Synthetic Peptide Analogue Differentially Alter the Binding Affinities of Cyclic Nucleotide Dependent Protein Kinases for Nucleotide Substrates‡

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Received June 10, 1987; Revised Manuscript Received October 16, 1987

ABSTRACT: Analogue of a synthetic heptapeptide substrate corresponding to the sequence around a phosphorylation site in histone H2B [Glass, D. B. & Krebs, E. G. (1982) J. Biol. Chem. 257, 1196-1200] were used to assess interactions between the peptide substrate and the ATP binding sites of cGMP-dependent protein kinase and the catalytic subunit of cAMP-dependent protein kinase. The affinity of each protein kinase for lin-benzo-ADP was determined in the absence and presence of substrate peptide by fluorescence anisotropy titrations [Bhatnagar, D., Roskoski, R., Jr., Rosendahl, M. S., & Leonard, N. J. (1983) Biochemistry 22, 6310-6317]. The Kd values of cGMP-dependent protein kinase for lin-benzo-ADP in the absence and presence of cGMP were 7.6 and 9.7 µM, respectively. Histone H2B(29-35) (Arg-Lys-Arg-Ser-Arg-Lys-Glu) had no effect on nucleotide affinity in either the absence or presence of cGMP. However, when lysine-34 located two residues after the phosphorylatable serine is replaced with an alanyl residue, the resulting [Ala34] histone H2B(29-35) and its analogue peptides interact with cGMP-dependent protein kinase and/or the nucleotide in a fashion that decreases nucleotide binding affinity approximately 3-fold. This amino acid replacement had previously been shown to cause an increase in Vmax and a decrease in the pH optimum for the phosphotransferase reaction. Replacement of positively charged residues at positions 30 and 31 of the peptide also decreased nucleotide affinity. Other analogues of histone H2B(29-35) failed to affect binding of lin-benzo-ADP to the active site of the cGMP-dependent enzyme. The effect of peptides to decrease nucleotide binding affinity was greater on ADP than on the fluorescent ligand. None of the histone peptide analogues significantly altered adenine nucleotide binding to the catalytic subunit of cAMP-dependent protein kinase. We conclude that histone H2B(29-35) peptides interact with the peptide or nucleotide binding sites differently in the two protein kinases, possibly because the dimeric cGMP-dependent protein kinase contains a regulatory domain.

The cGMP-dependent and cAMP-dependent protein kinases have been purified to homogeneity from various tissues and extensively characterized (Gill et al., 1977; Lincoln et al., 1977; Flockerzi et al., 1978). The critical differences between the two protein kinases are in the cyclic nucleotides that activate each enzyme and in the structural organization of their subunits. The cGMP-dependent protein kinase is a homodimer in which each polypeptide chain contains both cGMP-binding and catalytic domains. The holoenzyme is activated by cGMP binding without subunit dissociation (Gill et al., 1977; Lincoln et al., 1977; Corbin & Doskeland, 1983). On the other hand, binding of cAMP to the inactive cAMP-dependent protein kinase dissociates the heterotetrameric holoenzyme into a regulatory subunit dimer containing bound cAMP and two catalytically active subunits (Corbin et al., 1978). Primary sequence data of cGMP-dependent and cAMP-dependent protein kinases indicate that the cyclic nucleotide binding sites, the sites of autophosphorylation in the hinge regions, and the ATP binding sites show 25-47% percent sequence identity between the two enzymes (Shoji et al., 1981; Takio et al., 1983, 1984a,b). A lysine residue in the active site of each protein kinase has been labeled with [3-(N-morpholino)propanesulfonic acid. 3-(N-morpholino)propanesulfonic acid.

†This work was supported by U.S. Public Health Service Grants NS-15994 (R.R.), GM-28144 (D.B.G.), and GM-05829 (N.J.L.) and a fellowship (D.B.) from the American Heart Association, Louisiana Chapter.

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mologous proteins, numerous differences have been reported in the steady-state kinetic details of their substrate specificities (Glass & Krebs, 1979, 1982; Cohen, 1980; Geahlen & Krebs, 1980; Issinger et al., 1980; Aswad & Greengard, 1981; Zeilig et al., 1981; Glass, 1983; Glass & Smith, 1983; Glass et al., 1981, 1986). It is thought that at least some of these kinetic differences between the two cyclic nucleotide dependent protein kinases are due to dissimilarities in the active sites of the two enzymes.

We have previously shown (Bhatnagar et al., 1983, 1985) that the fluorescent "stretched-out" adenine nucleotide lin-benzo-ADP is a competitive inhibitor with respect to ATP of both the catalytic subunit of cAMP-dependent protein kinase and the cGMP-dependent protein kinase. In the latter study, we also observed that the preferred phosphoryl-accepting substrate of cGMP-dependent protein kinase, the synthetic peptide [Ala25]histone H2B(29-35), decreased the affinity for both lin-benzo-ADP and ADP. No such effect was observed with the cAMP-dependent enzyme. In this report we have used the fluorescent anisotropy titration technique (Bhatnagar et al., 1983, 1985) to examine the extent to which the individual amino acid residues of this peptide substrate are involved in interactions with the enzymes that alter the affinity of nucleotide binding.

