the kinase activities were pooled and loaded onto a cAMP–agarose (Sigma) column (1.5 × 5 cm). The column was preequilibrated with the buffer of 20 mM Tris-HCl at pH 8, 5 mM $MgCl_2$, 2.5 mM DTT, and 0.5 mM PMSF. After the enzyme sample was loaded, the column was washed with 30 mL of the buffer and subsequently eluted in 3-mL fractions at 1 mL/h with 90 mL of a linear 0–5 mM cAMP gradient in the same buffer.

Calculation of M_r. The native M_r value of diatom cAMP kinases was determined by gel filtration chromatography on Sephadex G-150 and sucrose density sedimentation. The Sephadex column described above was calibrated with known protein M_r markers. When the cAMP kinases were used for sucrose density sedimentation, the enzymes were eluted from DEAE-Sephacel, as described earlier, in the buffer without the addition of glycerol. Samples (0.2 mL) of the peak fraction for each kinase activity were loaded onto 5%–20% linear sucrose gradients and centrifuged for 18 h at 39000 rpm in a Beckman SW41Ti rotor at 4 °C. Fractions (0.17 mL) were collected from the bottom of the tubes. The sedimentation coefficients of the cAmP kinases were determined from the gradient by comparing their migration to BSA (Martin & Ames, 1961). The M_r of 67000 and sedimentation coefficient of 4.3 S for BSA were used for the calculation.

Assay Procedures. Protein kinase activity was assayed by measuring the radioactivity of [γ-^{32}P]ATP incorporated into TCA-precipitable material as described previously (Lin & Key, 1976). The standard reaction mixtures (0.2 mL) contained 20 mM Tris-HCl at pH 8, 5 mM $MgCl_2$, 2.5 mM DTT, 0.5 mM [γ-^{32}P]ATP (50–100 cpm/pmol), 50 μg of a sea urchin sperm histone H1, 50 μM cAMP when added, and indicated amounts of enzyme. Reactions were carried out at 28 °C for 5 min. cAMP-binding activity was determined by measuring the binding of [^{3}H]cAMP at pH 6.2 with the modified Millipore filter technique (Walter et al., 1977). The radioactivities of ^{32}P and ^{3}H samples were counted in a toluene-based scintillant (Lin & Key, 1976). Protein was determined by the method of Bradford (1976) except that histone concentrations were measured at 230 nm (Lin et al., 1973).

Photoaffinity Labeling. The experiments with 8-azido-[^{32}P]cAMP were performed under dim light as described by Walter et al. (1977). Reactions (0.4 mL) contained 50 mM MES at pH 6.2, 0.5 mM PMSF, 2.5 mM $MgCl_2$, 1.5 μM

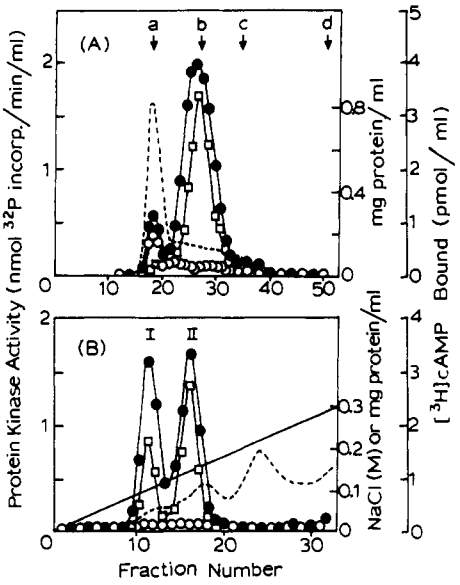

FIGURE 1: Fractionation of diatom cAMP kinases. (A) Chromatography of the 10000*g* supernatant obtained from two 1.5-L cultures (4×10^{10} cells) on Sephadex G-150. The column was calibrated with the following M_r markers: (a) blue dextran, 200000; (b) bovine serum albumin, 67000; (c) ovalbumin, 43000; (d) cytochrome *c*, 12200. (B) Fractionation of the cAMP kinase(s) eluted from gel filtration (fractions 23–32) on DEAE-Sephacel. As described under Experimental Procedures, aliquots (50 μL) were determined for the kinase activity in the presence (●) or absence (○) of 50 μM cAMP. Aliquots of 0.2 mL were assayed for cAMP-binding activity (□). Protein content (– –). NaCl gradient (—).

8-azido[^{32}P]cAMP (5000 cpm/pmol), other nucleotides when added, and the enzyme preparation to be assayed. After photoactivation by ultraviolet light irradiation, the proteins were denatured by boiling 2–3 min in 0.2% SDS, concentrated by lyophilization, and dialyzed against the sample buffer used for SDS–PAGE (10% gel) according to Laemmli (1970). Fluorography was performed according to Bonner and Laskey (1974).

