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Interaction of Guanosine Cyclic 3′,5′-Phosphate Dependent Protein Kinase with *lin*-Benzoadenine Nucleotides†

Deepak Bhatnagar,‡ David B. Glass,§ Robert Roskoski, Jr.,*,‡ Ralph A. Lessor,∥ and Nelson J. Leonard∥

Department of Biochemistry, Louisiana State University Medical Center, New Orleans, Louisiana 70119, Department of Pharmacology, Emory University School of Medicine, Atlanta, Georgia 30322, and Departments of Chemistry and Biochemistry, University of Illinois, Urbana, Illinois 61801
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ABSTRACT: Using the activated cGMP-dependent protein kinase in the presence of the phosphorylatable peptide [[Ala^{34}]histone H2B-(29–35)], we found that *lin*-benzoadenosine 5′-diphosphate (*lin*-benzo-ADP) was a competitive inhibitor of the enzyme with respect to ATP with a K_i (22 μM) similar to the K_d (20 μM) determined by fluorescence polarization titrations. The K_d for *lin*-benzo-ADP determined in the absence of the phosphorylatable peptide, however, was only 12 μM. ADP bound with lower affinity (K_i = 169 μM; K_d = 114 μM). With [Ala^{34}]histone H2B-(29–35) as phosphoryl acceptor, the K_m for *lin*-benzo-ATP was 29 μM, and that for ATP was 32 μM. The V_{max} with *lin*-benzo-ATP, however, was only 0.06% of that with ATP as substrate [0.00623 ± 0.00035 vs. 11.1 ± 0.17 μmol (min·mg)$^{-1}$]. Binding of *lin*-benzo-ADP to the kinase was dependent upon a divalent cation. Fluorescence polarization revealed that Mg^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, Ca^{2+}, Sr^{2+}, and Ba^{2+} supported nucleotide binding to the enzyme; Ca^{2+}, Sr^{2+}, and Ba^{2+}, however, did not support any measurable phosphotransferase activity. The rank order of metal ion effectiveness in mediating phosphotransferase activity was $Mg^{2+} > Ni^{2+} > Co^{2+} > Mn^{2+}$. Although these results were similar to those observed with the cAMP-dependent protein kinase [Hartl, F. T., Roskoski, R., Jr., Rosendahl, M. S., & Leonard, N. J. (1983) *Biochemistry 22*, 2347], major differences in the V_{max} with *lin*-benzo-ATP as substrate and the effect of peptide substrates on nucleotide (both *lin*-benzo-ADP and ADP) binding were observed.

The two cyclic nucleotide dependent protein kinases, namely, the adenosine cyclic 3′,5′-phosphate (cAMP)[1] dependent and the cGMP-dependent protein kinases, have been purified to homogeneity, and many characteristics have been defined. A comparison of the subunit structure, physicochemical properties, kinetic characteristics, and substrate specificity of these two protein kinases has been made (Gill & McCune, 1979; Glass & Krebs, 1980; Kuo & Shoji, 1982; Lincoln & Corbin, 1983). Both enzymes have similar phosphotransferase activities using ATP as a phosphoryl donor with similar, but not identical, substrate specificities (Glass & Krebs, 1980). The amino acid compositions are very similar (Lincoln & Corbin, 1977; Takio et al., 1983; Titani et al., 1983). The cGMP-dependent protein kinase and the type II cAMP-dependent enzyme both undergo an autophosphorylation reaction (Erlichman et al., 1974; Foster et al., 1981; de Jonge & Rosen, 1977). The molecular weights of the holoenzyme forms are almost the same. Structural homologies and common ancestral origins for the two cyclic nucleotide dependent protein kinases have also been hypothesized (Gill, 1977; Lincoln & Corbin, 1977, 1978, 1983) and are supported by recent sequence data (Takio et al., 1983; Titani et al., 1983).

The major differences reside in the specific nucleotide that binds to the enzyme to activate phosphotransferase activity and in the subunit structures. cAMP-dependent protein kinase is a tetramer that is activated by dissociation into a regulatory (R) subunit dimer containing bound cAMP and two catalytic (C) subunits (Corbin et al., 1978) as follows:

$$R_2C_2 + 4cAMP \rightleftharpoons R_2(cAMP)_4 + 2C$$

whereas cGMP-dependent protein kinase is a dimer that is activated without subunit dissociation (Corbin & Doskeland, 1983; Gill et al., 1977; Lincoln et al., 1977; Takai et al., 1976):

$$(cGMPdPK)_2 + 4cGMP \rightleftharpoons (cGMPdPK)_2\cdot(cGMP)_4$$

Walter et al. (1980) have reported that antiserum against the cGMP-dependent protein kinase does not cross-react with either catalytic or regulatory subunits of the type I or type II cAMP-dependent protein kinases and vice versa. Although amino acid sequence data indicate that a few regions of the two kinases do not correspond to each other, the ATP binding sites, the cyclic nucleotide binding sites, and the sites of autophosphorylation in the hinge regions show from 25 to 45% sequence identity between the two enzymes (Hashimoto et al., 1981, 1982; Takio et al., 1982; Titani et al., 1983). These results suggest that similarities exist in the functionally important regions such as the ATP-binding site. In order to ascertain the degree of these hypothesized similarities between the cAMP-dependent and cGMP-dependent protein kinases, we have used steady-state kinetics and a fluorescence polarization titration technique for characterizing the interaction of nucleotides with the cGMP-dependent protein kinase. For these determinations we have used the fluorescent *lin*-benzoadenine nucleotides, "stretched-out" analogues of adenine

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‡ Louisiana State University Medical Center.
§ Emory University School of Medicine.
∥ University of Illinois.

[1] Abbreviations: cGMP, guanosine cyclic 3′,5′-phosphate; cAMP, adenosine cyclic 3′,5′-phosphate; *lin*-benzo-ADP, *lin*-benzoadenosine 5′-diphosphate; Ser-peptide, Leu-Arg-Arg-Ala-Ser-Leu-Gly; [Ala^{34}]-histone H2B-(29–35), Arg-Lys-Arg-Ser-Arg-Ala-Glu; histone H2B-(29–35), Arg-Lys-Arg-Ser-Arg-Lys-Glu; Mops, 3-(*N*-morpholino)-propanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane.

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nucleotides (Leonard et al., 1978). We have previously shown that *lin*-benzo-ADP is a competitive inhibitor of the catalytic subunit of the cAMP-dependent protein kinase with respect to ATP with a K_i (8.0 μM) similar to the K_i for ADP (9.0 μM) (Hartl et al., 1983). The fluorescence polarization studies also showed that the catalytic subunit of that enzyme and the type II holoenzyme bind *lin*-benzo-ADP rigidily; *lin*-benzo-ATP, moreover, is a very good substrate for the phosphotransferase activity of the protein kinase with peptides, water, or type II regulatory subunit as phosphoryl acceptor.

