Adenosine Cyclic 3′,5′-Monophosphate Dependent Protein Kinase: A New Fluorescence Displacement Titration Technique for Characterizing the Nucleotide Binding Site on the Catalytic Subunit†

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ABSTRACT: We determined the dissociation constant (K_d) of a series of nucleotides for the bovine skeletal muscle type II catalytic subunit by displacing *lin*-benzoadenosine 5′-diphosphate (*lin*-benzo-ADP) with increasing concentrations of competing nucleotide. The K_d of each nucleotide was calculated from the decreases in the fluorescence polarization of *lin*-benzo-ADP that accompany its displacement from the catalytic subunit. We found that modifications of the adenine moiety reduce nucleotide affinity for the enzyme. The effect was most pronounced with modifications at position 6 of the base. Replacement of the 3′-hydroxyl group of ribose with a hydrogen increased the affinity of the nucleotide; addition of phosphate to the 2′- or 3′-hydroxyl groups, on the other hand, decreased nucleotide affinity. MgATP and MgADP exhibited K_d's of about 10 μM. AMP, which contains a negatively charged α-phosphate, bound with reduced affinity (643 μM). Adenosine, which lacks a charged α-phosphate, bound with a higher affinity (32 μM). To learn more about the nature of the α-phosphate binding site, a series of uncharged and positively charged derivatives of the 5′-position on the ribose moiety was prepared. The uncharged derivatives bound with much greater affinity than the negatively charged AMP. The K_d's for 5′-tosyladenosine and 5′-iodo-5′-deoxyadenosine were 30 and 32 μM, respectively. Like the negatively charged AMP, positively charged derivatives also bound less tenaciously than the neutral species. The positively charged 5′-amino-5′deoxyadenosine, for example, exhibited a 15-fold higher K_d (506 μM) than the neutral congenors. We hypothesized that the enzyme's site complementary to the α-phosphate is hydrophobic in nature. We found that adding hydrophobic groups to the positive charge at the 5′-position increased the binding affinity [K_d's for 5′-(ethylamino)-, 5′-(diethylamino)-, 5′-(triethylammonium)-, and 5′-(diallylamino)-5′-deoxyadenosine are 403, 284, 153 and 102 μM, respectively]. The binding of *lin*-benzo-ADP to the catalytic subunit of protein kinase is dependent upon a divalent cation. We tested several metals for their ability to promote binding and to support phosphotransferase activity. Fluorescence polarization studies revealed that Mg^{2+}, Mn^{2+}, Co^{2+}, Cd^{2+}, Ca^{2+}, and Sr^{2+} supported nucleotide binding to the catalytic subunit, whereas Ba^{2+}, Cr^{2+}, Fe^{2+}, Ni^{2+}, Zn^{2+}, Cu^{2+}, Gd^{3+}, and La^{3+} did not. Even though Ca^{2+} and Sr^{2+} promoted nucleotide binding, no measurable phosphotransferase activity was observed in their presence.

Adenosine cyclic 3′,5′-monophosphate (cAMP)[1] dependent protein kinases (ATP:protein phosphotransferase, EC 2.7.1.37) catalyze the phosphorylation of polypeptidic serine and threonine residues. These enzymes are generally classified as type I or type II according to their order of elution during chromatography on DEAE-cellulose (Corbin et al., 1975). Both isozymes are tetrameric proteins containing two monomeric catalytic subunits (C) and one dimeric regulatory subunit (R_2) (Beavo et al., 1975). The two isozymes differ in their interaction with ATP. The type II holoenzyme, for example, undergoes autophosphorylation in the presence of ATP (Erlichman et al., 1974). The type I enzyme, on the other hand, binds ATP with a K_d of 50 nM but does not undergo intramolecular autophosphorylation. The difference between the type I and II protein kinases resides mainly in the differences in their regulatory subunits (Haddox et al., 1972; Beavo et al., 1975; Zoller et al., 1979). The catalytic subunits are almost identical in terms of their molecular weight, amino acid composition, and substrate specificity regardless of whether they are purified from type I or type II isozymes (Hofmann et al., 1975, 1977; Sugden et al., 1976; Demaille et al., 1977; Peters et al., 1977; Zoller et al., 1979).

The requirement for a divalent metal cation (Mg^{2+}) for enzyme activity of the catalytic subunit is well established (Krebs, 1972; Walsh & Krebs, 1973). A combination of nuclear magnetic resonance (Granot et al., 1979, 1980) and steady-state kinetic studies (Armstrong et al., 1979a; Bolen et al., 1980; Cook et al., 1982) has led to the proposal of an inhibitory divalent metal site in addition to the metal's role in complex formation with nucleotide. Granot et al. (1980) reported that occupation of the inhibitory site by Mn^{2+} decreases the maximum velocity and increases the V/K for metal nucleotide as a result of an increased nucleotide affinity and decreased K_m. Our studies confirmed these findings (Cook et al., 1982).

One approach to aid in the understanding of the properties of protein kinase is to examine the specificity of nucleotide binding. We have developed a rapid, nondestructive and sensitive fluorescence polarization titration technique for characterizing the ATP binding site of the catalytic subunit of the type II cAMP-dependent protein kinase. The interaction of various divalent metal ions with the catalytic subunit with respect to nucleotide binding has also been investigated with the fluorescence technique. We have used fluorescent *lin*-benzoadenosine 5′-diphosphate, a "stretched-out" analogue of

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[1] Abbreviations: cAMP, adenosine cyclic 3′,5′-monophosphate; C, catalytic subunit; R, regulatory subunit; GTP, guanosine 5′-triphosphate; ITP, inosine 5′-triphosphate; Ser-peptide, Leu-Arg-Arg-Ala-Ser-Leu-Gly; AMP-PCP, adenosine 5′-(β,γ-methylenetriphosphate); Mops, 3-(*N*-morpholino)propanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; NBD-Cl, 7-chloro-4-nitro-2,1,3-benzoxadiazole.

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adenine nucleotide (Leonard et al., 1978), for these determinations. Hartl et al. (1983) have shown that *lin*-benzo-ADP is a competitive inhibitor of the catalytic subunit with respect to ATP with a K_i (8.0 μM) similar to the K_i for ADP (9.0 μM). These fluorescence polarization studies also showed that both the catalytic subunit and type II holoenzyme bind *lin*-benzo-ADP rigidly, so that there is little or no rotation of the *lin*-benzoadenine portion of the molecule within the nucleotide binding site. In contrast to 1,N^6-etheno-ATP, *lin*-benzo-ATP is a very good substrate for the phosphotransferase activity of protein kinase with peptides, water, or type II regulatory subunit as phosphoryl acceptors (Hartl et al., 1983).