Results

Fractionation of Diatom cAMP Kinases. Chromatography of the crude extract on Sephadex G-150 results in a single peak of cAMP kinase activity, which coelutes with cAMP-binding activity (Figure 1A). Assays with cGMP or total histones from either sea urchin sperm or calf thymus do not yield additional peaks of cyclic nucleotide dependent kinase activity. The cAMP kinase activity and cAMP-binding activity pooled from the gel filtration are cofractionated into two distinct peaks of activity on DEAE-Sephacel (Figure 1B). The enzymes elute at 0.10 and 0.15 M NaCl and are designated cAMP kinases I and II. The presence of 2.5–5 mM Mg^{2+} is essential for maximal protection of the kinase I from proteolytic degradation, as indicated by the observation that the presence of soybean trypsin inhibitor (0.25 mg/mL) prevents the enzyme from inactivation in the absence of the cation. Mn^{2+}, Ca^{2+}, Zn^{2+}, Na^+, K^+, or SiO_3^{2-} at 0.1–5 mM could not substitute for Mg^{2+}. For both enzymes, the overall recovery of the cAMP kinase and cAMP-binding activities from the ion-exchange column chromatography is greater than 90%. Kinases I and II eluted from DEAE-Sephacel are relatively stable for 3–4 days at 0–4 °C and catalyze the incorporation of about 22 and 17 nmol of ^{32}P, respectively, into histone H1 min^{-1} (mg of protein)$^{-1}$. Compared to the specific activities [about 10 μmol of phosphate incorporated min^{-1} (mg of protein)$^{-1}$] of purified cAMP kinases from other organisms, an additional 500-fold purification is probably necessary to obtain homogeneity of

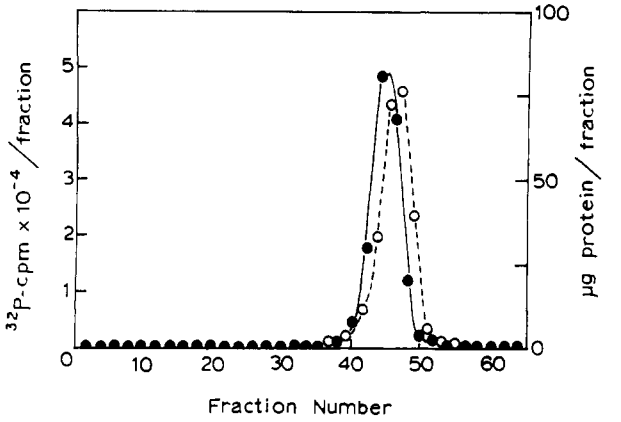

FIGURE 2: Sucrose density sedimentation of diatom cAMP kinase and BSA. To the peak fraction (0.2 mL, 0.02 mg of protein) of kinase II activity, as shown in Figure 1B, 0.4 mg of BSA was added and the mixture applied to a 5–20% sucrose gradient. Conditions of sedimentation are described under Experimental Procedures. Alternate fractions (0.17 mL) collected from the bottom of the tube were assayed for either protein content (○) or for protein kinase activity in the presence of 0.1 mM cAMP (●).

the two diatom kinases. However, attempts to further purify the kinases by cAMP affinity column chromatography have not succeeded. The cAMP–agarose resin has a N6 linker. Although there is no detectable free catalytic activity in the runoff fractions, the bound kinase and cAMP-binding activities of the two enzymes could not be recovered from the affinity column by elution with a 0–5 mM cAMP gradient. Furthermore, no enzyme activity could be recovered after rechromatography of the kinases (from DEAE-Sephacel) on the Sephadex G-150 column following preincubation and elution with 0.5 mM cAMP. This is due in part to the fact that the kinases are extremely labile ($t_{1/2} \leq 0.5$ h for the kinase I and $t_{1/2} \leq 2$ h for the kinase II at 0–4 °C) upon binding of cAMP in a dilute protein solution. Addition of BSA at 2–4 mg/mL only doubles the half-life.

M_r of Native Enzymes. The kinases I and II are coeluted slightly ahead of BSA from the calibrated Sephadex G-150 column (see Figure 1A). The estimated apparent M_r values for both kinases are about 75000. Removal of DTT from the buffers used for enzyme extraction and purification has no effect on the elution and recovery of the kinases from the Sephadex and DEAE-Sephacel columns. This shows that the formation of free sulfhydryl groups or disulfide linkages does not alter the apparent size of the kinases. Both diatom kinases from DEAE-Sephacel have the same sedimentation coefficient of 4.6 S, on the basis of their sedimentation positions compared to the position of BSA (4.3 S); only the result for the kinase II is shown here (Figure 2). The estimated M_r value for either of the kinases by sucrose density sedimentation is about 74000. Sucrose density sedimentation was also used for determining the effect of cAMP binding on the size of the kinases. Although only a 25% recovery in activity, kinase II does not dissociate into small species upon preincubation with 0.5 mM cAMP and 4 mg/mL BSA, and immediately followed by sedimentation in the sucrose density gradient containing the nucleotide (data not shown). The kinase I activity is too labile for such an experiment, following cAMP binding.