Our experiments show that the two enzymes exhibit many similarities in nucleotide binding and in metal ion requirements. There are, however, some distinctions. Phosphotransferase activity with the cGMP-dependent enzyme with *lin*-benzo-ATP as donor is very poor in contrast to that of the cAMP-dependent enzyme. The preferred synthetic acceptor peptide substrate of the cGMP-dependent enzyme decreases nucleotide affinity; no such effect is observed with the cAMP-dependent enzyme.

Materials and Methods

The synthetic heptapeptide (Ser-peptide) used as substrate (Leu-Arg-Arg-Ala-Ser-Leu-Gly) was purchased from Boehringer Mannheim Biochemicals, and Arg-Lys-Arg-Ser-Arg-Ala-Glu [[Ala^{34}]histone H2B-(29–35)] was synthesized as described by Glass & Krebs (1982). Carrier-free [γ-^{32}P]ATP and inorganic [^{32}P]phosphate were purchased from ICN, *lin*-benzo-[γ-^{32}P]ATP was synthesized from 20–25 nmol of *lin*-benzo-ADP and 15 mCi of inorganic [^{32}P]phosphate by the method of Walseth & Johnson (1979), and the product was purified as described for [γ-^{32}P]ATP by Palmer & Avruch (1981). The salts of metal ions were purchased from Aldrich Chemical Co. All other chemicals were purchased from Sigma Chemical Co. The concentrations of *lin*-benzoadenine nucleotides were measured by absorbance at 331 nm by using an extinction coefficient of 9750 M^{-1} cm^{-1} (Leonard et al., 1976).

Protein Kinase Preparations and Activity Measurements. The cGMP-dependent protein kinase was purified from bovine lung as described by Glass & Krebs (1979). The preparation had a specific activity of 5.5 μmol $(min \cdot mg)^{-1}$ by using 0.5 mg/mL histone H2B as substrate under the conditions described by Glass & Krebs (1979). The (–cGMP/+cGMP) activity ratio of these preparations of the enzyme using 25 μM histone H2B-(29–35) as substrate was 0.09, indicating an 11-fold stimulation of activity by cGMP.

The type II catalytic subunit of the cAMP-dependent protein kinase from bovine heart was purified as described by Hartl & Roskoski (1982). Phosphotransferase activity measurements of the catalytic subunit were performed as described by Hartl & Roskoski (1982) and Roskoski (1983). Protein concentrations were determined by the procedures of either Lowry et al. (1951) or Bradford (1976) using bovine serum albumin or ovalbumin as standards, respectively. For the determination of the molarity of the enzymes, the molecular weight of the catalytic subunit of the cAMP-dependent protein kinase was taken to be 40000 and that of the cGMP-dependent protein kinase as 154000.

Steady-State Kinetic Assays. Phosphotransferase activity of cGMP-dependent protein kinase was measured at 30 °C by using the phosphocellulose paper adsorption method of Roskoski (1983). *lin*-Benzo-[γ-^{32}P]ATP and [γ-^{32}P]ATP (10–100 μM) were used as the phosphoryl donors with specific activities of 100–200 and 40–150 cpm/pmol, respectively. Ser-peptide (1400 μM) or [Ala^{34}]histone H2B-(29–35) (200 μM) were used as the phosphoryl acceptors. The final volume (80 μL) of the reaction mixture contained 50 mM Mops, pH 7.0, 2 μM cGMP, 10 mM $MgSO_4$, 0.3 mg/mL bovine serum albumin, and 3 mM 2-mercaptoethanol. Assays were conducted with the amounts of cGMP-dependent protein kinase and for the times indicated in the tables and figure legends. These conditions were chosen on the basis of the substrates being used. Reactions were linear with respect to time and amount of enzyme. All values are the means and standard errors of three or more determinations.

Fluorescence Polarization Titrations. The fluorescence measurements were performed with a SLM4800 spectrofluorometer interfaced with a Hewlett-Packard HP 9825 A calculator. Fluorescence polarization was calculated by using a program supplied by SLM Instruments Inc. Polarization (P) and anisotropy (r) are defined as

$$P = \frac{I_\parallel - I_\perp}{I_\parallel + I_\perp} \qquad r = \frac{I_\parallel - I_\perp}{I_\parallel + 2I_\perp}$$

where, $I_\parallel$ and $I_\perp$ are the intensities observed parallel and perpendicular to the polarization of the exciting light, respectively. Polarization and anisotropy values of the *lin*-benzoadenine nucleotides were determined by using calcite polarizers. Excitation was at 334 nm, with 4-nm resolution, and emitted light was isolated with a Schott KV 389 filter.

Three types of polarization titrations were performed in order to determine the binding constants of various nucleotides for the cGMP-dependent protein kinase. The titrations were performed in 50 mM Mops (pH 7.0) and 100 mM NaCl in the presence or absence of 20 μM cGMP. This concentration of cGMP was sufficient to saturate the protein kinase even under the conditions of high concentration of the enzyme used in the fluorescence polarization studies. All experimental values represent the means and standard errors of at least three independent determinations.

(*a*) *Dilution Titration.* Polarization as a function of varying enzyme concentration (at constant [*lin*-benzoadenine nucleotide]) was measured as described in our earlier reports (Hartl et al., 1983; Bhatnagar et al., 1983) to determine P_b. P_b is the *polarization value* when all *lin*-benzoadenine nucleotide is bound to the protein kinase (at infinite enzyme concentration). The theoretical polarization (P_b) of *lin*-benzo-ADP rigidly bound to the protein kinase and the average angle of rotation (ω_p) of rigidly bound *lin*-benzo-ADP to the enzyme were calculated from Perrin's equation (Perrin, 1926) as described earlier for the cAMP-dependent enzyme (Hartl et al., 1983). The r_b, the *anisotropy value* when all *lin*-benzoadenine nucleotide is bound to the enzyme, was determined from anisotropy data obtained simultaneously. The theoretical anisotropy (r_b) of *lin*-benzo-ADP rigidly bound to the protein kinase was calculated from the following Perrin's equation (Lakowicz, 1983):

$$\frac{1}{r_b} = \frac{1}{r_0}\left(1 + \frac{RT\tau}{\eta V_0}\right)$$

where r_0 is the intrinsic anisotropy of the fluorophore, R is the universal gas constant $(erg \cdot mol^{-1} \cdot deg^{-1})$, T is the absolute temperature (K), η is the viscosity (centipoise), τ is the lifetime of the excited state (τ = 4.2 ns for *lin*-benzo-ADP; VanDerLijn et al., 1978a), and V_0 is the molecular volume of the fluorescent unit (cm^3/g).