Materials and Methods

Materials. The synthetic heptapeptide used as substrate (Leu-Arg-Arg-Ala-Ser-Leu-Gly) was purchased from Sigma Chemical Co. or Peninsula Laboratories. Carrier-free [γ-^{32}P]ATP was purchased from ICN. 5′-Iodo-5′-deoxyadenosine was obtained from Aldrich Chemical Co., and 2′-deoxyadenosine was purchased from U.S. Biochemical Corp. 5′-Amino analogues of adenosine [5′-amino-, 5′-(ethylamino)-, 5′-(diethylamino)-, 5′-(triethylammonium)-, and 5′-(diallylamino)-5′-deoxyadenosine] were synthesized from 5′-tosyladenosine (Aldrich Chemical Co.) as described by Schmidt et al. (1968) and Murayama et al. (1971). The salts of divalent metal ions were purchased from Aldrich Chemical Co. All other nucleoside and nucleotide analogues and chemicals used were purchased from Sigma Chemical Co. The concentration of *lin*-benzo-ADP was measured by absorbance at 331 nm with an extinction coefficient of 9750 M^{-1} cm^{-1} (Leonard et al., 1976). The extinction coefficients of ITP and GTP used for similar calculations were 12 200 M^{-1} cm^{-1} at 248.5 nm and 13 700 M^{-1} cm^{-1} at 252.5 nm, respectively (The Merck Index, 1976).

Protein Kinase Preparation and Activity Measurements. Type II catalytic subunit from bovine skeletal muscle was purified as described by Hartl & Roskoski (1982). Phosphotransferase activity of the catalytic subunit was measured in the presence of subsaturating amounts ($3K_{app}$) of metals that promoted the binding of the nucleotide to the enzyme under our experimental conditions. The Ser-peptide (K_m = 10 μM; Cook et al., 1982) and ATP (K_m = 10 μM; Cook et al., 1982) concentrations were 100 and 200 μM, respectively. The C subunit concentration was 7–10 nM. Time courses of enzyme activity were performed as described by Hartl & Roskoski (1982) and Roskoski (1983) in triplicate at 23 °C at pH 7.0 under identical buffer and salt concentrations as those used for the fluorescence polarization studies. Protein concentrations were determined by the procedure of Lowry et al. (1951).

Fluorescence Polarization Measurements. Fluorescence measurements were made with an SLM 4800 spectrofluorometer. Sample temperatures were maintained at 23 °C. This instrument was interfaced with a Hewlett-Packard desk calculator (HP 9825 A), which allowed spectral data to be stored digitally on magnetic tape. Fluorescence polarization was calculated with a program supplied by SLM Instruments, Inc. Polarization is defined as

$$P = (I_{\parallel} - I_{\perp})/(I_{\parallel} + I_{\perp})$$

where $I_{\parallel}$ and $I_{\perp}$ are the intensities observed parallel and perpendicular to the polarization of the exciting light, respectively, and P is polarization. Polarization of the fluorescent probe, *lin*-benzo-ADP, was determined with 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 catalytic subunit. The titrations were performed in 50 mM Mops (pH 7.0) and 100 mM NaCl.

(*a*) *Dilution Titration.* Polarization as a function of varying C subunit concentration (at constant *lin*-benzo-ADP) was performed as described by Hartl et al. (1983) to determine P_b. P_b is the polarization value when all *lin*-benzo-ADP is bound to the catalytic subunit (at infinite C subunit concentration).

(*b*) *Addition Titration.* Polarization (P_{obsd}) was recorded at each nucleotide concentration after addition of successive increments of *lin*-benzo-ADP with a constant C subunit concentration (6 μM). The dissociation constant (K_d) of *lin*-benzo-ADP for the C subunit was then determined as described by Hartl et al. (1983).

(*c*) *Displacement Titration.* The dissociation constant of various nucleotide analogues for the C subunit was determined by displacing the fluorescent nucleotide *lin*-benzo-ADP bound to the C subunit with other nucleotides. A brief description of the calculation of the K_d is given here. Its derivation is given by analogy to equations of steady-state enzyme kinetics and is plotted similarly to a Scatchard analysis where -1/slope yields the K_d. According to the Michaelis–Menten equation, the velocity of reaction is given by

$$v = [V][A]/(K_A + [A]) \quad (1)$$

where v = velocity, $[V] = V_{max}$, [A] = substrate concentration, and K_A = Michaelis constant. In the presence of a competitive inhibitor, the slope factor is added, and the equation becomes

$$v = \frac{[V][A]}{K_A(1 + [I]/K_I) + [A]} \quad (2)$$

where [I] = concentration of the inhibitor and K_I = the dissociation constant of the inhibitor (a thermodynamic binding constant or true K_d). Equation 2 can be transformed into an analogous form for use in the current study, i.e., in terms of *lin*-benzo-ADP bound to the enzyme and free in the medium:

$$B_{lin\text{-ADP}} = \frac{BF_{lin\text{-ADP}}}{K_{lin\text{-ADP}}(1 + [\text{inhibitor}]/K_I) + F_{lin\text{-ADP}}} \quad (3)$$

where $B_{lin\text{-ADP}}$ = concentration of *lin*-benzo-ADP bound at each concentration of inhibitor, B = concentration of *lin*-benzo-ADP bound in the absence of inhibitor, $K_{lin\text{-ADP}} = K_d$ for *lin*-benzo-ADP, $K_I = K_d$ for inhibitor or displacing nucleotide, $F_{lin\text{-ADP}}$ = concentration of free *lin*-benzo-ADP (i.e., total minus bound), and [inhibitor] = concentration of displacing nucleotide.