Activation by Cyclic Nucleotides. For most enzyme characterizations, the kinases purified through the DEAE-Sephacel stage were used. Kinases I and II are specifically activated by cAMP with the estimated K_a values of 0.6 and 1.0 μM (panels A and B of Figure 3). The cAMP analogue 8-azido-cAMP acts as cAMP while uridine 3′,5′-cyclic monophosphate and cytosine 3′,5′-cyclic monophosphate are less

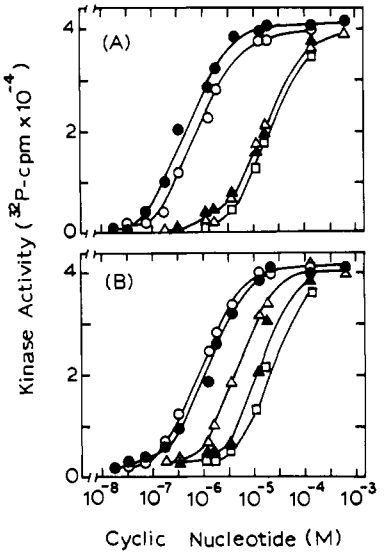

FIGURE 3: Effects of varying concentrations of cyclic nucleotides on the diatom kinases. The activities (A) kinase I (3 μg of protein) and (B) kinase II (4 μg of protein) eluted from DEAE-Sephacel were measured in reactions containing the indicated concentrations of cAMP (●), 8-azido-cAMP (○), cGMP (△), cUMP (▲), or cCMP (□), as described under Experimental Procedures.

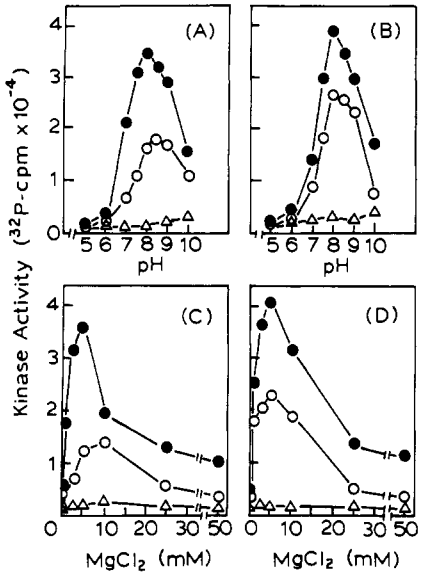

FIGURE 4: Activities of diatom cAMP kinases I and II as a function of pH and Mg^{2+} concentration. Aliquots (50 μL) of the peak fraction of the kinase I resolved on DEAE-Sephacel were assayed under standard conditions except that (A) pH was varied with 100 mM potassium acetate (pH 5.6) or 100 mM Tris (pH 7–10) and (C) the concentration of Mg^{2+} was varied in the presence of 10 μM cAMP (●) or 10 μM cGMP (○) or in the absence of the nucleotides (△). For 0 mM Mg^{2+}, the reactions contained 3 mM EDTA. Assays of the effects of (B) pH and (D) Mg^{2+} concentrations on the kinase II activity were as that described for the kinase I activity except that the peak fraction of the kinase II resolved on DEAE-Sephacel was used.

effective. Optimum concentrations of cAMP and cGMP have no additive effect on the activation. The kinases are not activated by the 2′,3′ derivatives of the cyclic nucleotides, GTP, 5′-AMP, adenosine, and adenine, nor are they sensitive to EGTA, Ca^{2+}/calmodulin, and Ca^{2+}/diacylglycerol.

General Properties. With histone H1 as protein substrate, both diatom kinases activated by either cAMP or cGMP have temperature optima at 23–30 °C and pH optima at 8.0 (Figure 4A,B). They require 5 mM Mg^{2+} for maximal activity, and the cation concentrations at ≥10 mM are inhibitory (Figure 4C,D). Mn^{2+} alone at 0.1–10 mM could not support kinase I activity. When assayed with the optimum concentration of Mg^{2+}, kinase I activity is inhibited 50% and 60% by 0.25 mM Mn^{2+} and 25 μM Ca^{2+}, respectively. However, Mn^{2+} alone at 0.25 mM could support about 65% of the maximal kinase II activity. Ca^{2+} at 0.25–1.0 mM showed no effect on kinase II activity. Both kinases prefer ATP (K_m = 0.1 mM) as phosphate donor; neither GTP, UTP, or CTP could substitute for ATP.

Table I: Protein Substrate Specificity of Diatom cAMP Kinases[a]

protein substrate	activity (^{32}P cpm incorporated/5 min) kinase I	kinase II
histone H1	46 400	40 900
histone H2B	7 200	6 900
histone H3	4 400	4 200
histone H2A + H4	15 300	3 700
protamine	49 100	27 000
casein	178	125
phosvitin	430	187

[a] Kinase activity was measured, as described under Experimental Procedures, in reactions containing 50 μM cAMP, 3 μg of protein of the kinase I preparation or 4 μg of protein of the kinase II preparation, and 0.25 mg/mL of histones purified from sea urchin sperm or 0.5 mg/mL of casein or phosvitin.