The value of r_0 for *lin*-benzo-ADP in the above equation was determined by measuring the fluorescence anisotropy in solutions of varying viscosity with sucrose or glycerol; $1/r$ was plotted vs. T/η, and $1/r_0$ was obtained from the ordinate intercept where $\eta \rightarrow \infty$. The value of r_0 was calculated to be

Table I: Binding Constants of *lin*-Benzo-ADP for the cGMP-Dependent Protein Kinase

nucleotide	[cGMP] (μM)	[peptide][c] (μM)	K_d (μM)[a]	$\bar{n}$[a]	K_i (μM)[b]
lin-benzo-ADP	0	0	7.6 ± 0.88	1.92	
	20	0	10.6 ± 1.21	1.84	
	20	200	23.3 ± 1.63	1.94	21.5 ● 0.35
ADP	20	0	12.2 ± 0.96		
	20	200	114 ± 3.9		169 ± 5.4

[a] K_d values and number of binding sites per enzyme ($\bar{n}$) were determined from Scatchard plots of data obtained from fluorescence polarization addition or displacement titrations as described under Materials and Methods. [b] The K_i was determined by steady-state kinetics. The enzyme (0.4 μg/mL) was incubated at 30 °C with [γ-^{32}P]ATP concentrations from 10 to 100 μM at fixed concentrations of *lin*-benzo-ADP (0–33.2 μM) or ADP (0–1000 μM) and activity measured after 2 min as described under Materials and Methods. [c] [Ala^{34}]histone H2B-(29–35) was the acceptor substrate.

0.301 for *lin*-benzo-ADP at 334 nm. The average angle of rotation (ω_r) of *lin*-benzo-ADP rigidly bound to the cGMP-dependent protein kinase was calculated from anisotropy (r_b) data according to the following equation (Lakowicz, 1983):

$$\frac{1}{r_b} = \frac{1}{r_0}\left(\frac{3}{\cos^2 \omega_r - 1}\right)$$

(*b*) *Addition Titration.* Polarization (P_{obsd}) was recorded as described previously (Hartl et al., 1983; Bhatnagar et al., 1983) at each nucleotide concentration after addition of successive increments of *lin*-benzoadenine nucleotide to a constant protein kinase concentration. The dissociation constant (K_d) of *lin*-benzoadenine nucleotide for the enzyme was then determined by Scatchard analysis (Scatchard, 1949).

(*c*) *Displacement Titration.* The dissociation constant (K_d) of ADP for the cGMP-dependent protein kinase was determined by displacing the fluorescent *lin*-benzo-ADP bound to the enzyme with ADP as previously described for other nucleotides (Bhatnagar et al., 1983).

Results

Interaction of lin-Benzo-ADP with cGMP-Dependent Protein Kinase. The enzyme velocity was determined with varying ATP concentrations (10–100 μM) at a fixed, near saturating [Ala^{34}]histone H2B-(29–35) concentration (200 μM; Glass & Krebs, 1982) in the presence of various fixed concentrations of *lin*-benzo-ADP (0, 8.3, 16.6, 24.9, and 33.2 μM). Mg-*lin*-benzo-ADP was a competitive inhibitor with respect to MgATP (not shown). A slope vs. [*lin*-benzo-ADP] replot was linear and gave a K_i for *lin*-benzo-ADP of 21.5 ± 0.35 μM. The K_i for ADP obtained in parallel experiments was 169 ± 5.4 μM (Table I). Thus, *lin*-benzo-ADP, which is 2.4 Å wider in the adenine moiety, interacts with the active site of the protein kinase with a higher affinity than does the natural reaction product, ADP.

[Ala^{34}]histone H2B-(29–35) was selected as the phosphate acceptor for the cGMP-dependent protein kinase in these studies since it contains only one site of phosphorylation (as compared to various histones) and is one of the best known substrates (low K_m and high V_{max}) for the enzyme (Glass et al., 1981; Glass & Krebs, 1982). The peptide is an analogue of the sequence around serine-32 in histone H2B in which an alanine residue has been substituted for the lysine normally present at position 34. Both peptides have similar K_m values (20–30 μM), but the Ala^{34} replacement analogue has a 4–5-fold higher V_{max} [20 μmol (min·mg)$^{-1}$] than that of the parent peptide, histone H2B-(29–35), at pH 7.4 in Tris buffer (Glass & Krebs, 1982). The pH optimum (Figure 1) for the phosphorylation of histone H2B-(29–35) is rather high [see also Glass & Krebs (1979)]. One possible reason for this is that the lysine in position 34 is a negative determinant (Glass & Krebs, 1982) when in the protonated form (Glass, unpublished results). The pH profile of [Ala^{34}]histone H2B-(29–35), which lacks lysine-34, does not, therefore, show this behavior (Figure 1). Since we wished to perform our studies in Mops buffer at pH 7.0 (for comparison with our earlier studies with the cAMP-dependent enzyme), [Ala^{34}]histone H2B-(29–35) was the more suitable substrate. Ser-peptide was not routinely used for the steady-state kinetic studies because, even though the cGMP-dependent protein kinase has a high V_{max} with Ser-peptide, its K_m is high as well (approximately 200 μM; Glass & Krebs, 1979; Glass et al., 1981). This meant that we would have had to use Ser-peptide at a concentration of well over 1 mM to even approach saturation.

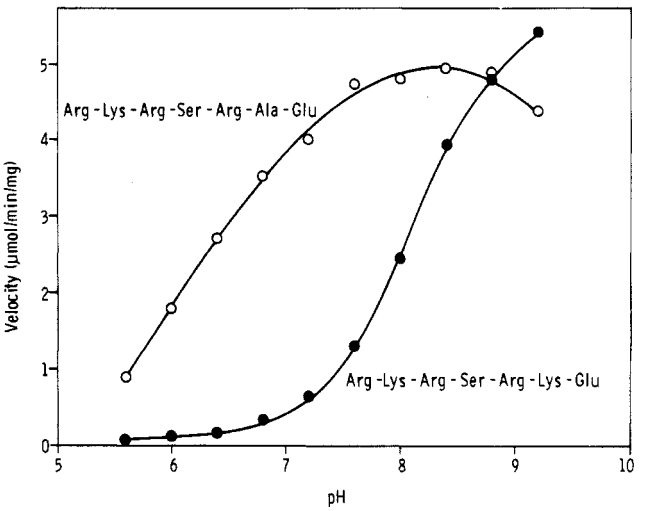

FIGURE 1: Effect of pH on the phosphorylation of histone H2B-(29–35) (●) and [Ala^{34}]histone H2B-(29–35) (○) by cGMP-dependent protein kinase. Both synthetic peptides were used at a concentration of 250 μM. The enzyme was used at a concentration of 0.5 μg/mL. Reaction velocities were determined under standard assay conditions as described under Materials and Methods except that the pH was changed. Reactions were maintained at the indicated pH with the appropriately adjusted MES/Tris (50 mM/50 mM) buffers. Reactions were conducted for 5 min.