MATERIALS AND METHODS

The peptide substrates were synthesized as described by Glass and Krebs (1982). All peptides were purified and characterized by amino acid analysis. Chemicals were purchased from Sigma Chemical Co. Concentrations of lin-benzoadenine nucleotides were measured spectrophotometrically 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 and Krebs (1979). Preparations had specific activities of 4-6 umol-(min-mg)^-1 using 0.5 mg/mL of native histone H2B as substrate under conditions described by Glass and Krebs (1979). The -cGMP/+cGMP activity ratios of these preparations of enzyme with 25 µM histone H2B(29-35) as substrate were 0.08-0.09, indicating an approximately 12-fold stimulation of activity by cGMP.

The catalytic subunit from type II bovine cardiac muscle was purified as described by Zoller et al. (1979). Specific activity measured by the method of Roskoski (1983) using 100 µM Ser-peptide and 200 µM [γ-32P]ATP (Cook et al., 1982) was 12.6 umol-(min-mg)^-1. Protein concentrations were determined according to Bradford (1976) using ovalbumin as standard. For the determination of enzyme molarity, molecular weights of cGMP-dependent protein kinase and the catalytic subunit of cAMP-dependent protein kinase were taken to be 154 000 and 40 000, respectively.

Fluorescence Anisotropy Titrations. Fluorescence measurements were performed with an SLM 4800 spectrofluorometer interfaced with a Hewlett-Packard 9825A calculator. Fluorescence anisotropy (r) was calculated from polarization values (P) according to the following equation obtained from a program supplied by SLM Inc.:

\[ r = 2P / (3 - P) \]

(Lakowicz, 1983). Anisotropy is defined as

\[ r = (I_4 + I_2) / (I_4 + 2I_2) \]

where I_4 and I_2 are the intensities observed parallel and perpendicular to the polarization of the exciting light, respectively. Polarization and anisotropy values of lin-benzo-adenine nucleotides were determined by using calcite polarizers. Excitation was at 334 nm, with a 4-nm resolution, and emitted light was isolated with Schott KV 389 filters.

Three types of anisotropy titrations were performed in order to determine the binding constants of lin-benzo-ADP and ADP for the protein kinases. Titrations were conducted with protein kinase and lin-benzo-ADP in 50 mM Mops (pH 7.0) and 100 mM NaCl in the presence or absence of the indicated peptides. In the case of CAMP-dependent protein kinase, titrations were performed in the presence or absence of 20 µM cGMP. This concentration of CAMP was sufficient to fully activate the cAMP-dependent enzyme even when used at concentrations of 3-4 µM in the fluorescence anisotropy studies. Cyclic AMP was not routinely added to the catalytic subunit of cAMP-dependent protein kinase, but in several titration experiments the addition of 10 µM CAMP had, as expected, no effect on any of the binding constants. Lin-Benzo-ADP was not a substrate for the phosphotransferase reactions catalyzed by either protein kinase. Experimental values represent the means of three to five independent determinations. Standard errors were routinely 3-5% of the mean values.

Anisotropy of the free fluorescent ligand (r_f) was obtained from polarization measurements in protein- or peptide-free buffer. Because binding titrations were carried out in the presence of enzymes and/or peptides, which increased the bulk viscosity of the medium and could thereby potentially affect the polarization of the free ligand, r_f was determined in the presence of enzymes or peptides as well. In these control experiments, measurements were performed in the presence of high concentrations of competing nonfluorescent ligand (ADP) so that the contribution to anisotropy by bound species was negligible; i.e., [lin-benzo-ADP]_free ≫ [lin-benzo-ADP]_bound. The r_f values for lin-benzo-ADP or Mg-lin-benzo-ADP determined in the presence of enzymes or peptides alone were almost identical with those measured in protein- or peptide-free buffer. Therefore, these changes in viscosity of the medium had little or no effect, and the peptide analogues were not directly interacting with lin-benzo-ADP or Mg-lin-benzo-ADP in solution at the concentrations used.