Phosphorylation of Histone H1. The identity of endogenous protein substrates for the two diatom cAMP kinases is at present not known. Of the various proteins tested, histone H1 (sea urchin sperm) and protamine are the most effective substrates for both enzymes (Table I). The estimated K_m value for histone H1 is 0.1 μM (0.1 mg/mL). The site of histone H1 phosphorylation catalyzed by either of the diatom kinases in the presence of cAMP and [γ-^{32}P]ATP was determined. The majority of ^{32}P is associated with one peptide derived from the N-terminal fragment of CNBr-cleaved ^{32}P-labeled histone H1, following digestion by the mouse submaxillary endoproteinase, Arg C, and separation by electrophoresis and chromatography on a thin-layer cellulose plate. Analysis of the ^{32}P-labeled peptide by automatic spinning-cup sequencing and manual Edman degradation (Hunter et al., 1984) shows the sequence to be Arg-Lys-Gly-Ser(^{32}P)-Ser-Asn-Ala-Arg. This is the same sequence in sea urchin sperm histone H1 that is phosphorylated in vivo in the sperm or in vitro by the sperm cAMP kinase (Porter et al., 1988).

cAMP Binding and Photoaffinity Labeling. The cAMP concentrations for half-maximal binding of kinases I and II are 0.4 and 0.7 μM as determined by binding of the enzymes to various concentrations of [^{3}H]cAMP with the Millipore filter technique (Walter et al., 1977). These values are comparable to the K_a values of cAMP for activation of the kinases. Table II shows that nonradioactive cAMP and 8-azido-cAMP are effective in competing for binding of both kinases with [^{3}H]cAMP and that nonradioactive cGMP is a weak competitor. The cAMP analogue 8-azido[^{32}P]cAMP was used as the photoaffinity probe in conjunction with SDS–PAGE for identifying the cAMP-binding proteins of the diatom kinases. Figure 5A shows that in the kinase I preparation a single protein band of about 78 kDa is photolabeled with 1.5 μM 8-azido[^{32}P]cAMP (lane 1). Labeling of the protein is effectively inhibited by cAMP (lane 2), slightly reduced by ATP (lane 3), and not affected by 5′-AMP (lane 4), when these nonradioactive nucleotides are added in excess amounts prior to photoactivation. Nonradioactive cAMP, however, does not affect the labeling if it is added after photoactivation (lane 5). The labeled protein is slightly degraded to a 65-kDa polypeptide under the experimental conditions (see lanes 1 and

Table II: Effects of Nonradioactive cAMP, cGMP, and 8-Azido-cAMP on the Binding of Diatom cAMP Kinases with [^{3}H]cAMP[a]

competing compd	% inhibition of [^{3}H]cAMP binding	
	kinase I	kinase II
cAMP		
5 μM	90	90
10 μM	99	98
cGMP		
5 μM	10	20
10 μM	30	54
8-azido-cAMP		
5 μM	90	90
10 μM	99	97

[a] cAMP-binding assays were performed, as described under Experimental Procedures, in reactions containing 0.5 μM [^{3}H]cAMP (5000 cpm/pmol), the indicated concentrations of nonradioactive cyclic nucleotides, and 12 μg of protein of the kinase I or 25 μg of protein of the kinase II preparation. The values of 100% residual [^{3}H]cAMP binding for kinases I and II are 680 and 2500 cpm. The data represent the average of three experiments.

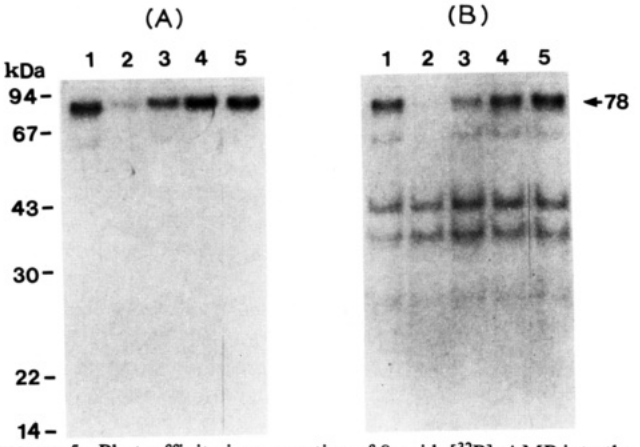

FIGURE 5: Photoaffinity incorporation of 8-azido[^{32}P]cAMP into the diatom cAMP kinases. Autoradiograms of SDS–PAGE on which (A) kinase I preparation, 12 μg of protein , and (B) kinase II preparation, 25 μg of protein, were separated after photolabeling with the kinase II preparation with 1.5 μM 8-azido[^{32}P]cAMP (lane 1) and with the addition of 0.15 mM nonradioactive cAMP (lane 2), ATP (lane 3), or 5′-AMP (lane 4). Nonradioactive cAMP at 0.15 mM was added after photoactivation (lane 5). Samples were boiled in the presence of 5% 2-mercaptoethanol before electrophoresis. The protein M_r markers used: phosphorylase *b*, 94 000; bovine serum albumin, 67 000; ovalbumin, 43 000; carbonic anhydrase, 30 000; soybean trypsin inhibitor, 22 000; lysozyme, 14 000.