Measurement of the K_d for lin-Benzo-ADP and ADP by Fluorescence Polarization and Anisotropy Titrations. For characterization of the interaction of *lin*-benzo-ADP with the cGMP-dependent protein kinase in the presence and absence of cGMP, fluorescence polarization titrations were performed. *lin*-Benzo-ADP was used instead of *lin*-benzo-ATP because the protein kinase demonstrated intrinsic ATPase activity (Glass and O'Neill, unpublished observations) for which *lin*-benzo-ATP was a substrate (not shown). No difference was observed in the fluorescence emission spectrum of *lin*-benzo-ADP in the presence or absence of the protein kinase with or without saturating cGMP (not shown). Binding of the nucleotide to the enzyme, however, was accompanied by an increase in polarization. The binding constants of *lin*-benzo-ADP for the cGMP-dependent protein kinase were measured by fluorescence polarization dilution and addition titrations and those of ADP by fluorescence polarization displacement titrations as described under Materials and Methods.

The polarization (P_b) of *lin*-benzo-ADP bound to the protein kinase was determined by dilution titration (Figure 2A). At constant *lin*-benzo-ADP concentration in the presence of 10 mM Mg^{2+}, the fluorescence–polarization (P_{obsd}) increases with increasing enzyme concentration as more ligand is bound. P_{obsd} ranges from P_f (zero protein concentration) to P_b (all ligand

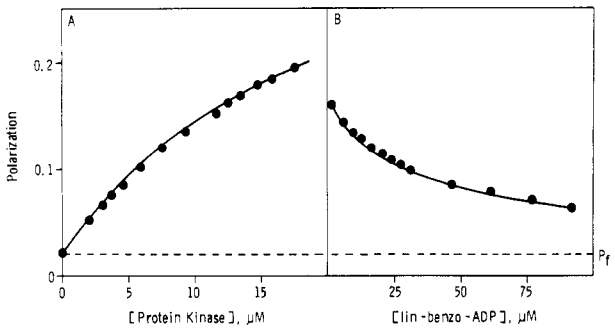

FIGURE 2: Polarization titrations of the cGMP-dependent protein kinase with *lin*-benzo-ADP. Measurements were performed at 23 °C in 50 mM Mops (pH 7.0), 100 mM NaCl, and 10 mM $MgSO_4$. (Panel A) Dilution titration. The protein kinase was varied (17.5–3.8 μM) at a constant (2 μM) *lin*-benzo-ADP concentration in the presence of 10 mM $MgSO_4$ and 20 μM cGMP. (Panel B) Addition titration. *lin*-Benzo-ADP was varied at a constant (3 μM) protein kinase concentration in 150-μL total volume by addition of 1-μL portions of a concentrated *lin*-benzo-ADP solution. The titration shown was performed in the presence of 20 μM cGMP and 200 μM phosphorylatable peptide [Ala^{34}]histone H2B-(29–35). Corrections for the changes in volume and ligand concentration were routinely made.

Table II: Fluorescence Polarization and Anisotropy of *lin*-Benzo-ADP Bound to the cGMP-Dependent Protein Kinase

	theoretical[a]		experimental[b]	
condition	P_b	ω_p (deg)	P_b	ω_p (deg)
–cGMP	0.3669	14	0.354 ± 0.017	17
+cGMP[c]	0.3669	14	0.350 ± 0.012	18
	theoretical[a]		experimental[b]	
condition	r_b	ω_r (deg)	r_b	ω_r (deg)
–cGMP	0.2787	14	0.267 ± 0.011	17
+cGMP[c]	0.2787	14	0.264 ± 0.008	18

[a] P_b, ω_p, r_b, and ω_r were calculated from Perrin's equations for polarization and anisotropy, respectively, as described under Materials and Methods. [b] Values for P_b and r_b were obtained from polarization dilution titrations (Figures 2A and 3). The values for average angle of rotation (ω_p and ω_r) were calculated from P_b and P_o (0.392) and r_b and r_o (0.301) for *lin*-benzo-ADP, respectively, as described under Materials and Methods. [c] The final concentration of cGMP was 20 μM.

is bound to the protein). Extrapolation of a plot of (polarization)$^{-1}$ vs. [protein kinase]$^{-1}$ to zero resulted in a value of P_b of nearly 0.35 (Figure 3). This corresponds well to a P_b value of 0.370 calculated by the Perrin equation (Perrin, 1926) for rigidly bound *lin*-benzo-ADP (Table II). The values of P_b obtained in the absence or presence of 20 μM cGMP were similar (Table II). The observed P_b was used to calculate the average angle of rotation (ω_p) of *lin*-benzo-ADP bound to the cGMP-dependent protein kinase, in the absence or presence of 20 μM cGMP, during the lifetime of its excited state (4.2 ns; VanDerLijn et al., 1978a). The values of 17° and 18° for ω_p obtained in either the absence or presence of cGMP, respectively, are very close to the theoretical value of 14° calculated for the rotation of the protein from the Perrin equation. P_0, the intrinsic polarization of the fluorophore, and P_{bound}, the polarization in the bound state, are similar within experimental error. Thus, most or all of the observed rotation of *lin*-benzo-ADP bound to the enzyme is due to the rotation of the protein itself. This also indicates that *lin*-benzo-ADP binds rigidly to the cGMP-dependent protein kinase in both the absence or presence of cGMP, so that there is little or no rotation within the adenine binding site.

Anisotropy values were also calculated (by the definition given under Materials and Methods) from the fluorescence intensity data obtained during the titration. The values of r_b,

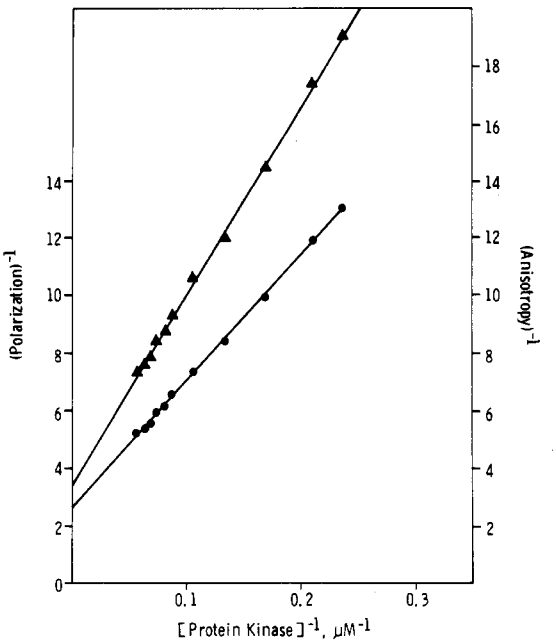

FIGURE 3: Determination of fluorescence polarization (P_b) and anisotropy (r_b) for the protein kinase. Dilution titrations were performed with the protein kinase in the presence of 20 μM cGMP as described in the legend to Figure 2 and under Materials and Methods. From the ordinate intercepts of a plot of (polarization)$^{-1}$ (●) or (anisotropy)$^{-1}$ (▲) vs. [protein kinase]$^{-1}$, a P_b value of 0.350 and an r_b value of 0.264 were obtained for the protein kinase in the presence of 20 μM cGMP.