(a) Dilution Titrations. Anisotropy as a function of varying enzyme concentration (at constant lin-benzo-ADP concentration) was measured as described earlier (Bhatnagar et al., 1985) to determine r_0, which is the anisotropy value when all lin-benzo-ADP is bound to the protein kinase (i.e., at infinite enzyme concentration). The theoretical value of r_0 and the average angle of rotation ω for lin-benzo-ADP rigidly bound to the protein kinases were calculated from Perrin's equations as described earlier by using the value of r_0 as 0.301 for lin-benzo-ADP (Bhatnagar et al., 1985) and the following anisotropy relationship (Lakowicz, 1983):

\[ r = r_0 (3 \cos^2 \omega - 1) / 2 \]

(b) Addition Titrations. Lin-Benzo-ADP (1-2 µM) was added to a solution of 3-5 µM protein kinase, and the anisotropy was recorded (r). In the absence of Mg^2+ this anisotropy value (r_t) was the same in the presence or absence of enzyme, indicating that little or no binding of the nucleotide occurs in the absence of metal ion under these conditions. MgSO_4 was then added to a final concentration of 10 mM, and the increased value of anisotropy, due to the binding of metal-nucleotide complex to the enzyme, was recorded (r_max). Anisotropy (r_max) was then recorded, as previously described (Bhatnagar et al., 1983, 1985), at each nucleotide concentration after addition of successive increments (approximately 1-2 µM) of lin-benzo-ADP to a constant protein kinase concentration, under the specified conditions of each experiment.
The concentrations of bound and free nucleotide after each addition were calculated as follows:

\[ [B] = \frac{r_{\text{obsd}} - r_f}{r_b - r_f} [\text{lin-benzo-ADP}]_t \]

and

\[ [F] = [\text{lin-benzo-ADP}]_t - [B] \]

where \([B]\) and \([F]\) are the concentrations of \(\text{lin-benzo-ADP}\) bound to the enzyme and free in solution, respectively, at the total concentration of the ligand, \([\text{lin-benzo-ADP}]_t\), in the cuvette and \(r_b\) (determined from the dilution titration), \(r_f\) and \(r_{\text{obsd}}\) are the anisotropy values as described above.

(c) Displacement Titration. The dissociation constant \(K_d\) of ADP for the protein kinases was determined by displacing the fluorescent \(\text{lin-benzo-ADP}\) bound to the enzymes with ADP as previously described (Bhatnagar et al., 1983, 1985). The respective value of the \(K_d\) of \(\text{lin-benzo-ADP}\) in the presence of each peptide was used for the calculation of the \(K_d\) of ADP in the presence of that peptide.

**RESULTS**

Properties of \(\text{lin-Benzo-ADP} \) Binding to Cyclic Nucleotide Dependent Protein Kinases. The fluorescence emission spectrum of \(\text{lin-benzo-ADP}\) was identical in the presence or absence of the enzymes with or without near-saturating amounts of their respective cyclic nucleotides (data not shown). This indicates that the modified adenine base of the nucleotide does not interact with aromatic residues in the nucleotide binding site and that the quantum yields of free and bound ligand are the same. The present approach is also substantiated by the fact that the \(K_d\) values of \(\text{lin-benzo-ADP}\) determined by fluorescence measurements agree with the corresponding \(K_i\) values determined by steady-state kinetic measurements (Bhatnagar et al., 1983, 1985).

The anisotropy \(r_f\), when all \(\text{lin-benzo-ADP}\) is bound to the protein kinase, was determined by dilution titrations (not shown) similar to those described earlier (Bhatnagar et al., 1983, 1984, 1985). Within the lifetime of the excited state, experimental values of \(r_f\) and \(\omega_f\) for both \(\text{cGMP-dependent protein kinase}\) and the catalytic subunit of \(\text{cAMP-dependent protein kinase}\) (Table I) were in good agreement with theoretical values of \(r_f\) and \(\omega_f\) calculated from Perrin’s equations for \(\text{lin-benzo-ADP}\) rigidly bound to the respective enzymes. This indicates that \(\text{lin-benzo-ADP}\) binds rigidly to these protein kinases under the experimental conditions and that the observed rotation of \(\text{lin-benzo-ADP}\) bound to the respective enzyme is due to the rotation of the protein per se. Therefore, the value of \(r_f\) obtained from the dilution titrations can be used to calculate the \(K_d\) of \(\text{lin-benzo-ADP}\) for these enzymes from addition titrations as described under Materials and Methods.

The binding of \(\text{lin-benzo-ADP}\) to each protein kinase in the presence or absence of synthetic peptides was determined by fluorescence anisotropy addition titrations. A typical fluorescence titration for \(\text{cGMP-dependent protein kinase}\) in the presence of \(\text{Ala}^{34}\)histone H2B(29-35) is shown in Figure 1A, including the Scatchard (1949) analysis of data in Figure 1B. This peptide decreased the affinity of the protein kinase for \(\text{lin-benzo-ADP}\) by 2.3-fold. That result was consistent with our earlier observation using another peptide analogue, \([\text{Ala}^{34}]\)histone H2B(29-35) (Bhatnagar et al., 1985). Therefore, we chose to examine the influence of an entire series of peptide analogues of histone H2B(29-35) on the binding affinity of \(\text{cAMP-dependent protein kinase}\) for the nucleotide.