2). Figure 5B shows that photoaffinity labeling of the kinase II preparation with the cAMP analogue also results in the specific labeling of a 78-kDa protein band as described for the kinase I preparation. In the kinase II preparation two additional protein bands (35 and 40 kDa) are also labeled, but the labeling is not inhibited by nonradioactive cAMP, ATP, or 5′-AMP added prior to photoactivation. These two labeled proteins or polypeptides apparently are not the degraded products of the 78-kDa protein. No difference in the labeling protein bands occurs for the enzyme samples boiled in the presence or absence of 5% 2-mercaptoethanol before electrophoresis. Thus, the cAMP-binding proteins in both kinase preparations have apparent M_r values comparable to that determined by gel filtration and sucrose density sedimentation for the native enzymes. Attempts to identify the catalytic subunits (or domains) of the kinases by photoaffinity labeling with 8-azido[α-^{32}P]ATP were unsuccessful because of the contamination of ^{32}P in the γ-position of ATP purchased from ICN and the phosphorylation of the 78-kDa proteins by the

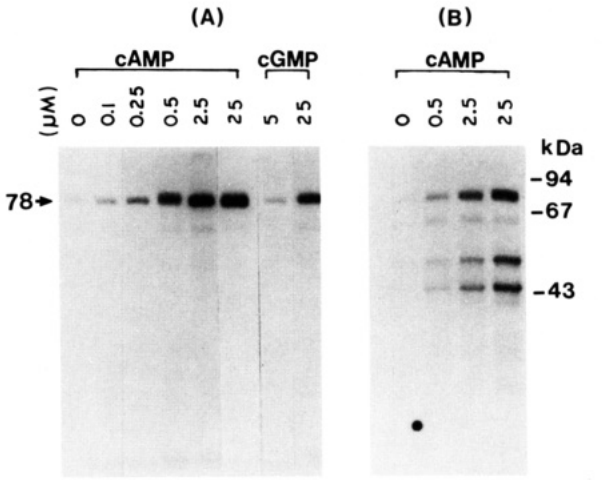

FIGURE 6: cAMP-dependent self-phosphorylation of the diatom cAMP kinases. Autoradiograms of SDS–PAGE on which (A) 8 μg of protein of the kinase I preparation and (B) 18 μg of the protein of the kinase II preparation were separated after phosphorylation at 28 °C for 5 min in reactions containing 0.5 mM [γ-^{32}P]ATP (200 cpm/pmol) and the indicated concentrations of cAMP or cGMP. Samples were boiled in the presence of 5% 2-mercaptoethanol before electrophoresis. The protein M_r markers used are as described in Figure 5.

nucleotide. Otherwise, this experiment should provide new insights into the subunit compositions of the enzymes.

Self-Phosphorylation. Incubation of the kinase I preparation with [γ-^{32}P]ATP results in the incorporation of the radioactivity from [γ-^{32}P]ATP into a single 78-kDa protein, and the phosphorylation is dependent on cAMP concentration (Figure 6A). cGMP is less effective in supporting the protein phosphorylation. Dilutions of the same amount of kinase I solution by 2–4-fold do not significantly reduce the rate of phosphorylation of the 78-kDa protein in the presence of cAMP. The rate of the phosphorylation is linear up to 7 min of reaction. Evidently, the phosphorylation is independent of the enzyme concentration and thus is an intramolecular reaction. This indicates that the phosphorylated protein has to be the kinase itself. cAMP-dependent phosphorylation also occurs on a 78-kDa protein in the kinase II preparation (Figure 6B). The phosphorylation also is independent of enzyme concentration. Thus, like the kinase I, the kinase II also is undergoing self-phosphorylation upon activation by cAMP. Additionally, two other proteins (45 and 50 kDa) in the kinase II preparation also contain ^{32}P after 5-min reaction, but whether they are the degraded products of the 78-kDa protein is not clear.

Inhibition of Selected Compounds/Proteins. Figure 7A shows that the heat-stable protein kinase modulator ($\leq$10 μM), isolated from rabbit muscle by the method of Cohen et al. (1977), slightly increases the measured enzyme activities of the two diatom cAMP kinases toward histone H1. As a positive control, the modulator at 10 μM completely inhibits the activity of rabbit muscle cAMP kinase. The phosphorylation of lysine-rich histone H1 catalyzed by either of the diatom kinases is completely inhibited by the presence of arginine-rich histone H3 at a mass ratio of about 0.5 for H3/H1 (Figure 7B). The two diatom kinases, however, respond differently to arachidonic acid. The unsaturated long-chain fatty acid at 20 μM inhibits kinase I activity 50% while at $\leq$50 μM it slightly increases kinase II activity (Figure 7C). Furthermore, the activities of two diatom kinases show slight differences in inhibition by microbial alkaloids, staurosporine, and the K-252 compounds (Table III). Both diatom enzymes are sensitive to nanomolar concentrations of staurosporine isolated from *Streptomyces* sp.; the K_i values