anisotropy when all ligand is bound to the protein (obtained from the dilution titration, Figure 3), and ω_r, the average angle of rotation of *lin*-benzo-ADP bound to the enzyme (calculated from r_b by the Perrin equation), are listed in Table II. These values are in good agreement with the values of r_b and ω_r calculated with the Perrin equation (Lakowicz, 1983) for *lin*-benzo-ADP rigidly bound to the protein kinase in the absence and presence of 20 μM cGMP. The conclusions from this comparison (theoretical vs. observed) are similar to those made from polarization data.

The K_d values of *lin*-benzo-ADP for the cGMP-dependent protein kinase were determined by fluorescence polarization addition titrations (Figure 2B). *lin*-Benzo-ADP does not bind to the enzyme in the absence of Mg^{2+}, which is indicated by a low polarization value (P_f = 0.019). Following addition of Mg^{2+}, however, there is an increase in polarization, indicating binding of the ligand to the protein (Figure 2B) and reflecting a certain [bound]/[free] ratio of ligand in solution. With successive additions of the ligand, this ratio is lowered, and there is a corresponding decrease in the polarization value. Scatchard analysis (not shown) of the data obtained in such addition titrations gives the K_d (23 μM) of *lin*-benzo-ADP for the cGMP-dependent protein kinase in the presence of 20 μM cGMP and 200 μM [Ala^{34}]histone H2B-(29–35).

The K_d of ADP for the cGMP-dependent protein kinase was determined by fluorescence polarization displacement titrations. *lin*-Benzo-ADP, bound to the enzyme in the presence of 10 mM Mg^{2+} (polarization value = P_{max}), was displaced with increasing concentrations of ADP as demonstrated by a concomitant decrease in polarization. The polarization observed at any given concentration of the competing nucleotide was designated as P_{obsd}. The bound and free *lin*-benzo-ADP concentrations at each concentration of ADP were calculated from P_{max}, P_{obsd}, and P_f as described in Bhatnagar et al. (1983).

The K_d's of *lin*-benzo-ADP for the cGMP-dependent protein kinase in the absence or presence of cGMP are similar (Table

Table III: Kinetic Parameters of cGMP-Dependent Protein Kinase with ATP and *lin*-Benzo-ATP as Substrates

substrate	K_m (μM)[a]	V_{max}[a] [μmol (min·mg)$^{-1}$]
ATP	32.2 ± 0.57	11.1 ± 0.17
lin-benzo-ATP	28.5 ± 2.3	0.00623 ± 0.00035

[a] Determined by radioisotopic assay with [Ala^{34}]histone H2B-(29–35) as phosphoryl acceptor as described under Materials and Methods. Reactions were performed at a fixed, near saturating concentration of the peptide substrate (200 μM) and Mg^{2+} concentration of 10 mM. Nucleotide concentrations were varied from 10 to 100 μM. When ATP was the substrate, assays were conducted for 2 min at a protein kinase concentration of 0.3–0.4 μg/mL. When *lin*-benzo-ATP was the substrate, assays were conducted for 10–15 min with 8–15 μg/mL enzyme. K_m and V_{max} were determined both from double-reciprocal plots (v_0^{-1} vs. [nucleotide]$^{-1}$) and from plots of v_0 vs. v_0/[nucleotide] according to Hofstee (1952).

I) with a stoichiometry of 2 mol of *lin*-benzo-ADP bound per mol of the enzyme dimer. The K_i value of *lin*-benzo-ADP, however, is 2-fold greater than the K_d value of *lin*-benzo-ADP determined in the absence of the [Ala^{34}]histone H2B-(29–35) (Table I). Addition of this peptide substrate to the fluorescence titration decreases the binding affinity of *lin*-benzo-ADP for the enzyme 2-fold (from 11 to 23 μM) while the number of binding sites remains constant (2 mol/mol of enzyme dimer). The K_d of *lin*-benzo-ADP of 23 μM determined in the presence of [Ala^{34}]histone H2B-(29–35) then corresponds well with the K_i for *lin*-benzo-ADP of 22 μM. The K_d of *lin*-benzo-ADP is in good agreement with K_d of ADP for the enzyme when both K_d's are obtained in the absence of the peptide substrate (Table I). But in the presence of [Ala^{34}]-histone H2B-(29–35) the K_d of ADP for the enzyme is 5-fold higher than that of *lin*-benzo-ADP and is in agreement with the K_i of 169 μM of ADP for the cGMP-dependent protein kinase (Table I).

The K_d values of *lin*-benzo-ADP and ADP under various conditions were calculated on the basis of anisotropy as well. The maximum difference between these values obtained from anisotropy and polarization data was within experimental error (less than 2%). The determinations in this study have been based on polarization values to maintain continuity with earlier work (Hartl et al., 1983; Bhatnagar et al., 1983).

lin-Benzo-ATP as a Substrate for cGMP-Dependent Protein Kinase. To determine the effectiveness of *lin*-benzo-ATP as a phosphoryl donor for the reaction catalyzed by the cGMP-dependent protein kinase, steady-state kinetic studies were performed with *lin*-benzo-ATP or ATP and [Ala^{34}]histone H2B-(29–35) as phosphoryl acceptor. *lin*-Benzo-ATP has a K_m of 29 μM which corresponds well with the K_m for ATP of 32 μM (Table III). However, the V_{max} with *lin*-benzo-ATP is over 3 orders of magnitude less than that with ATP as substrate (Table III). Similar results were observed when Ser-peptide was used as the phosphoryl acceptor.

Metal Ion Dependence of Nucleotide Binding and Phosphotransferase Activity. As mentioned earlier, nucleotide binding to the protein kinase is dependent on the presence of a divalent cation. On addition of metal ion, *lin*-benzo-ADP binds to the enzyme, resulting in an increase in polarization. With increasing Ca^{2+} concentration, for example, the fluorescence polarization is enhanced, which indicates that an increasing amount of *lin*-benzo-ADP is bound to the enzyme (Figure 4). The binding of the nucleotide to the cGMP-dependent protein kinase is half-maximum at 1.0 mM Ca^{2+} and plateaus at approximately 10 mM. The K_d of 1.2 mM for Ca^{2+} was determined from a plot of (polarization)$^{-1}$ vs. [Ca^{2+}]$^{-1}$ (Figure 4, inset).