Effect of Synthetic Peptide Analogues on Binding of \(\text{lin-Benzo-ADP}\) to \(\text{cGMP-Dependent Protein Kinase}.\) Series of
Table II: Effect of Peptide Analogues on Binding of lin-Benzo-ADP to cGMP-Dependent Protein Kinasea

<table>
<thead>
<tr>
<th>no.</th>
<th>peptide</th>
<th>apparent $K_d$ or $K_i$ (μM)</th>
<th>$K_d$ (μM)b</th>
<th>$K_i$ (μM)b</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Arg-Lys-Arg-Ser-Arg-Lys-Glu¹</td>
<td>22d</td>
<td>7.8 (1.98)</td>
<td>10.3 (1.94)</td>
</tr>
<tr>
<td>2</td>
<td>Ac-Arg-Lys-Arg-Ser-Arg-Lys-Glu</td>
<td>47</td>
<td>7.1 (2.21)</td>
<td>9.4 (1.97)</td>
</tr>
<tr>
<td>3</td>
<td>Ala-Lys-Arg-Ser-Arg-Lys-Glu</td>
<td>204</td>
<td>7.4 (2.36)</td>
<td>8.1 (2.09)</td>
</tr>
<tr>
<td>4</td>
<td>Arg-Ala-Arg-Ser-Arg-Lys-Glu</td>
<td>64 (10.0)</td>
<td>6.9 (2.11)</td>
<td>9.7 (1.86)</td>
</tr>
<tr>
<td>5</td>
<td>Arg-Lys-Ala-Ser-Arg-Lys-Glu</td>
<td>200</td>
<td>9.2 (1.78)</td>
<td>16.2 (1.54)</td>
</tr>
<tr>
<td>6</td>
<td>Arg-Lys-Ala-Arg-Lys-Glu</td>
<td>86 (1.2)</td>
<td>6.8 (1.53)</td>
<td>15.4 (1.92)</td>
</tr>
<tr>
<td>7</td>
<td>Arg-Lys-Arg-Thr-Arg-Lys-Glu</td>
<td>24 (0.5)</td>
<td>6.4 (2.06)</td>
<td>7.0 (2.25)</td>
</tr>
<tr>
<td>8</td>
<td>Arg-Lys-Arg-Ser-Ala-Lys-Glu</td>
<td>105</td>
<td>7.1 (1.79)</td>
<td>11.1 (1.88)</td>
</tr>
<tr>
<td>9</td>
<td>Arg-Lys-Arg-Ser-Ala-Ala-Glu</td>
<td>29 (0.5)</td>
<td>7.3 (2.12)</td>
<td>8.5 (2.06)</td>
</tr>
<tr>
<td>10</td>
<td>Arg-Lys-Ala-Ser-Ala-Ala-Glu</td>
<td>267</td>
<td>7.8 (1.91)</td>
<td>10.5 (1.93)</td>
</tr>
<tr>
<td>11</td>
<td>Arg-Lys-Arg-Ser-Lys-Ala</td>
<td>26 (0.5)</td>
<td>7.8 (1.75)</td>
<td>12.2 (1.79)</td>
</tr>
<tr>
<td>12</td>
<td>Arg-Lys-Arg-Ser-Lys-Glu-Ser-Tyr-Ser-Val</td>
<td>10 (21.4)</td>
<td>6.4 (1.59)</td>
<td>10.8 (1.88)</td>
</tr>
</tbody>
</table>

¹ The dissociation constants ($K_d$'s) of lin-benzo-ADP for the protein kinase with or without 20 μM cGMP were determined by fluorescence anisotropy addition titrations as described under Materials and Methods. When present, peptides were at a fixed concentration of 200 μM. Numbers in parentheses (n) refer to the number of lin-benzo-ADP binding sites per protein kinase dimer. Values of $K_d$ and n are the means of three to five different experiments. 
² The dissociation constants are taken from Glass and Krebs (1979, 1982) and Glass (1983).

Table III: Effect of Concentration of Selected Peptide Analogues on Binding of lin-Benzo-ADP to cGMP-Dependent Protein Kinasea