By rearrangement of eq 3

$$B_{lin\text{-ADP}}\left(1 + \frac{K_{lin\text{-ADP}}}{F_{lin\text{-ADP}}}\right) = (-1/K_I)\left(B_{lin\text{-ADP}}\frac{[\text{inhibitor}]}{F_{lin\text{-ADP}}}K_{lin\text{-ADP}}\right) \quad (4)$$

Equation 4 is in the form $y = mx + c$, the equation for a straight line, where

$$x = B_{lin\text{-ADP}}([\text{inhibitor}]/F_{lin\text{-ADP}})K_{lin\text{-ADP}} \quad (5)$$

$$y = B_{lin\text{-ADP}}(1 + K_{lin\text{-ADP}}/F_{lin\text{-ADP}}) \quad (6)$$

$$\text{slope} = -1/K_I$$

and

$$\text{intercept} = B$$

Therefore, the negative of the reciprocal of the slope (determined by least-squares fit) of the x and y plot will give the K_d of the inhibitor or the competing nucleotide. The K_d for *lin*-benzo-ADP was measured from the addition titration. To solve for x and y at varying concentrations of the displacing nucleotide, [inhibitor], the concentrations of bound, $B_{lin\text{-ADP}}$, and free, $F_{lin\text{-ADP}}$, *lin*-benzo-ADP at a given concentration of the displacing nucleotide were determined experimentally as follows.

Arbitrarily, a concentration of *lin*-benzo-ADP was selected to give a polarization (P_{max}) between 0.13 and 0.14 for 6 μM C subunit. P_{max} is the polarization value corresponding to the (maximum) amount of ligand that binds to 6 μM C subunit in the absence of inhibitor. It is more convenient to describe the binding in terms of the saturation fraction of ligand, $\bar{S}$, the concentration of ligand bound to the enzyme divided by the total concentration of ligand added to the medium. If the polarization is used as a measure of the saturation fraction of ligand, then experimentally

$$\bar{S} = \frac{P_{max} - P_f}{P_b - P_f} \qquad 0 \le \bar{S} \le 1$$

where P_{max} = maximum polarization observed at the selected concentration of ligand (*lin*-benzo-ADP), P_b = polarization when all ligand is bound to the C subunit (obtained from dilution titration), and P_f = minimum polarization observed corresponding to that of free or unbound *lin*-benzo-ADP (when no ligand is bound to the C subunit).

The displacement titration was performed as follows. A low concentration (1–2 μM) of *lin*-benzo-ADP (to give a polarization, P_{max}, of about 0.13–0.14) was added to 6 μM catalytic subunit in a total volume of 150 μL. The fluorescence polarization was recorded (P_f). $MgSO_4$ (10 mM final) was then added in a volume of 1 μL to promote binding of the *lin*-benzo-ADP to the catalytic subunit, and the enhancement in polarization was recorded (P_{max}). Then increasing concentrations of the competing ligand were added (1 μL at a time), which displaced some of the *lin*-benzo-ADP bound to the enzyme. After equilibration, the polarization following each addition was then recorded (P_{obsd}). Bound ($B_{lin\text{-ADP}}$) and free ($F_{lin\text{-ADP}}$) *lin*-benzo-ADP were calculated by the following equations at each concentration of the displacing ligand, [inhibitor]:

$$B_{lin\text{-ADP}} = \frac{P_{obsd} - P_f}{P_{max} - P_f}\bar{S}[lin\text{-benzo-ADP}_{total}]$$

$$F_{lin\text{-ADP}} = [lin\text{-benzo-ADP}_{total}] - B_{lin\text{-ADP}}$$

All determinations were performed in triplicate. The standard deviation was less than 10% of the respective K_d value.

Results

At constant *lin*-benzo-ADP concentration, the fluorescence polarization (P_{obsd}) increases with increasing catalytic subunit concentration as more ligand is bound (Figure 1A). This ranges from P_f (zero protein concentration) to P_b (all ligand is bound to the protein). P_b is obtained from extrapolating a plot of P^{-1} vs. [catalytic subunit]$^{-1}$ to zero (infinite C subunit concentration). The value of P_b thus obtained by dilution titration is 0.289 ± 0.011. This agrees well with 0.297 calculated for *lin*-benzo-ADP rigidly bound to C subunit with the Perrin equation (Hartl et al., 1983).

Little or no binding of *lin*-benzo-ADP to the catalytic subunit occurs in the absence of Mg^{2+}, which is indicated by a low polarization value of 0.020 (P_f). On addition of Mg^{2+}, however, there is an increase in polarization, indicating binding of the ligand to the protein (Figure 1B). This polarization value reflects a certain [bound] to [free] ratio of the ligand in solution. With successive additions of ligand to the protein solution the [bound]/[free] ratio of the ligand is altered, and the polarization decreases with each addition of *lin*-benzo-ADP. The volume of ligand added is small so that the protein concentration is nearly constant. To eliminate the possible error due to dilution or time-dependent changes in the C subunit during the binding studies, a new protein solution was also used at the higher *lin*-benzo-ADP concentrations in pilot experiments. There was a maximum of 3% difference between the polarization values recorded under the two conditions and this procedure was therefore not routinely used. The K_d of *lin*-benzo-ADP of 10.6 ± 1.1 μM for the free catalytic subunit determined by Scatchard analysis (Table I) from data obtained in these titrations agrees well with our previously determined K_i of 11.6 ± 3.1 μM (Hartl et al., 1983).

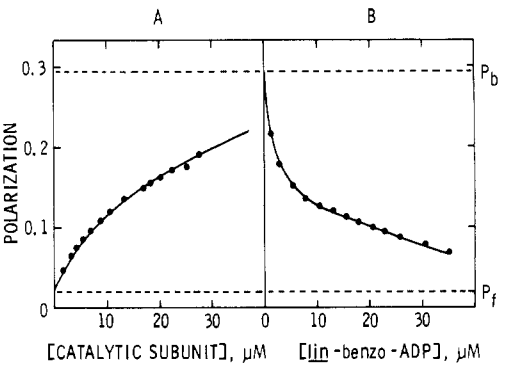

FIGURE 1: Polarization titrations of catalytic subunit with *lin*-benzo-ADP. Measurements were performed at 23 °C in 50 mM Mops (pH 7.0)–0.1 M NaCl–10 mM $MgSO_4$. (A) Dilution titration. Catalytic subunit was varied (2–25 μM) at constant (2 μM) total *lin*-benzo-ADP concentration in the presence of 10 mM $MgSO_4$ in a total volume of 150 μL. (B) Addition titration. *lin*-Benzo-ADP was varied (2–25 μM) at constant (6 μM) catalytic subunit concentration in 150-μL total volume by addition of 1-μL portions of *lin*-benzo-ADP solution. Corrections for the changes in volume and ligand concentrations were routinely made. Polarization was volume independent.