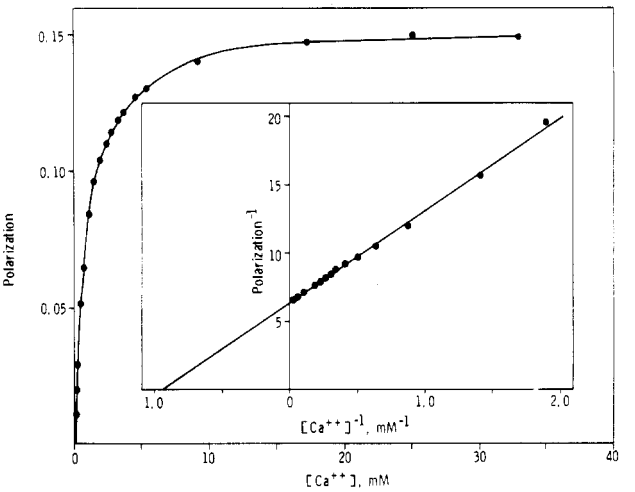

FIGURE 4: Metal ion requirement of nucleotide binding to the cGMP-dependent protein kinase. Polarization titrations were performed at 23 °C in 50 mM Mops (pH 7.0), 100 mM NaCl, and 20 μM cGMP. Fluorescence polarization is enhanced with an increase in Ca^{2+} concentration (0–32 mM) as a result of increased binding of *lin*-benzo-ADP to the protein kinase. (Inset) A double-reciprocal plot of (polarization)$^{-1}$ vs. [Ca^{2+}]$^{-1}$ of the data. The x intercept of the plot corresponds to $-1/K_d$ for Ca^{2+}.

Table IV: Dependence of Nucleotide Binding and Phosphotransferase Activity of cGMP-Dependent Protein Kinase on Metal Ions

metal ion	K_d (mM)[a]	phosphotransferase activity (%)[b]
none	ND[c]	<0.6[d]
Mg^{2+}	1.4 ± 0.18	100.0
Mn^{2+}	0.144 ± 0.008	6.5 ± 0.1
Co^{2+}	0.723 ± 0.012	23.7 ± 0.6
Cd^{2+}	ND	<0.2[d]
Ca^{2+}	1.2 ± 0.10	<0.5[d]
Sr^{2+}	4.23 ± 0.82	<0.3[d]
Ba^{2+}	4.64 ± 0.66	<0.6[d]
Fe^{2+}	ND	ND
Ni^{2+}	1.6 ± 0.12	30.4 ± 0.9
Zn^{2+}	ND	<0.2[d]
Cu^{2+}	ND	ND
Gd^{3+}	ND	ND
La^{3+}	ND	ND

[a] Determined by fluorescence polarization enhancement procedure as described in the legend of Figure 4. [b] For catalytic activity determinations, all metal ions were added as their Cl^- salts (except for Cd^{2+}, which was added as $CdSO_4$). The metal ions that supported nucleotide binding were used at final concentrations of 3 times their apparent K_d values. Metal ions that did not support binding were used at a concentration of 10 mM. Activities are expressed as a percentage of the activity in the presence of 4.2 mM $MgCl_2$, which corresponds to a catalytic activity of 14.2 ± 0.7 μmol (min·mg)$^{-1}$ (n = 7). The activity measurements were made in the presence of 400 μM [γ-^{32}P]ATP and 1400 μM Ser-peptide with 1.2 μg/mL protein kinase for 2 min. [c] ND, not detectable. [d] The radioactive product measured in the assays was less than twice that of the background (minus enzyme blank).

A number of metal ions (Mg^{2+}, Mn^{2+}, Co^{2+}, Ca^{2+}, Sr^{2+}, Ba^{2+}, and Ni^{2+}) promoted nucleotide binding to the enzyme as demonstrated by an enhancement in fluorescence polarization (Table IV). There was no increase in fluorescence polarization caused by these metal ions in the presence of *lin*-benzo-ADP without the enzyme. On the other hand, Cd^{2+}, Fe^{2+}, Zn^{2+}, Cu^{2+}, Gd^{3+}, and La^{3+} failed to promote any binding under our experimental conditions. Cd^{2+}, moreover, seemed to denature and precipitate the enzyme since addition of 5 mM Mg^{2+}, following a Cd^{2+} titration, failed to promote nucleotide binding.

Subsaturating metal ion concentrations (3 times the K_d of metal ions) were used to determine which metals support

phosphotransferase activity. Under these conditions, the catalytic activity was maximal [14.2 μmol (min·mg)$^{-1}$] in the presence of 4.2 mM Mg^{2+} (Table IV). Of the other metal ions tested Ni^{2+} and Co^{2+} were most effective. At a 2.2 mM concentration, Co^{2+} sustained a catalytic activity of 3.37 μmol (min·mg)$^{-1}$; with 4.8 mM Ni^{2+}, an activity of 4.32 μmol (min·mg)$^{-1}$ was recorded. Mn^{2+} (432 μM) supported less than 7% of the activity of the enzyme as compared to that supported by 4.2 mM Mg^{2+}. Although Ca^{2+}, Sr^{2+}, and Ba^{2+} promoted binding of the nucleotide to the enzyme (Table IV), no significant phosphotransferase activity of the cGMP-dependent enzyme was measured in the presence of any of these metal ions at concentrations 3 times their respective K_d values under our experimental conditions. Cd^{2+}, Fe^{2+}, Zn^{2+}, Cu^{2+}, Gd^{3+}, and La^{3+} at concentrations of 10 mM failed to support detectable phosphotransferase activity.

The cGMP-dependent protein kinase is similar to the cAMP-dependent enzyme in that increasing the concentration of Mg^{2+} lowers the K_m for ATP but also lowers the V_{max} of the reaction (Glass & Krebs, 1979; Cook et al., 1982). For example, in our experiments with 200 μM [Ala^{34}]histone H2B-(29–35) at 3 mM Mg^{2+}, the K_m for ATP was 44.9 μM and the V_{max} was 13.6 μmol (min·mg)$^{-1}$ as compared with the K_m of ATP of 32.2 μM and V_{max} of 11.1 μmol (min·mg)$^{-1}$ at 10 mM Mg^{2+}. The effect on the cAMP-dependent enzyme, however, was more dramatic.