<table>
<thead>
<tr>
<th>no.</th>
<th>peptide</th>
<th>[peptide] (μM)</th>
<th>$K_d$ (μM)b</th>
<th>$K_i$ (μM)b</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Arg-Lys-Arg-Ser-Arg-Lys-Glu¹</td>
<td>100 (7.4 (2.03))</td>
<td>9.1 (2.06)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Arg-Ala-Arg-Ser-Arg-Lys-Glu</td>
<td>1000 (10.1 (1.76))</td>
<td>ND</td>
<td>9.8 (1.88)</td>
</tr>
<tr>
<td>3</td>
<td>Arg-Lys-Arg-Ser-Ala-Lys-Glu</td>
<td>1000 (10.6 (1.84))</td>
<td>ND</td>
<td>15.6 (1.41)</td>
</tr>
<tr>
<td>4</td>
<td>Arg-Lys-Arg-Ser-Ala-Ala-Glu</td>
<td>1000 (7.9 (1.97))</td>
<td>ND</td>
<td>16.2 (1.55)</td>
</tr>
<tr>
<td>5</td>
<td>Arg-Lys-Arg-Ser-Ala-Ala-Glu</td>
<td>150 (10.6 (1.84))</td>
<td>ND</td>
<td>26.2 (1.74)</td>
</tr>
<tr>
<td>6</td>
<td>Arg-Lys-Arg-Ser-Ala-Ala-Glu</td>
<td>1000 (16.1 (1.86))</td>
<td>ND</td>
<td>25.1 (1.81)</td>
</tr>
<tr>
<td>7</td>
<td>Arg-Lys-Arg-Ser-Ala-Ala-Glu</td>
<td>1000 (15.3 (1.82))</td>
<td>ND</td>
<td>22.1 (2.12)</td>
</tr>
<tr>
<td>8</td>
<td>Arg-Lys-Arg-Ser-Ala-Ala-Glu</td>
<td>150 (14.8 (1.79))</td>
<td>ND</td>
<td>23.0 (1.94)</td>
</tr>
<tr>
<td>9</td>
<td>Arg-Lys-Arg-Ser-Ala-Ala-Glu</td>
<td>500 (12.4 (1.91))</td>
<td>ND</td>
<td>24.4 (1.78)</td>
</tr>
<tr>
<td>10</td>
<td>Arg-Lys-Arg-Ser-Ala-Ala-Glu</td>
<td>500 (14.2 (1.89))</td>
<td>ND</td>
<td>22.7 (2.08)</td>
</tr>
<tr>
<td>11</td>
<td>Ac-Arg-Lys-Arg-Ser-Ala-Ala-Glu</td>
<td>1000 (7.9 (1.97))</td>
<td>ND</td>
<td>16.2 (1.84)</td>
</tr>
</tbody>
</table>

² The dissociation constants ($K_d$'s) of lin-benzo-ADP for the protein kinase with or without 20 μM cGMP were determined as described under Materials and Methods in the presence of peptide analogues at concentrations approximately 5 (or greater) times those of their respective $K_d$ or $K_i$ values. These kinetic constants are listed in Table II, except for peptides 13–15. Numbers in parentheses (n) refer to the number of lin-benzo-ADP binding sites per protein kinase dimer. Values of $K_d$ and n are the means of three to five different experiments. 
² Numbers in parentheses (n) refer to the number of lin-benzo-ADP binding sites per protein kinase dimer. ND, not determined. 
² The dissociation constants are taken from Glass and Krebs (1979, 1982) and Glass (1983).
were manifest only in the presence of cGMP.

The $K_d$ values of cGMP-dependent protein kinase for the series of peptides varies considerably (Table II), and it seems reasonable to expect that the $K_d$'s of the peptides would also vary. Therefore, we measured the $K_d$ of lin-benzo-ADP at additional, selected concentrations of several of the peptides. These included near-saturating concentrations on the basis of the known $K_m$ values. A 1000 nM concentration of histone H2B(29-35) still failed to change the $K_d$ for lin-benzo-ADP (Table III). Concentrations of 1000 nM of the Ala30, Ala31, or Ala34 analogues produced no further decreases in lin-benzo-ADP affinity (Table III) than did 200 nM peptide (Table II). A high concentration of [Ala32,Ala34]histone H2B(29-35) maximally decreased nucleotide affinity (Table III), an effect not seen at 200 nM (Table II), which is below the $K_m$ value of this peptide. Three additional peptides in this series (peptides 13-15), which all contain the Ala34 substitution and have kinetic constants similar to [Ala34]histone H2B(29-35), also decreased the affinity for lin-benzo-ADP. High concentrations of all of the Ala34-substituted peptides (peptides 9, 10, and 13-15) also caused minor decreases in binding of nucleotide in the absence of cGMP. This is consistent with the ability of high concentrations of synthetic peptides to activate cGMP-dependent protein kinase in the absence of cGMP.

**Effect of Synthetic Peptide Analogues on Binding of lin-Benzo-ADP to cAMP-Dependent Protein Kinase**. The parent peptide used in studies with cGMP-dependent protein kinase is also a substrate for the catalytic subunit of cAMP-dependent protein kinase (Glass & Krebs, 1979), although it has a $V_{max}$ only 5% that of Ser-peptide, a preferred substrate for the latter enzyme. Histone H2B(29-35) or its analogues with alanine replacements of basic residues at either position 30 or position 34 had no effect on lin-benzo-ADP affinity of catalytic subunit even at concentrations 5-fold above their $K_m$ or $K_d$ values (Table IV). Ser-peptide and an inhibitor analogue of Ser-peptide (peptides 16 and 17) also had no effect on the affinity of cAMP-dependent protein kinase for nucleotide. The stoichiometry of binding of lin-benzo-ADP to the enzyme was 1 mol/mol of protein kinase in all cases.