Table I: Dissociation Constant (K_d) of *lin*-Benzo-ADP for the Catalytic Subunit[a]

[Mg^{2+}] (mM)	K_d (μM)	$\bar{n}$
10	10.6 ± 1.1	0.97 ± 0.11
3.5	9.2 ± 0.9	0.95 ± 0.23

[a] K_d values and number of binding sites per mole of enzyme ($\bar{n}$) were determined from Scatchard plots of data obtained from fluorescence polarization addition and dilution titrations at two Mg^{2+} concentrations.

Determination of Dissociation Constant (K_d) of Nucleotides for Catalytic Subunit. The dissociation constants for various ATP analogues have been determined by displacing the fluorescent nucleotide *lin*-benzo-ADP with increasing concentrations of the competing nucleotide. The titration for AMP-PCP is given in Figure 2 (inset). In the absence of Mg^{2+}, *lin*-benzo-ADP does not bind to the catalytic subunit, and a low polarization value of 0.0196 (P_{min}) is observed. Following addition of Mg^{2+} (10 mM final), the polarization value increases to 0.139 (P_{max}), indicating binding of the nucleotide to the enzyme. *lin*-benzo-ADP is then displaced with increasing concentrations of AMP-PCP with a concomitant decrease in polarization. The polarization observed at any given concentration of the competing nucleotide is designated as P_{obsd}. After calculation of the concentrations of bound and

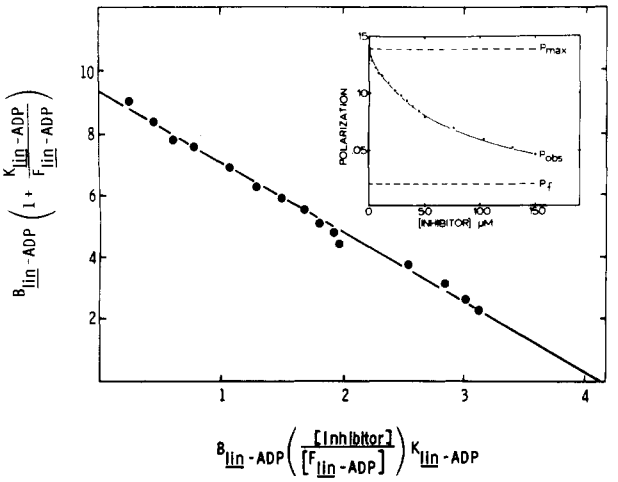

FIGURE 2: Determination of K_d for AMP-PCP by displacement analysis. As given in text, the dissociation constant is calculated as the negative of the reciprocal of the slope of the plot derived from the data obtained from the fluorescence displacement titration (inset). The abbreviations are given under Materials and Methods. (Inset) Fluorescence displacement titration of *lin*-benzo-ADP with AMP-PCP. Experimental details are given under Materials and Methods. Increasing concentrations of AMP-PCP decrease the observed polarization (P_{obsd}). P_f is measured in the absence of added Mg^{2+} (or without enzyme). P_{max} is recorded following the addition of 10 mM Mg^{2+} in the presence of enzyme.

Table II: Comparison of Dissociation Constant (K_d) Determined by Fluorescence Polarization Titrations and Inhibition Constant (K_i) Determined by Steady-State Kinetic Measurements of ATP Analogues

analogue	K_d (μM)[a]	K_i (μM)
adenosine	32	35[b]
adenosine 5′-monophosphate	642	640[b]
adenosine 5′-diphosphate	10	9.4[b]
adenosine 5′-(β,γ-imidotriphosphate)	54	60,[c] 54[d]
inosine 5′-triphosphate	14200	13000[d]
8-bromoadenosine 5′-triphosphate	117	150[d]
2′-deoxyadenosine 5′-triphosphate	2	1.5[d]

[a] K_d's were determined as described under Materials and Methods. [b] Cook et al. (1982). [c] Sugden et al. (1976). [d] Hoppe et al. (1978).

free *lin*-benzo-ADP at each concentration of AMP-PCP from P_{max}, P_{obsd}, and P_f, as described under Materials and Methods, a K_d of 46 μM for AMP-PCP was determined from the slope of the plot (Figure 2).

The K_d's determined by our fluorescence procedure are in good agreement with the K_i's determined by other investigators using steady-state kinetic measurements (Table II). This agreement exists for analogues with dissociation constants ranging from 2 μM (2′-deoxy-ATP) to 14 mM (ITP). We have determined the dissociation constants of additional ATP analogues by the fluorescence displacement titration method. These compounds result from modification of the adenine ring, the ribose moiety, or the triphosphate chain of the ATP molecule.

We find that modification of the adenine part of ATP brings about a reduction in its affinity for the catalytic subunit. Introduction of bromine at position 8, for example, results in a significant increase in K_d (analogues **2** and **3** of Table IIIA). A large increase in K_d results from modification at position 6 of the base. Replacing the amino group at position 6 of ATP with a keto group (ITP, analogue **4**) produces a nucleotide with a very low affinity for the catalytic subunit (K_d = 14200 μM). GTP (analogue **5**) also exhibits a very high K_d. 1,N^6-etheno-ATP (analogue **6**) fails to exhibit any measurable

Table III: Dissociation Constant (K_d) Values of Various ATP Analogues for Catalytic Subunit[a]

no.	analogue	K_d (μM)
	(A) Modifications of Adenine Ring	
1	adenosine 5′-triphosphate	10[b]
2	8-bromoadenosine 5′-triphosphate	117
3	8-bromoadenosine 5′-diphosphate	204
4	inosine 5′-triphosphate	14200
5	guanosine 5′-triphosphate	3700
6	1,N^6-ethenoadenosine 5′-triphosphate	>20000[c]
7	6-amino-2-chloropurine riboside	1430
	(B) Modifications of Ribose Moiety	
8	2′-deoxyadenosine 5′-triphosphate	18
9	3′-deoxyadenosine 5′-triphosphate	2
10	2′-deoxyadenosine	29
11	adenosine 2′-monophosphate	1060
12	adenosine 3′-monophosphate	792
13	adenosine cyclic 2′,3′-monophosphate	801
	(C) Modifications of Triphosphate Moiety	
14	adenosine 5′-(α,β-methylenetriphosphate)	34
15	adenosine 5′-(β,γ-methylenetriphosphate)	46
16	adenosine 5′-(β,γ-imidotriphosphate)	54
17	adenosine 5′-diphosphate	10
18	adenosine 5′-monophosphate	643
19	adenosine	32
	(D) Modifications at 5′-Position of Ribose Moiety	
20	5′-iodo-5′-deoxyadenosine	32
21	5′-*O*-(*p*-toluenesulfonyl)adenosine	30
22	5′-amino-5′-deoxyadenosine	506
23	5′-(ethylamino)-5′-deoxyadenosine	403
24	5′-(diethylamino)-5′-deoxyadenosine	284
25	5′-(triethylammonium)-5′-deoxyadenosine	153
26	5′-(diallylamino)-5′-deoxyadenosine	102