Discussion

We have studied the interaction of *lin*-benzoadenine nucleotides with the cGMP-dependent protein kinase. *lin*-Benzo-ADP, which is stretched out by 2.4 Å over ADP in its adenine moiety (Scopes et al., 1977), is a linear competitive inhibitor (Cleland, 1970) with respect to Mg·ATP as is ADP (as described under Results). In addition, the K_d for *lin*-benzo-ADP determined by fluorescence polarization studies (in the absence of peptide substrate) is very close to the K_d determined for ADP under similar conditions (Table I). Thus, *lin*-benzo-ADP binds to the active site of the enzyme as does ADP. However, *lin*-benzo-ADP binds to the protein kinase [in the presence of peptide substrate [Ala^{34}]histone H2B-(29–35)] with over 6-fold tighter affinity than does ADP (Table I). For the cAMP-dependent protein kinase catalytic subunit, on the other hand, the K_i of *lin*-benzo-ADP (from inhibition studies) and its K_d (from fluorescence polarization studies) were found to be very close to the kinetically determined K_i for ADP (Hartl et al., 1983) with Ser-peptide as substrate. Unlike the cGMP-dependent enzyme, the K_d of *lin*-benzo-ADP for the cAMP-dependent protein kinase catalytic subunit was not altered by 200 μM [Ala^{34}]histone H2B-(29–35) (K_d = 10.2 μM in the absence of peptide and 9.8 μM in the presence of peptide). These K_d values of *lin*-benzo-ADP for the cAMP-dependent enzyme correspond well with the K_d of 8.9 μM of ADP in the presence of 200 μM [Ala^{34}]histone H2B-(29–35).

The polarization data in Table II indicate that the *lin*-benzoadenine moiety is rigidly bound to the cGMP-dependent protein kinase within the limits of detection. This parallels our observations for the cAMP-dependent protein kinase catalytic subunit (Hartl et al., 1983; Bhatnagar et al., 1983). Granot et al. (1979) showed by NMR that there is a strong interaction between the catalytic subunit of cAMP-dependent protein kinase and the adenosine portion of $Co(NH_3)_4ATP$. This differs from the binding of *lin*-benzo-ATP to *Escherichia coli* aspartate transcarbamylase (Chien & Weber, 1973; VanDerLijn et al., 1978b) and pyruvate kinase (Barrio et al., 1973). In the latter two cases, *lin*-benzo-ATP is bound in a manner such that there is appreciable rotational freedom within the nucleotide binding site as demonstrated by fluorescence polarization.

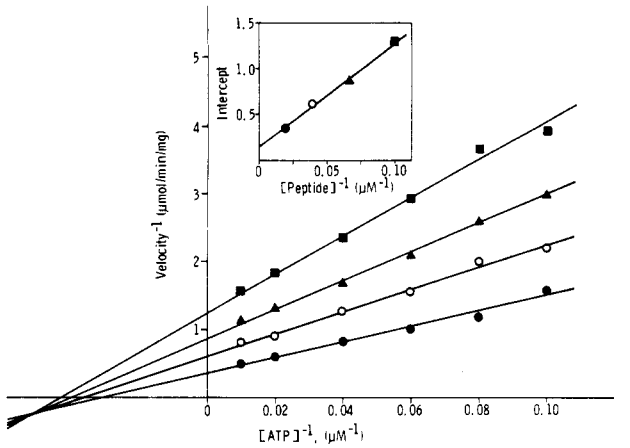

FIGURE 5: Initial velocity pattern of cGMP-dependent protein kinase using ATP and histone H2B-(29–35) as substrates. The enzyme (1.0 μg/mL) was incubated for 2 min at 30 °C with [γ-^{32}P]ATP concentrations from 10 to 100 μM at fixed histone H2B-(29–35) concentrations of 10 (■), 15 (▲), 25 (○), and 50 (●) μM. Activity was measured as described under Materials and Methods except the buffer was 30 mM Tris-HCl (pH 7.4) and the $MgSO_4$ concentration was 3 mM. (Inset) Replot of the velocity^{-1}–axis intercept vs. [peptide]$^{-1}$.

As summarized in Table I, there is a significant effect of the presence of the peptide substrate, [Ala^{34}]histone H2B-(29–35), on the binding affinity of both ADP and *lin*-benzo-ADP to the cGMP-dependent protein kinase, but this is not so for the cAMP-dependent protein kinase (not shown). In addition, *lin*-benzo-ADP binds to the cGMP-dependent enzyme with greater affinity than does ADP (Table I); *lin*-benzo-ADP and ADP, on the other hand, bind to the catalytic subunit of the cAMP-dependent protein kinase with essentially equal affinities (Hartl et al., 1983; Bhatnagar et al., 1983). The large difference between the K_i values for *lin*-benzo-ADP and ADP itself (for the cGMP-dependent enzyme; Table I) was not due merely to the use of [Ala^{34}]histone H2B-(29–35) as the phosphate-accepting substrate. When Ser-peptide (1400 μM) was used as the substrate in the presence of 10 mM Mg^{2+}, the K_i for *lin*-benzo-ADP was 15.7 μM and the K_i for ADP was 132 μM. The K_i of ADP of 169 μM, moreover, is similar to the value of 265 μM previously reported (Glass et al., 1981) when a different buffer and pH were used. Even at lower Mg^{2+} (3 mM), the K_i of *lin*-benzo-ADP was 17.7 μM and that of ADP was 233 μM when [Ala^{34}]histone H2B-(29–35) was used as the substrate.

The decrease in binding affinity of adenine nucleotides due to the peptide could be because [Ala^{34}]histone H2B-(29–35) is so positively charged that it interacts ionically with either the adenine nucleotide or the enzyme in such a way so as to disturb the binding of the nucleotide to the active site of the enzyme. In any event, the conclusion that the peptide decreases the affinity of the enzyme for adenine nucleotide is consistent with earlier reports of the pattern of the initial velocity kinetics (Glass et al., 1981). Steady-state kinetic studies using the parent peptide histone H2B-(29–35) as the acceptor and ATP as the donor show that the lines intersect at a point *below* the *x* axis (Figure 5). As the concentration of the histone peptide is increased, the apparent K_m value of ATP is increased (i.e., a decreased affinity). Varying the concentration of ATP affects the apparent K_m values of histone peptide in a similar fashion (data not shown). This type of initial velocity plot is not seen with all peptide substrates of the cGMP-dependent protein kinase. Specifically, the kinetics

performed with ATP and a peptide corresponding to the autophosphorylation site in the enzyme (Glass & Smith, 1983) yield initial velocity lines that intersect above the x axis (Glass, unpublished observations). In this case, as the concentration of one substrate is increased, the apparent K_m of the other substrate is decreased.