**Effect of Synthetic Peptide Analogues on Binding of ADP to Cyclic Nucleotide Dependent Protein Kinases**

<table>
<thead>
<tr>
<th>no.</th>
<th>peptide</th>
<th>[peptide] (μM)</th>
<th>[cGMP] (μM)</th>
<th>cGMP-dep protein kinase (μM)</th>
<th>cAMP-dep protein kinase (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Arg-Lys-Arg-Ser-Arg-Lys-Glu&lt;sup&gt;1&lt;/sup&gt;</td>
<td>100</td>
<td>20</td>
<td>11.6</td>
<td>9.0</td>
</tr>
<tr>
<td>4</td>
<td>Arg-Ala-Arg-Ser-Arg-Lys-Glu</td>
<td>150</td>
<td>20</td>
<td>10.6</td>
<td>9.0</td>
</tr>
<tr>
<td>6</td>
<td>Arg-Lys-Arg-Ala-Ser-Arg-Lys-Glu</td>
<td>200</td>
<td>20</td>
<td>11.6</td>
<td>9.0</td>
</tr>
<tr>
<td>9</td>
<td>Arg-Lys-Arg-Ser-Arg-Ala-Glu</td>
<td>200</td>
<td>20</td>
<td>11.6</td>
<td>9.0</td>
</tr>
<tr>
<td>13</td>
<td>Ala-Arg-Lys-Arg-Ser-Arg-Ala-Glu</td>
<td>200</td>
<td>20</td>
<td>11.6</td>
<td>9.0</td>
</tr>
<tr>
<td>16</td>
<td>Leu-Arg-Arg-Ala-Ser-Leu-Gly&lt;sup&gt;4&lt;/sup&gt;</td>
<td>1000</td>
<td>20</td>
<td>10.6</td>
<td>9.0</td>
</tr>
<tr>
<td>17</td>
<td>Leu-Arg-Arg-Ala-Ala-Leu-Gly</td>
<td>5000</td>
<td>20</td>
<td>11.6</td>
<td>9.0</td>
</tr>
</tbody>
</table>

<sup>1</sup> Same as footnote 1 in Table II. 
<sup>4</sup> Same as footnote 4 in Table II. 
<sup>5</sup> Peptide 16 corresponds to the sequence at the site of phosphorylation in t-type pyruvate kinase catalyzed by cAMP-dependent protein kinase (Kemp et al., 1977). When the serine in this parent peptide has been substituted by an alanine, the latter is underlined. 
<sup>6</sup> A 1000 nM concentration of histone H2B(29-35) or its analogues with alanine replacements of basic residues at either position 30 or position 34 had no effect on lin-benzo-ADP affinity of catalytic subunit even at concentrations 5-fold above their $K_m$ or $K_d$ values (Table IV). Ser-peptide and an inhibitor analogue of Ser-peptide (peptides 16 and 17) also had no effect on the affinity of cAMP-dependent protein kinase for nucleotide. The stoichiometry of binding of lin-benzo-ADP to the enzyme was 1 mol/mol of protein kinase in all cases.

**Table IV: Effect of Peptide Analogues on Binding of lin-Benzo-ADP to the Catalytic Subunit of cAMP-Dependent Protein Kinase**