[a] Determined by the fluorescence polarization titration technique described under Materials and Methods. [b] Determined by steady-state kinetic measurements. [c] Exhibited no measurable binding under our experimental conditions.

binding under our experimental conditions. A considerable decrease in affinity is also observed upon introduction of a chlorine atom at position 2 of the adenine base (analogue **7**, Table IIIA).

Modifications of the hydroxyl groups at positions 2′ and 3′ of the ribose moiety produce different changes in the binding of ATP to the catalytic subunit (Table IIIB). For example, replacement of the 2′-hydroxyl by hydrogen (analogue **8**) decreases the binding affinity almost 2-fold. By contrast, introduction of hydrogen in place of the 3′-hydroxyl increases the binding affinity 5-fold (analogue **9**). Replacement of the 2′- or 3′-hydroxyl group by a phosphate group (analogues **11**, **12**, and **13**) produces a large decrease in binding affinity as shown by high K_d values (approximately 1 mM) for these analogues.

Replacement of the α,β-oxygen by a methylene group (analogue **14**, K_d = 34 μM) or the β,γ-oxygen by a methylene (analogue **15**, K_d = 46 μM) or imido group (analogue **16**, K_d = 54 μM) moderately decreases binding of the ATP molecule to the enzyme (Table IIIC). No reduction in binding affinity occurs upon removal of the γ-phosphate of the ATP molecule (ADP, K_d = 10 μM). However, removal of the β-phosphate from ADP greatly decreases the binding affinity between the resulting analogue (AMP, analogue **18**) and the catalytic subunit (K_d = 643 μM). But if the α-phosphate from AMP is removed, the binding between the analogue (adenosine, analogue **19**) and the enzyme becomes much tighter (K_d = 32 μM). These findings parallel our previous results obtained by steady-state enzyme kinetics (Cook et al., 1982). We proposed that the nucleotide binding site complementary to

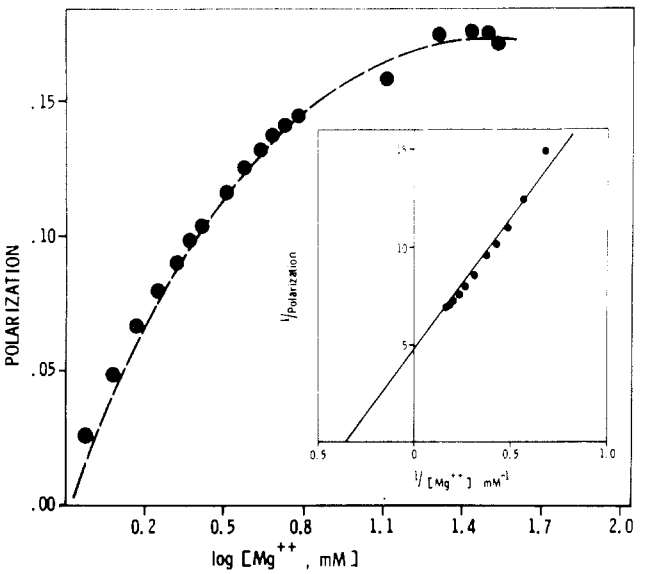

FIGURE 3: Divalent metal ion requirement of nucleotide binding to catalytic subunit. Polarization titrations were performed at 23 °C in 50 mM Mops (pH 7.0)–100 mM NaCl. Fluorescence polarization is enhanced with increase in Mg^{2+} concentration (1–30 mM) as a result of increased binding of *lin*-benzo-ADP to the catalytic subunit. (Inset) A double-reciprocal plot of polarization vs. $[Mg^{2+}]$ of the data. The x intercept of the plot corresponds to $-1/K_{app}$ for Mg^{2+}.

the α-phosphate may be negatively charged or hydrophobic. We have synthesized uncharged and neutral analogues to test these possibilities.

We find (Table IIID) that if no charge is present at the 5′-position of the ribose moiety (analogues **19**, **20**, and **21**), then there is an increased affinity between the analogue and the catalytic subunit compared with AMP. This occurs even with the large hydrophobic toluenesulfonyl group (analogue **21**) in this position. However, introduction of either a negative (analogue **18**) or a positive charge (analogue **22**) at the α-phosphate position causes the binding affinity to decrease 20-fold as compared to that of adenosine (analogue **19**). Addition of hydrophobic groups onto the positive charge at the 5′-position progressively decreases the K_d of the analogues from 506 μM for 5′-amino-5′-deoxyadenosine to 102 μM for 5′-(diallylamino)-5′-deoxyadenosine (analogues **22–26**). These results suggest that the enzyme site complementary to the α-phosphate is hydrophobic in nature.

Metal Ion Dependence of Nucleotide Binding and Phosphotransferase Activity. As mentioned previously, *lin*-benzo-ADP requires a divalent cation to bind to the catalytic subunit of the protein kinase. An increasing concentration of effective metal increases the fluorescence polarization as more *lin*-benzo-ADP binds to enzyme. With increasing Mg^{2+} concentration, for example, the fluorescence polarization increases, indicating that an increasing amount of *lin*-benzo-ADP is bound to the catalytic subunit (Figure 3). The binding of the nucleotide to the C subunit is half-maximum at 3 mM Mg^{2+} and plateaus at 9–10 mM. The K_{app} of 2.85 mM for Mg^{2+} is determined from a plot of polarization^{-1} vs. $[Mg^{2+}]^{-1}$ (Figure 3, inset).