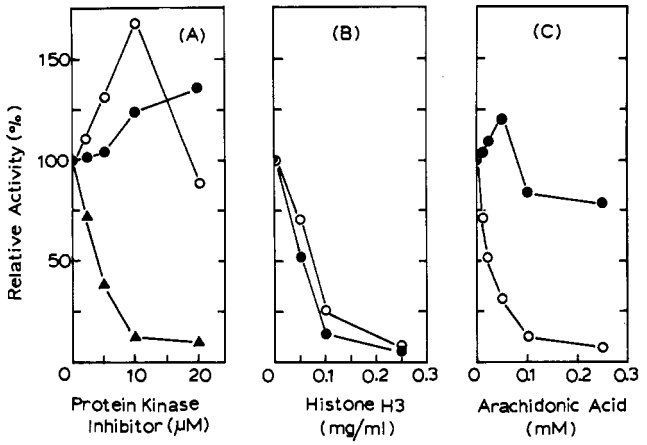

FIGURE 7: Effects of (A) rabbit muscle protein kinase modulator, (B) sea urchin sperm histone H3, and (C) arachidonic acid on protein kinase activity. Activities of the diatom cAMP kinases I (○) and II (●) resolved on DEAE-Sephacel (Figure 1B) and the C subunit of rabbit muscle cAMP kinase (▲) were determined under standard conditions with the additions as indicated. The values of 100% activity for the diatom kinases I and II and the subunit of rabbit muscle kinase were about 56 000, 63 000, and 96 000 cpm of ^{32}P incorporated into histone H1 per 5 min.

Table III: Inhibition of Diatom cAMP Kinases I and II by Staurosporine and the K-252 Compounds[a]

inhibitor	inhibition constant, K_i (nM)	
	kinase I	kinase II
staurosporine	18	6
K-252a	8	18
K-252b	25	100
KT 5720	50	120
KT 5822	12	20

[a] Kinase activity was measured in the standard reaction mixtures containing various concentration of indicated inhibitors. For determination of K_i values, various concentrations of [γ-^{32}P]ATP were used at a fixed concentration of histone H1 as described under Experimental Procedures. K-252b, KT 5720, and KT 5822 are the 9-carboxylic acid, the 9-*n*-hexyl ester, and the 9-methoxy derivatives of K-252a, respectively.

are comparable to 2.7–8.5 nM reported for inhibition of the cAMP kinase from rabbit muscle, the cGMP kinase from pig lung, and the protein kinase C from rat brain (Tamaoki et al., 1986; Kase et al., 1987). The diatom kinase I is more sensitive than the kinase II to inhibition by the K-252 compounds. Interestingly, KT 5720 (the 9-*n*-hexyl ester derivative of K-252a) appears to be a potent inhibitor only for cAMP kinases as indicated by K_i values to 50 and 120 nM for the diatom cAMP kinases I and II, 60 nM for the cAMP kinase from rabbit muscle (Kase et al., 1987), and >2000 nM for either the cGMP kinase from pig lung or the protein kinase C from rat brain (Kase et al., 1987).

Discussion

This study establishes the presence of two novel cAMP-dependent protein kinases in the marine diatom *C. fusiformis*; they differ significantly in subunit composition, self-phosphorylation, and mode of activation from cyclic nucleotide dependent protein kinases described previously. In contrast to the tetrameric subunit composition known to the holoenzymes of other cAMP kinases (Rosen et al., 1977; Edelman et al., 1987), the diatom enzymes appear to be monomeric as judged by the following observations. In both diatom enzyme preparations the cAMP-binding proteins have apparent M_r values of about 78 000 as determined by SDS–PAGE following photoaffinity labeling with 8-azido[^{32}P]cAMP. These values are roughly in agreement with those (about M_r 75 000) determined by gel filtration and sucrose density sedimentation for the native enzymes, indicating the possibilities that either of the holoenzymes could be a monomer containing the R and C domains or that the catalytic and cAMP-binding activities could be located on two separate proteins of about the same size. The latter seems unlikely because the catalytic and cAMP-binding activities are not separable by cAMP affinity column chromatography or sucrose density sedimentation in the presence of cAMP following preincubation with the nucleotide. The affinity column has been frequently used to separate the C and R subunits of other cAMP kinases (Rosen et al., 1977; Edelman et al., 1987). Furthermore, phosphorylation of the diatom enzyme preparations in the presence of ATP and cAMP occurs on the proteins which have the same M_r values as that of the cAMP-binding proteins upon SDS–PAGE. The protein phosphorylation is cAMP dependent, but the rate of reaction is independent of the enzyme concentration, indicating that the diatom enzymes can catalyze self-phosphorylation upon activation by cAMP. These results also strongly argue that the self-phosphorylation is not catalyzed by the diatom cAMP kinases with separable R and C subunits. Moreover, intermolecular disulfide linkages, which engage in dimerization of mammalian cGMP kinase (Gill et al., 1977), cannot be demonstrated in the two diatom enzymes. Thus, the diatom enzymes behave as monomers with M_r values comparable to that of the monomeric protein of cGMP kinase from the cilia of *Paramecium tetraurelia* (Miglietta & Nelson, 1988), the monomers of the homodimeric mammalian cGMP kinases (Gill et al., 1976, 1977; Takai et al., 1976; Miglietta & Nelson, 1988), or the combination of one R subunit and one C subunit of the tetrameric cAMP kinases (Rosen et al., 1977; Edelman et al., 1987). Binding to cAMP evidently does not dissociate the diatom enzymes into smaller species.