<table>
<thead>
<tr>
<th>no.</th>
<th>peptide</th>
<th>[peptide] (μM)</th>
<th>apparent $K_m$ or $K_d$ (μM)</th>
<th>$V_{max}$ [pmol/min/mg]</th>
<th>$K_d$ (μM) (n)&lt;sup&gt;4&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>none</td>
<td>none</td>
<td>none</td>
<td>none</td>
<td>none</td>
</tr>
<tr>
<td>2</td>
<td>Arg-Lys-Arg-Ser-Arg-Lys-Glu&lt;sup&gt;1&lt;/sup&gt;</td>
<td>200</td>
<td>113&lt;sup&gt;4&lt;/sup&gt;</td>
<td>1.2</td>
<td>10.6 (1.11)</td>
</tr>
<tr>
<td>4</td>
<td>Arg-Ala-Arg-Ser-Arg-Lys-Glu</td>
<td>500</td>
<td>13&lt;sup&gt;4&lt;/sup&gt;</td>
<td>&lt;0.1</td>
<td>7.2 (1.16)</td>
</tr>
<tr>
<td>9</td>
<td>Arg-Lys-Arg-Ser-Arg-Ala-Glu</td>
<td>1000</td>
<td>13&lt;sup&gt;4&lt;/sup&gt;</td>
<td>&lt;0.1</td>
<td>9.1 (1.06)</td>
</tr>
<tr>
<td>13</td>
<td>Ala-Arg-Lys-Arg-Ser-Arg-Ala-Glu</td>
<td>500</td>
<td>13&lt;sup&gt;4&lt;/sup&gt;</td>
<td>&lt;0.1</td>
<td>12.3 (0.86)</td>
</tr>
<tr>
<td>16</td>
<td>Leu-Arg-Arg-Ala-Ser-Leu-Gly&lt;sup&gt;4&lt;/sup&gt;</td>
<td>1000</td>
<td>13&lt;sup&gt;4&lt;/sup&gt;</td>
<td>&lt;0.1</td>
<td>7.6 (1.08)</td>
</tr>
<tr>
<td>17</td>
<td>Leu-Arg-Arg-Ala-Ala-Leu-Gly</td>
<td>5000</td>
<td>13&lt;sup&gt;4&lt;/sup&gt;</td>
<td>&lt;0.1</td>
<td>7.8 (1.11)</td>
</tr>
</tbody>
</table>

<sup>1</sup> Peptide analogues at the specified concentrations were added to 6 μM catalytic subunit of cAMP-dependent protein kinase, and the dissociation constants ($K_d$'s) of lin-benzo-ADP for the enzyme were determined by fluorescence anisotropy addition titrations as described under Materials and Methods.
<sup>4</sup> Same as footnote 4 in Table II.
lin-benzo-ADP, use of the fluorescent probe enabled us to
detect and quantitate differences in the $K_d$ of a ligand whose
binding was altered.

**DISCUSSION**

lin-Benzo-ADP and lin-benzo-ATP are useful probes of
nucleotide binding to both the cAMP-dependent and cGMP-
dependent protein kinases. The kinetic constants and binding
affinities of the former enzyme for natural and lin-benzo-
adenine nucleotides are similar (Hartl et al., 1983; Bhatnagar
et al., 1985). In the case of cGMP-dependent protein kinase,
however, there are substantive differences between fluorescent
and natural adenine nucleotides in their interactions with the
enzyme. For example, lin-benzo-ATP is an extremely poor
substrate for cGMP-dependent protein kinase compared with
ATP (Bhatnagar et al., 1985).

A major finding of the present study was the difference in
nucleotide binding to the catalytic subunit of cAMP-dependent
protein kinase and the cGMP-dependent enzyme in the
presence of peptide substrates. Histone H2B(29–35) is an
unusual substrate in that all the residues other than the
phosphate-accepting serine are charged. It is a favorable
substrate for cGMP-dependent protein kinase, however, having
better kinetic constants than several other peptide and protein
substrates (Glass & Smith, 1983; Glass et al., 1986; Hemmings
et al., 1984). Although the $K_m$ and $v_{max}$ values differ sub-
stantially, histone H2B(29–35) and its analogues are also
substrates for cAMP-dependent protein kinase (Glass &
Krebs, 1982). None of the histone peptides, nor the widely
used Ser-peptide substrate of cAMP-dependent protein kinase,
affected the binding affinities of catalytic subunit for either
ADP or lin-benzo-ADP. On the other hand, peptide substrates
had clear effects on nucleotide binding to cGMP-dependent
protein kinase. In the absence of peptide substrate, lin-
benzo-ADP and ADP bound to activated cGMP-dependent
protein kinase (plus cGMP) with comparable affinities of
about 10 $\mu$M. Histone H2B(29–35) decreased the affinity of
the enzyme for ADP by 5-fold, but had no effect on binding of
lin-benzo-ADP. Other peptide analogues uniformly de-
creased the binding affinity for ADP, but most had no effect on
the dissociation constant for lin-benzo-ADP. However,
alogues of histone H2B(29–35) with alanine substituted in
positions 30, 31, or 34 (peptides 4, 5, and 9) also decreased
the affinity of the cGMP-dependent enzyme for lin-benzo-
ADP compared with the absence of peptide. Of these peptides,
the Ala34-substituted analogues decreased nucleotide binding
affinities the most. The greater hydrophobic bonding of lin-
benzo-ADP to the enzyme compared with ADP may account
for the higher affinity of the former nucleotide in the presence of
peptide substrates.