Other metal ions (Mn^{2+}, Co^{2+}, Cd^{2+}, Ca^{2+}, Sr^{2+}) also promote binding of the nucleotide to C as demonstrated by an increase in fluorescence polarization in the presence of these metals. On the other hand, Ba^{2+}, Cr^{2+}, Fe^{2+}, Ni^{2+}, Zn^{2+}, Cu^{2+}, Gd^{3+}, and La^{3+} fail to promote any binding under our experimental conditions. This is reflected by a lack of an increase in fluorescence polarization in the presence of these metals (Table IV).

Table IV: Dependence of Nucleotide Binding and Phosphotransferase Activity of Catalytic Subunit on Metal Ions

metal ion	K_{app} (mM)[a]	phosphotransferase activity[b]
no metal added	nil	ND[d]
Mg^{2+}	2.82 ± 0.42	100
Mn^{2+}	0.031 ± 0.003	61
Co^{2+}	0.059 ± 0.008	52
Cd^{2+}	2.1 ± 0.35	10
Ca^{2+}	3.3 ± 0.85	ND
Sr^{2+}	6.75 ± 1.1	ND
Ba^{2+}[c]	nil	ND
Cr^{2+}[c]	nil	ND
Fe^{2+}[c]	nil	ND
Ni^{2+}[c]	nil	ND
Zn^{2+}[c]	nil	ND
Cu^{2+}[c]	nil	ND
Gd^{3+}[c]	nil	ND
La^{3+}[c]	nil	ND

[a] Determined by fluorescence polarization enhancement procedure as described in legend of Figure 3. [b] For catalytic activity determinations, all metal ions were added as their Cl^- salts (except for Zn^{2+} and Cu^{2+}, which were added as $ZnSO_4$ and $CuSO_4$) to produce a concentration in the assay of $3K_{app}$. Activities are expressed as a percentage of the activity in the presence of 8.5 mM $MgCl_2$, which corresponds to a catalytic activity of 15 μmol of P_i transferred (mg of protein)$^{-1}$ min^{-1}. [c] Catalytic activities were determined with 15 mM of these metal ions in the assay. [d] ND, not detectable (less than 0.002% of the activity observed in presence of 8.5 mM $MgCl_2$).

The dependence of phosphotransferase activity of the catalytic subunit of these metal ions has also been investigated. The assays were performed under subsaturating metal ion concentrations ($3K_{app}$ of metals) (Table IV). Under these conditions, the catalytic activity is maximal [15 μmol (mg of protein)$^{-1}$ min^{-1}] in the presence of 8.5 mM Mg^{2+}. Of the other metal ions tested, Mn^{2+} is most effective at a 100 μM concentration, sustaining a catalytic activity of 9.2 μmol (mg of protein)$^{-1}$ min^{-1}. With 180 μM Co^{2+}, an activity of 7.8 μmol (mg of protein)$^{-1}$ min^{-1} is recorded. $CdCl_2$ (6 mM) supports only 10% of the activity of the catalytic subunit as compared to that supported by 8.5 mM $MgCl_2$. Although Ca^{2+} and Sr^{2+} promote binding of the nucleotide to the catalytic subunit, no measurable phosphotransferase activity of C subunit occurs in the presence of either 10 mM $CaCl_2$ or 20 mM $SrCl_2$ under our experimental conditions. Ba^{2+}, Cr^{2+}, Fe^{2+}, Ni^{2+}, Zn^{2+}, Cu^{2+}, Gd^{3+}, and La^{3+} at concentrations of 15 mM fail to support detectable phosphotransferase activity.

Discussion

Using a wide range of substituted nucleotide analogues, Hoppe et al. (1977, 1978) have studied the topography of the ATP binding site of the catalytic subunit of the type I cAMP-dependent protein kinase from rabbit skeletal muscle. Others have utilized chemical modification of the C subunit to characterize further the ATP binding site on the free C subunit (Kupfer et al., 1979; Hoppe & Friest, 1979; Witt & Roskoski, 1975; Pal et al., 1975; Wyatt & Colman, 1977; Hixson & Krebs, 1979; Taylor et al., 1981; Corbin et al., 1981; Hartl & Roskoski, 1982). We have developed a fluorescence polarization titration procedure for measuring the dissociation constant of various nucleotide analogues of ATP. This is achieved by displacing the fluorescent nucleotide *lin*-benzo-ADP (Leonard et al., 1975, 1976; Scopes et al., 1977), bound to the C subunit, with the analogue whose K_d is to be determined. *lin*-Benzo-ATP was not used for these studies because the catalytic subunit of cAMP-dependent protein kinase

possesses ATPase activity (Sugden et al., 1976; Armstrong et al., 1979b) for which *lin*-benzo-ATP is also a substrate (Hartl et al., 1983).

The specificity of nucleotide binding to the free catalytic subunit of the type II cAMP-dependent protein kinase has been determined with respect to the K_d's of several ATP congeners. On the basis of these binding affinities, the parts of the ATP molecule that play an essential role in the binding of ATP to the catalytic subunit have been determined.

We find that the binding affinities for the type II catalytic subunit (bovine skeletal muscle) agree well with those determined by Hoppe et al. (1978) for the type I catalytic subunit (rabbit skeletal muscle). Our studies indicate that modifications of the adenine part of ATP bring about a considerable reduction in the binding affinity (i.e., high K_d) between the catalytic subunit and the resulting analogue, the effect being most pronounced with modifications at position 6 on the adenine base. Hoppe et al. (1978) have suggested that the adenine-binding portion of the nucleotide binding site has a high specificity for an unsubstituted amino group at position 6. This is illustrated by the remarkable increase in K_i that they observed upon increasing the bulkiness of the substituent on this amino group by methylation (K_i = 180 μM), dimethylation (K_i = 2600 μM), and benzylation (K_i > 20 000 μM). Furthermore, we also find that replacing the amino group at position 6 with a keto group results in an analogue (ITP) with extremely low affinity (K_d = 14 200 μM) for the catalytic subunit. We postulated that this amino group on the ATP molecule is associated in an interaction with the catalytic subunit as a hydrogen-bond donor (Cook et al., 1982). This concept is supported by the observation that the decrease in binding affinity due to modifications at position 8 of the base (analogues **2** and **3**) is not as pronounced as that for modification at position 6. That the adenine moiety of ATP plays a major role in the binding of the ATP to the catalytic subunit is similar to the case of horse liver phosphoglycerokinase (Blake & Evans, 1974). In addition, the predominant effect of the amino group at position 6 of the adenine base is similar to results obtained with tRNA synthetases from *Escherichia coli* and from bakers' yeast (Freist et al., 1976a,b; Marutzky et al., 1976). On the basis of nuclear magnetic resonance studies, Granot et al. (1979) found substantial changes in the torsional angle of glycosidic linkage, which also suggests that the enzyme interacts strongly with the adenosine portion of the nucleotide.