Like most cAMP kinases, the diatom enzymes are acidic in nature and are half-maximally activated by micromolar cAMP. In general, the cGMP kinases are activated at much lower concentrations of cGMP (10^{-7}–10^{-8} M), reflecting the low level of the nucleotide in the cell (Rosen et al., 1977; Gill & McCune, 1979; Miglietta & Nelson, 1988). Additionally, the diatom enzymes catalyze specifically the phosphorylation of histone H1 from ATP on a serine residue in a peptide containing the most favored sequence (basic-basic-X-Ser) for cAMP kinases, where basic is either Arg or Lys and X is usually a small hydrophobic amino acid (Edelman et al., 1987; Taylor, 1987). For the diatom enzymes, the sequence is Arg-Lys-Gly-Ser. The specificity of cAMP for the diatom enzymes is independent of pH, temperature, Mg^{2+} concentration, and protein substrate (histone species). Despite their similarities in structure and differences in cyclic nucleotide specificity, the diatom cAMP kinases are further distinguished from the cGMP kinase of *Paramecium*. The *Paramecium* kinase appears to be a basic protein that can use either ATP or GTP to phosphorylate its preferred protein substrate casein on both serine and threonine residues (Miglietta & Nelson, 1988). Although the diatom kinases I and II resolved on DEAE-Sephacel respond differently to several compounds (e.g., arachidonic acid, staurosporine, and the K-252 compounds), they may represent different isozymes as a result of phosphorylation/dephosphorylation, minor proteolytic degradation, and/or binding to other proteins. However, the physicochemical relationship, if any, between the diatom kinases is at present not clear. Multiple peaks of mammalian cAMP kinase on DEAE-cellulose, however, are due to different

isozymic forms of the R subunit (Rosen et al., 1977; Edelman et al., 1987). Since many protein kinases so far analyzed have a conserved catalytic core (Gill & McCune, 1979; Taylor, 1987), the diatom cAMP kinases may contain different isozymic forms of the R domain. Cloning and sequencing the gene(s) coded for the kinases should provide new insights into the structural nature of the R domains.

Apart from their unique molecular structure, another striking feature of the two diatom enzymes is the cAMP-dependent self-phosphorylation. As discussed above, self-phosphorylation is independent of enzyme concentration, indicating an intrapeptide reaction as has been described for the monomeric protein kinase C (Newton & Koshland, 1987). Self-phosphorylation is common to a variety of protein kinases but for other cAMP and cGMP kinases is independent of cAMP and cGMP (Rosen et al., 1977; Gill & McCune, 1979; Lincoln & Corbin, 1983; Edelman et al., 1987; Miglietta & Nelson, 1988). The differences in self-phosphorylation between the diatom enzymes and other cyclic nucleotide dependent protein kinases could be determined at the structural level. For the tetrameric cAMP kinases and the homodimeric cGMP kinases, many investigators have proposed that the R and C subunits (or domains) interact maximally with each other, involving an inhibitory substrate analogue site on the R subunit and the catalytic site on the C subunit (Corbin et al., 1978; Gill & McCune, 1979; Lincoln & Corbin, 1983). Through this antiparallel interaction, the kinases are inactive toward outside protein substrates, but the protein substrate site on the R subunit is vulnerable for phosphorylation by the C subunit. Activation by cyclic nucleotide binding results in exposure of the protein substrate site as well as the catalytic site. However, this model involving an allosteric mechanism is not applicable to monomeric proteins such as the diatom cAMP kinases. We propose that in the diatom enzymes the protein substrate analogue site(s) on the R and/or C domain(s) are hidden, as is the catalytic site of the C domain, and that binding of cAMP exposes the catalytic site and the closer proximity of the protein substrate site(s) to the catalytic site. Following such conformational changes the enzymes are active in catalyzing both self-phosphorylation and the phosphorylation of outside protein substrates. Thus, competition for the catalytic site between the protein substrate analogue site on the R domain and an outside protein substrate would be expected. In fact, the addition of histone H1 or protamine, the preferred protein substrate for the enzymes, partially reduces the cAMP-dependent self-phosphorylation; the rate of self-phosphorylation is comparable to that of histone H1 or protamine phosphorylation (unpublished data). The physiological role of self-phosphorylation for the diatom enzymes is unclear. Since only the activated enzymes are modified, a downregulation by self-phosphorylation could be the mechanism by which the nonactivated diatom kinases are able to respond to other signals. Such a mechanism has been proposed for Ca^{2+}/diacylglycerol-dependent self-phosphorylation of protein kinase C, also a monomeric protein (Newton & Koshland, 1987).