There was no correlation between $K_m$ or $K_v$ values of the
peptides and their ability to alter nucleotide affinity. Histone
H2B(29–35) and [Ala34]histone H2B(29–35), for example, both
exhibit low $K_m$ and relatively high $v_{max}$ values with
cGMP-dependent protein kinase, yet the latter peptide sig-
nificantly decreased lin-benzo-ADP affinity and the former
was without effect. [Ala34]histone H2B(29–35) was chosen as
a substrate for cGMP-dependent protein kinase in the or-
original lin-benzo-ADP studies because the enzyme had a pH
optimum close to 7 with this peptide (Bhatnagar et al., 1985).
The parent peptide, histone H2B(29–35), has an appreciably
higher pH optimum (Glass & Krebs, 1979). Replacement of
lysine-34 in the peptide with alanine also increases the $v_{max}$
of the reaction (Glass & Krebs, 1982). High pH is needed to
deprotonate lysine-34 in histone H2B(29–35), effectively
removing this positively charged, adverse determinant of
specificity. An alanine substitution accomplishes the same
thing.

An exact understanding of the mechanism by which histone
peptides affect the nucleotide binding affinity of cGMP-de-
dpendent protein kinase remains unknown and may require
solution of the crystal structure of the active site of the enzyme.
However, this enzyme can be compared with the catalytic
subunit of cAMP-dependent protein kinase. The CAMP-de-
dpendent protein kinase retains its regulatory and catalytic
domains in the same polypeptide chain, which makes the ac-
tivated holoenzyme unlike the free catalytic subunit of the
CAMP-dependent enzyme. Native histones are known to in-
teract with a polyarginine binding site in the regulatory domain
of the former enzyme to produce a time-dependent loss of
does not act like intact histones in this respect (Walton & Gill,
1981). However, interactions of basic peptides at this anionic
region of the regulatory domain might result in conformational
changes in the nucleotide binding portion of the active site.
The peptide could also influence nucleotide binding affinity
through a cooperative interaction of the identical subunits of
the dimeric cGMP-dependent protein kinase. Binding of
cGMP to the two subunits of this enzyme is known to display
positive cooperativity (McCune & Gill, 1979). A firm con-
clusion as to whether or not the effects of peptides on nu-
cleotide binding to cGMP-dependent protein kinase involve
the regulatory domain or a cooperative subunit interaction (or
both) must await studies with a stable catalytic fragment of
the enzyme. An alternate explanation is that interactions of
histone H2B(29–35) analogues, Mg-lin-benzo-ADP, or
Mg-ADP and residues within the active sites are fundamentally
different in the two cyclic nucleotide dependent protein kinases.
The cGMP-dependent enzyme is obviously able to differentiate
between histone H2B(29–35) and its Ala34 analogue when
lin-benzo-ADP binding is measured. This dissimilarity be-
tween the two peptides could be due to intrapeptide charge
interactions that produce different conformations of the active
site of cGMP-dependent protein kinase. It is also plausible
that lysine-34 of histone H2B(29–35) is directly involved in
anionic interaction with the $\beta$-phosphate of lin-benzo-ADP
or ADP that stabilizes nucleotide binding in the active site of
the enzyme. Replacement of lysine-34 with alanine would then
decrease nucleotide affinity. A lysine residue (lysine-389) is
known to be present in the nucleotide binding site of cGMP-
dependent protein kinase and is thought to interact with the
$\gamma$-phosphate of ATP (Hashimoto et al., 1982; Puri et al.,
1985).

In the absence of cGMP, the peptides had no significant
effect on nucleotide binding to cGMP-dependent protein ki-
nase. Autophosphorylation of the enzyme, but not phos-
phorylation of exogenous substrates, occurs under this condition
(Foster et al., 1981). As previously suggested for inhibition of
cAMP-dependent protein kinase activity by its regulatory
subunit (Witt & Roskoski, 1980; Granot et al., 1980), the
peptides were probably unable to interact with the cGMP-
dependent protein kinase in the absence of activating cGMP
because the regulatory domain covers the active site. Ex-
tremely high concentrations of [Ala34]histone H2B(29–35)
were able to decrease nucleotide affinity in the absence of
cGMP. With millimolar concentrations of this peptide, the
enzyme exhibits $\text{cGMP}+/\text{cGMP}$ activity ratios of 0.7–0.8,
indicating that high concentrations of peptide can compete with
and displace the regulatory domain from the active site.
Parallel but more dramatic changes were previously observed
for the holoenzyme of cAMP-dependent protein kinase (Hartl et al., 1983). The fact that activation of cGMP-dependent protein kinase by cGMP caused about a 25% increase in the $K_i$ for lin-benzo-ADP indicates that the regulatory domain of the enzyme may help stabilize nucleotide binding in the absence of cGMP.

Although the two cyclic nucleotide dependent protein kinases share a high degree of sequence homology, details of the substrate binding and phosphotransferase reactions catalyzed at their active sites exhibit a number of differences. It is not yet clear whether these differences between the enzymes are due to amino acid substitutions in their primary sequences or to their different subunit structures. These dissimilarities may be eventually exploited in the development of selective inhibitors of each enzyme or may be useful in understanding their interactions with natural protein substrates.

REFERENCES