The ribose moiety of the ATP molecule plays a less important, though definite, role in its binding to the enzyme. Replacement of the 2'- and 3'-hydroxyl groups with hydrogen (analogues **8** and **9**) did not affect binding as significantly as the modification at position 6 on the adenine base. It is not yet possible to determine the reason for the 80–100-fold increase in K_d's of analogues **11–13** as a result of introduction of a phosphate group at the 2'- and 3'-positions of the ribose moiety. This significant decrease in affinity could be due to either the large size of the phosphate moiety in the analogue as compared to the OH group at the 2'- and 3'-position on the ATP molecule or the introduction of a negative charge at these positions.

The triphosphate moiety of ATP contributes to the binding of ATP to the catalytic subunit as can be seen from the relative affinity of ATP analogues for C. If one divides the K_m (10 μM) of the free catalytic subunit for ATP (which is close to its K_d value; Moll & Kaiser, 1977) by the K_d of an ATP analogue, one obtains a corresponding K_d'. When this K_d' is evaluated thermodynamically, one obtains a $\delta\Delta G$ value that reflects the change in affinity produced by the specific modification. As described by Hoppe et al. (1978), the $\delta\Delta G$ can be calculated from

$$\delta\Delta G = -RT \ln \frac{K_d(\text{ATP})}{K_d(\text{analogue})}$$

where R is the gas constant (8.31432 J deg^{-1} mol^{-1}) and T is the absolute temperature. By this method, all binding constants are related to those of ATP as determined under the same experimental conditions. The $\delta\Delta G$ values (kJ/mol) for the analogues with modifications at the triphosphate part of the ATP molecular are as follows: −3.01 for AMP–CPP (analogue **14**), −3.78 for AMP–PCP (analogue **15**), −4.15 for AMP–PNP (analogue **16**), 0 for ADP (analogue **17**), −10.54 for AMP (analogue **18**), and −2.86 for adenosine (analogue **19**). These $\delta\Delta$G values indicate that a small reduction in affinity is observed upon replacing either the α,β or the β,γ-oxygen by a methylene or an imido group (analogues **14–16**). However, a large decrease in affinity is observed by the removal of both the γ- and β-phosphates from the ATP molecule (analogue **18**, AMP). The removal of the α-phosphate on AMP restores the higher affinity of the resulting analogue (adenosine, analogue **19**) with the catalytic subunit. These observations suggested that either a negative charge or a hydrophobic site was present in the region of the α-phosphate in the nucleotide binding site to repulse the two formal negative charges present on the α-phosphate of AMP. The binding strength of MgADP and adenosine may involve only interaction of the nucleoside portion of the molecule since adenosine lacks the α-phosphate while the α-phosphate is not charged in MgADP. At high Mg^{2+} concentrations (10 mM) the formal negative charge present on the α-phosphate in MgATP is also neutralized (Cook et al., 1982).

A plot of the $\delta\Delta G$ values of ATP analogues with modifications at the 5'-position of the ribose moiety is given in Figure 4. The data in this plot represent the change (kJ/mol) in nucleotide binding resulting from the chemical alteration of the ligand with respect to adenosine instead of ATP. We found that no charge at the 5'-position of the ribose moiety was associated with high affinity of the analogues and the catalytic

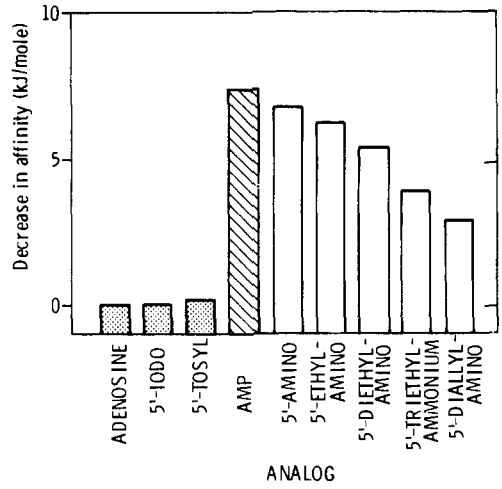

FIGURE 4: Decrease in binding affinity (kJ/mol) of analogues for the catalytic subunit as a result of substitution of various charge groups at the 5'-position of the ribose moiety of adenosine. AMP refers to adenosine 5'-monophosphate, and all other analogue abbreviations refer to the substituted groups at the 5'-position of adenosine. In order to compare the binding affinity of analogues modified at the α-phosphate position of the ATP molecule, $\delta\Delta G$ values were determined as given in the text except that the K_d' values were determined by dividing the K_d of adenosine by that of the analogue. The stippled bars refer to uncharged analogues; the clear bars refer to positive analogues; the lined bar refers to the negative analogue.

subunit (analogues **19–21**). On the other hand, introduction of a negative charge (AMP, analogue **18**) or a positive charge (5′-amino-5′-deoxyadenosine, analogue **22**) at the 5′-position decreased the binding affinity by 7 kJ/mol. This observation suggests that the region on the catalytic subunit where the α-phosphate of the ATP molecule (i.e., the 5′-position of the ribose moiety) resides is not negatively charged but hydrophobic.

The hydrophobicity on the catalytic subunit has been further characterized by the addition of hydrophobic groups onto the positive charge of the 5′-amino-5′-deoxyadenosine (analogues **23–26**). There was a progressive increase in the binding affinity observed with the increase in hydrophobicity in the group attached to the 5′-amino residue on the ribose moiety (Figure 4). We believe that this further substantiates the suggestion that the region of the α-phosphate in the nucleotide binding site on the catalytic subunit is hydrophobic. However, other possibilities exist. For example, the hydrophobic substituents in the analogues might interact with a hydrophobic subsite not normally used in nucleotide binding.