In higher eukaryotes, the R subunit of the cAMP kinases contains at least four functional sites, namely, for interaction with the C subunit, for dimerization, and for the low and the high affinity of cAMP binding. Multzel et al. (1977) have proposed that the evolution of R subunits started from an ancestral low-affinity cyclic nucleotide binding protein and proceeded through a series of genetic events involving gene duplication, gene fusion, and point mutation. As single polypeptide chains, the lower eukaryotic diatom cAMP kinases do not have the functional site for dimerization in their R domains. This may also be the case for the monomeric cGMP kinase from *Paramecium*. However, unlike the cGMP kinase, the R domains of diatom kinases apparently contain only site(s) for the low affinity of cAMP binding as indicated by the fact that K_a or K_d values for cAMP are in the micromolar range, but not in the nanomolar range. Thus, if the cAMP and cGMP kinases have evolved from a common ancestral protein, the diatom enzymes may more closely represent such an ancestor. Although further purification of the enzymes to homogeneity is needed, the results presented strongly suggest that either of the diatom cAMP kinases is a monomeric protein containing one R domain and one C domain as part of a contiguous polypeptide chain. Activation of the enzymes is simply by cAMP binding. The stoichiometry of cAMP binding remains to be determined. The role of these enzymes in the silicon-regulated cell cycle of the diatom is currently under investigation.

ACKNOWLEDGMENTS

We thank Drs. Victor D. Vacquier and Jean Y.-J. Wang for discussions and Dr. Donald C. Porter for help in analyzing the site of histone H1 phosphorylation.

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Salt Effects on Histone Subunit Interactions As Studied by Fluorescence Spectroscopy†

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Received December 29, 1988; Revised Manuscript Received April 26, 1989

ABSTRACT: The salt concentration dependence of the aggregation properties of calf thymus and chicken erythrocyte histones has been investigated by using fluorescence spectroscopy. The isolated H2A/H2B and H3/H4 subunit preparations were labeled with 5-(dimethylamino)naphthalene-1-sulfonyl (dansyl). This long-lived fluorescence probe allows for the observation of rotations due to tumbling of the particle and thus is a probe for changes in the size of macromolecular assemblies. The fluorescence polarization and lifetime were measured as a function of salt concentration for these isolated preparations. Next, each labeled preparation was reconstituted with its unlabeled complement, and the salt concentration dependence of histone core octamer interactions was investigated in the same manner. Salt-induced core particle formation was observed by monitoring the dansyl-labeled dimers for both the calf thymus and chicken erythrocyte preparations. Evidence for subunit dissociation of the isolated H2A–H2B preparations was also found, as well as aggregation of the isolated H3/H4 subunits to at least dimers of tetramers. The calf thymus H3/H4 preparation was in aggregated form under all conditions studied, whereas the chicken erythrocyte H3/H4 only formed aggregates at high protein or salt concentrations. We have found evidence that the dimer can displace the tetramer from the higher order aggregate in order to form core particles. Such competition between the subunit interfaces in the histone system suggests that they may play a regulatory role in histone–DNA interactions.

Histones are small proteins that complex and condense eukaryotic chromosomal DNA [for a recent review, see McGhee and Felsenfeld (1980)]. Histones have been well characterized, and sequence studies have shown strong homology between species (Isenberg, 1979). Four major histones, H2A (14.5 kDa), H2B (13.8 kDa), H3 (15.3 kDa), and H4 (11.3 kDa), aggregate to an octamer that interacts with approximately 165 base pairs of DNA forming a nucleosome. The nucleosome consists of an $(H3)_2(H4)_2$ tetramer center flanked by two H2A–H2B[1] dimers in a tripartite arrangement (Richmond et al., 1984; Burlingame et al., 1985) with DNA looping twice around the outside of the protein core. A fifth histone, H1, binds to the DNA regions between the nucleosomes.

Due to the high content of basic amino acids, nucleosomes undergo a variety of structural changes with ionic strength [e.g., see Park and Fasman (1987)]. Below 200 mM salt, the two H2A–H2B dimers only weakly interact with the tetramer while remaining bound to DNA (Martinson et al., 1979), and at salt concentrations of approximately 2 M, DNA dissociates from the intact core octamer (Yager & van Holde, 1984). The loss of dimer–tetramer contacts has been postulated to account for the increased nuclease sensitivty of DNA in active genes and thus has been implicated in playing a role in nucleosome opening during transcription (Chao et al., 1979; Simpson & Stafford, 1983). In this work, we use fluorescence techniques to focus on histone–histone interactions and how these change as a function of ionic strength.

Much of our knowledge of nucleosome structure and possible dissociation mechanisms has been contributed by fluorescence studies. D'Anna and Isenberg (1974) and Libertini and Small (1982), using the intrinsic fluorescence of histone core particles, found evidence of a two-step H2A–H2B dimer dissociation from the octamer as the salt concentration

† This project was supported in part by BRSG S07 RR05396 to S.F.S. by the Biomedical Research Support Grant Programs Division of Research Resources, NIH. Additional support was provided in part by the University of Illinois at UC and by NIH Grants R29 GM399690 (C.A.R.) and P41 RR03155-01 (C.A.R. and R.M.R.).

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[1] Abbreviations: H2A–H2B, histone "dimer" subunits; H3/H4, histone "tetramer" subunits; DNS or dansyl, 5-(dimethylamino)-naphthalene-1-sulfonyl; CT, calf thymus; CE, chicken erythrocyte; SDS, sodium dodecyl sulfate; SAS, species-associated spectra; TBS, Tris-buffered saline; PBS, phosphate-buffered saline.

0006-2960/89/0428-6631$01.50/0