The large difference in the affinities of cGMP-dependent protein kinase for *lin*-benzo-ADP as compared to ADP (Table I) in the presence of [Ala^{34}]histone H2B-(29–35) might be due to a conformational change induced by the peptide. A comparable phenomenon has been extensively studied with hexokinase (Bennett & Steitz, 1980) where glucose binds to the enzyme prior to ATP and brings about a conformational change of the two lobes of the hexokinase molecule so as to place the ATP molecule in a suitable position for the phosphorylation of glucose. In the case of the cGMP-dependent protein kinase, the K_m values are essentially the same with ATP or *lin*-benzo-ATP as substrate, but the V_{max} with *lin*-benzo-ATP is very low, over 3 orders of magnitude less than that with ATP. This also suggests that in the presence of the peptide the γ-phosphate of *lin*-benzo-ATP is not in a suitable position for phosphotransferase activity to occur possibly due to the lateral stretching of 2.4 Å of the adenine ring system (in *lin*-benzo nucleotides). Most enzymes that utilize *lin*-benzo-ATP as substrate exhibit a lower V_{max} with a K_m comparable to that seen with ATP (Leonard et al., 1978). Alternative mechanisms, however, might be responsible for the low V_{max} seen with the cGMP-dependent protein kinase. To assure that the low V_{max} values were not due to technical problems with the *lin*-benzo-[γ-^{32}P]ATP preparations, they were tested with the catalytic subunit of the cAMP-dependent enzyme. The K_m of catalytic subunit for *lin*-benzo-ATP was 14.9 μM, and the V_{max} was 4.03 μmol (min·mg)$^{-1}$ with the *lin*-benzo-[γ-^{32}P]ATP synthesized for this study. These values are in good agreement with our previous results [11.3 μM for K_m and 5.0 μmol (min·mg)$^{-1}$; Hartl et al., 1983].

The K_i's of *lin*-benzo-ADP or ADP for the catalytic subunit of the cAMP-dependent protein kinase are not altered either in the presence of [Ala^{34}]histone H2B-(29–35) (as stated earlier) or with Ser-peptide (Hartl et al., 1983; Bhatnagar et al., 1983). With that enzyme *lin*-benzo-ATP, *lin*-benzo-ADP, ATP, and ADP have the same affinity for the enzyme (Hartl et al., 1983; Bhatnagar et al., 1983). Also, *lin*-benzo-ATP is a good substrate for the catalytic subunit, with a K_m equal to that of ATP and a V_{max} 20% that of the V_{max} of ATP. Therefore, unlike the cGMP-dependent enzyme, the cAMP-dependent protein kinase appears to accommodate *lin*-benzo-ADP and *lin*-benzo-ATP more like ADP and ATP, respectively, both in the absence or in the presence of the peptide substrate.

The requirement of a divalent metal cation for the enzyme activity of the cGMP-dependent protein kinase is well established (Kuo et al., 1978). As stated under Results, binding of *lin*-benzo-ADP is enhanced (increased fluorescence polarization) by increasing the concentration of metal ion. This further shows that the metal ion is required for nucleotide binding to the cGMP-dependent enzyme. We suggest that the apparent K_d values (Table IV) reflect the affinities of the metal ions for an inhibitory binding site on the enzyme similar to that discussed earlier for cAMP-dependent protein kinase catalytic subunit (Granot et al., 1979; Bhatnagar et al., 1983). The rank order of metal ion effectiveness in supporting phosphotransferase activity was $Mg^{2+} > Ni^{2+} > Co^{2+} > Mn^{2+}$. To our knowledge this is the first report as to the ability of Ni^{2+} to support nucleotide binding and catalysis of the cGMP-dependent protein kinase. Otherwise, these results are generally consistent with most of the early reports on the *mammalian* cGMP-dependent protein kinase: i.e., Co^{2+} is the next best metal after Mg^{2+} in supporting catalysis, and Mn^{2+} will do so only weakly (Hofmann & Sold, 1972; Nakazawa & Sano, 1975; Kuo et al., 1976; Shoji et al., 1977a,b; Gill et al., 1977). In an early report with the *arthropod* enzyme, however, the rank order was $Co^{2+} > Mn^{2+} > Mg^{2+}$ in supporting catalysis (Kuo & Greengard, 1970). More recently, Hixson & Krebs (1981) have reported that Co^{2+} increases the pseudo-first-order rate constant of inactivation of the cGMP-dependent protein kinase by 5′-[*p*-(fluorosulfonyl)-benzoyl]adenosine compared to the rate constant in the absence of metal ion; Co^{2+} was better than Mg^{2+} in doing so. No correlation between the ionic radii or the electronic configurations of the metal ions could be established with either nucleotide binding or catalytic activity.

The metal ion requirement of the cGMP-dependent protein kinase, apart from the actual K_d values of metal ions for the enzymes, differs from that of the catalytic subunit of the cAMP-dependent enzyme in the effectiveness of the metal ions in supporting catalysis. The rank order for the catalytic subunit of the cAMP-dependent enzyme was $Mg^{2+} > Mn^{2+} > Co^{2+} > Cd^{2+}$ (Bhatnagar et al., 1983). Ni^{2+} and Ba^{2+} did not support either catalysis or nucleotide binding in the case of the cAMP-dependent protein kinase (Bhatnagar et al., 1983), whereas Ni^{2+} supported both catalysis and nucleotide binding and Ba^{2+} only nucleotide binding to the cGMP-dependent protein kinase (Table IV). Cd^{2+}, on the other hand, did not support either catalysis or nucleotide binding with the cGMP-dependent enzyme but was effective for both functions with the catalytic subunit of the cAMP-dependent enzyme.

In conclusion, the interactions of *lin*-benzoadenine di- and triphosphates with the cGMP-dependent protein kinase (in this study) were similar for most part to those observed with the cAMP-dependent protein kinase (Hartl et al., 1983; Bhatnagar et al., 1983). The major differences were in the V_{max} for *lin*-benzo-ATP and the effect of peptide substrates on nucleotide binding. There appears to be some conformational change in the cGMP-dependent protein kinase on peptide substrate binding which seems to distort the nucleotide binding site, especially the adenine binding region. The determinants within the histone peptide producing such an effect might be conveniently studied by determining the influences of a series of synthetic peptide analogues (Glass & Krebs, 1982) on the binding of *lin*-benzo-ADP. This conformational change may be responsible for the extremely low V_{max} (almost one-two thousandth of that with ATP) observed with *lin*-benzo-ATP (Table III). The K_m values for *lin*-benzo-ATP and ATP were similar. There is no evidence for any such effect within the catalytic subunit of the cAMP-dependent protein kinase where the V_{max} for *lin*-benzo-ATP is quite comparable to that for ATP (Hartl et al., 1983; this study) and the K_m values for *lin*-benzo-ATP and ATP are very similar. The binding affinity of *lin*-benzo-ADP for the holoenzyme form (−cGMP) of the cGMP-dependent enzyme is quite similar to that for the catalytic form of the enzyme (+cGMP) (Table I). By contrast, with the cAMP-dependent protein kinase, the presence of the regulatory subunit with the catalytic subunit (in the type II holoenzyme) results in a 3-fold increase in affinity of *lin*-benzo-ADP for the enzyme when compared with that of the catalytic subunit alone (Hartl et al., 1983).

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