Metal Ion Requirement of Catalytic Subunit. Nuclear magnetic resonance and steady-state kinetic studies of the catalytic subunit of type II cAMP-dependent protein kinase from bovine heart have established that the catalytically active complex is an enzyme–ATP–metal bridge (Granot et al., 1980). At high concentrations, both Mn^{2+} and Mg^{2+} occupy a site on the enzyme and the β and γ or α, β, and γ positions of metal–ATP. K_{app} values of various divalent metal ions for the C subunit determined in this study by fluorescence titrations are listed in Table IV. The dissociation constant of Mg^{2+} determined from a plot of polarization^{-1} against [free Mg^{2+}]$^{-1}$ is in good agreement with the dissociation constants of 2–3 mM (Cook et al., 1982) and 2.3 mM (Armstrong et al., 1979) for Mg^{2+} reported by other investigators. The K_{app} of 31 μM for Mn^{2+} is also in good agreement with the dissociation constants of 21–30 μM reported by Armstrong et al. (1979) and 17–19 μM demonstrated by Granot et al. (1979) using nuclear magnetic resonance techniques. A K_{app} for Co^{2+} was found to be 60 μM from our fluorescence studies whereas electron paramagnetic resonance measurements of Granot et al. (1980) have yielded a dissociation constant of 280 μM for $Co(NH_3)_4$-AMP-PCP. These comparisons suggest that the fluorescence polarization determination of dissociation constants of metals described in this study is sensitive and accurate. The K_{app} values for Cd^{2+}, Ca^{2+}, and Sr^{2+} (Table IV), which promoted binding of nucleotide to the catalytic subunit in our study, have not been determined previously. The other metal ions tested failed to support nucleotide binding to the C subunit. The K_{app} values listed in Table IV for metal ions that promoted nucleotide binding are much higher than the concentrations required to form metal–ATP complexes (Taqui Khan & Martell, 1966). Therefore, the values determined in this study reflect the affinity of divalent metal ions for the inhibitory binding site on the enzyme.

The metal–ATP complexes of Mg^{2+}, Mn^{2+}, Co^{2+}, and Cd^{2+}, which bound to the catalytic subunit, supported significant phosphotransferase activity (Table IV) although the extent varied with the metal. Similar observations have been reported by Sugden et al. (1976). However, their results differ from ours in that they have reported lower catalytic activity for Mn^{2+} and significantly higher activity in the presence of Ni^{2+}. These distinctions may be accounted for by different concentrations of free metal in the reaction medium or in the phosphorylatable substrate. There is agreement between our results and those of Sugden et al. (1976) in that Ca^{2+} and Sr^{2+} failed to support the catalytic activity of the protein kinase. This was in spite of our observation that both Ca^{2+} and Sr^{2+} promote binding of nucleotide to the catalytic subunit. Ca^{2+} is a competitive inhibitor with respect to Mg^{2+} for pyruvate kinase (Mildvan & Cohn, 1965; Kachmar & Boyer, 1953). It is known to occupy rather than alter the Mg^{2+} binding sites on the enzyme. A similar situation may exist in our case for the protein kinase. No correlation between the ionic radius or the electronic configuration of the metal ions could be established with either nucleotide binding or catalytic activity.

The finding that Mg^{2+} is required for nucleotide binding to enzyme explains some of our previous studies on substrate protection against chemical modification and inactivation. ATP alone failed to protect protein kinase against inactivation by ethoxyformic anhydride (Witt & Roskoski, 1975), Cibacron Blue F3GA (Witt & Roskoski, 1980), and NBD-Cl (Hartl & Roskoski, 1982). MgATP, on the other hand, decreased the rates of inactivation in each case.

We have demonstrated with NBD-Cl-modified inactive catalytic subunit that chemical modification decreases nucleotide binding (Hartl et al., 1983). Analysis of nucleotide binding to the catalytically inactive holoenzymes is also possible with this technique but not with steady-state kinetic models. This becomes particularly important when binding constants of nucleotides for the catalytic subunit have to be compared with those of holoenzyme. One such case is the study of Hoppe et al. (1978) in which they have compared two sets of binding constants that in principle need not always be comparable. In their report, they have stated that while in the case of the undissociated enzyme the K_i values were obtained from competitive binding experiments (the undissociated enzyme being catalytically inactive), in the case of free catalytic subunit the K_i values were obtained from activity measurements. Since their measurements were carried out in the presence of protein substrate (histone), which may alter the geometry of the active site during catalysis, they suggested that their binding affinities may be perturbed. Our measurements, which agree well with theirs, are not determined during catalysis. Taken together, this suggests that neither the protein substrate nor catalysis alter the specificity of nucleotide binding.

Our procedure for determining the binding affinity of a nucleotide analogue for the catalytic subunit and binding constants for metals is easier and more rapid than the use of steady-state kinetic methods. The K_d's (over a wide range of binding affinities) determined by this technique compare very well with the K_i's determined by other investigators (Table II). Our technique is nondestructive, and the enzyme can be recovered by simple dialysis. This method, moreover, does not require synthesis of each radiolabeled nucleotide. The mathematical analysis for the calculation of the K_d of one agent displacing another is general and does not require a fluorescent probe. The K_d of the probe and the ability to quantitate the amount of binding at a known concentration of inhibitor or displacing ligand are required. We have used this procedure, for example, to determine the K_d for methacholine for the cholinergic muscarinic receptor by displacing labeled quinuclidinyl benzilate (Roskoski et al., 1982).

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Registry No. **1**, 56-65-5; **2**, 23567-97-7; **3**, 23600-16-0; **4**, 132-06-9;

5, 86-01-1; **6**, 37482-17-0; **7**, 146-77-0; **8**, 1927-31-7; **9**, 73-04-1; **10**, 958-09-8; **11**, 130-49-4; **12**, 84-21-9; **13**, 634-01-5; **14**, 7292-42-4; **15**, 3469-78-1; **16**, 25612-73-1; **17**, 58-64-0; **18**, 61-19-8; **19**, 58-61-7; **20**, 4099-81-4; **21**, 5135-30-8; **22**, 14365-44-7; **23**, 87830-56-6; **24**, 87830-57-7; **25**, 87830-58-8; **26**, 87830-59-9; *lin*-benzo-ADP, 61925-59-5; Mg, 7439-95-4; Mn, 7439-96-5; Co, 7440-48-4; Cd, 7440-43-9; Ca, 7440-70-2; Sr, 7440-24-6; Ser-peptide, 65189-71-1; MgATP, 1476-84-2; MgADP, 7384-99-8; protein kinase, 9026-43-1.

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