Synthetic Peptide Analogues Differentially Alter the Binding Affinities of Cyclic Nucleotide Dependent Protein Kinases for Nucleotide Substrates†

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ABSTRACT: Analogues 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 K_d 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 [Ala^{34}]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 V_{max} 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 [*p*-(fluorosulfonyl)benzoyl]-adenosine (Hixson & Krebs, 1979, 1981; Zoller & Taylor, 1979) and with *o*-phthalaldehyde (Puri et al., 1985a,b) and is thought to be involved in binding of the nucleoside triphosphate substrate.

The substrate specificities of the cGMP-dependent and cAMP-dependent protein kinases exhibit similarities when assayed in vitro (Hashimoto et al., 1976; Lincoln & Corbin, 1977; Edlund et al., 1977; Khoo et al., 1977; Blumenthal et al., 1978). Synthetic oligopeptides have been extensively used to investigate the substrate specificities of these two protein kinases (Krebs & Beavo, 1979; Glass & Krebs, 1980; Bramson et al., 1984). A kinetically favorable peptide substrate for cGMP-dependent protein kinase is histone H2B(29-35)[1] and its analogues (Glass & Krebs, 1979, 1982), while a well-studied model substrate of cAMP-dependent protein kinase is Ser-peptide, corresponding to the site of phosphorylation in L-type pyruvate kinase (Kemp et al., 1977; Feramisco et al., 1980; Rosevear et al., 1984). Although the two enzymes are ho-

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[1] Abbreviations: histone H2B(29-35), Arg-Lys-Arg-Ser-Arg-Lys-Glu; Ser-peptide, Leu-Arg-Arg-Ala-Ser-Leu-Gly; *lin*-benzo-ADP, *lin*-benzoadenosine 5'-diphosphate; Mops, 3-(*N*-morpholino)propanesulfonic acid.

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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 [Ala^{34}]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 μmol·(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-^{32}P]ATP (Cook et al., 1982) was 12.6 μmol·(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

$$r = (I_{\parallel} + I_{\perp})/(I_{\parallel} + 2I_{\perp})$$

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 *lin*-benzoadenine 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 cGMP-dependent protein kinase, titrations were performed in the presence or absence of 20 μM cGMP. This concentration of cGMP was sufficient to fully activate the cGMP-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., $[lin\text{-benzo-ADP}]_{free} \gg [lin\text{-benzo-ADP}]_{bound}$. 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_b, 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_b and the average angle of rotation ω_r 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):

$$r_b = r_0[(3 \cos^2 \omega_r - 1)/2]$$

(*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_f). In the absence of Mg^{2+} this anisotropy value (r_f) 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_{obsd}) 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.

Table I: Fluorescence Anisotropy of *lin*-Benzo-ADP Bound to Cyclic Nucleotide Dependent Protein Kinases

enzyme	condition	theoretical[a]		experimental[b]	
		r_b	ω_r (deg)	r_b	ω_r (deg)
cGMP-dependent protein kinase	−cGMP	0.279	13	0.271 ± 0.006	15
	+20 μM cGMP	0.279	13	0.268 ± 0.008	16
catalytic subunit, cAMP-dependent protein kinase	−cAMP	0.220	25	0.216 ± 0.005	26
	+10 μM cAMP	0.220	25	0.222 ± 0.006	25

[a] r_b and ω_r were calculated from Perrin's equations for anisotropy as described under Materials and Methods. [b] Values for r_b were obtained from anisotropy dilution titrations. Values for average angle of rotation (ω_r) were calculated from r_0 for *lin*-benzo-ADP as described under Materials and Methods.

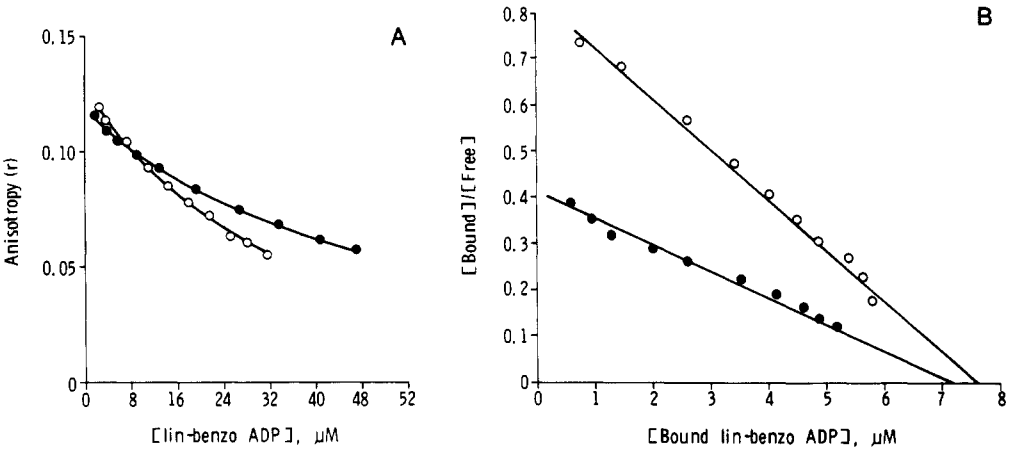

FIGURE 1: (A) Fluorescence anisotropy addition titrations of *lin*-benzo-ADP with cGMP-dependent protein kinase in the presence of 20 μM cGMP, in the absence (○) and presence (●) of the peptide [Ala[31]]histone H2B(29–35) at a concentration of 1 mM. *lin*-Benzo-ADP was varied at a constant (4 μM) protein kinase concentration by addition of aliquots of a concentrated solution of *lin*-benzo-ADP as described under Materials and Methods. (B) Scatchard plots of data in panel A. K_d values for *lin*-benzo-ADP of 8.0 and 18.4 μM in the absence (○) and presence (●), respectively, of the peptide were calculated from the slope. The number of moles of *lin*-benzo-ADP bound per mole of cGMP-dependent protein kinase holoenzyme were calculated from the x intercept and were 1.8 and 1.9 in the absence (○) and presence (●) of the peptide, respectively.

The concentrations of bound and free nucleotide after each addition were calculated as follows:

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

and

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

where [B] and [F] are the concentrations of *lin*-benzo-ADP bound to the enzyme and free in solution, respectively, at the total concentration of the ligand, $[lin\text{-benzo-ADP}]_t$, in the cuvette and r_b (determined from the dilution titration), r_f, and r_{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 *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 *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 lin-Benzo-ADP Binding to Cyclic Nucleotide Dependent Protein Kinases. The fluorescence emission spectrum of *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 *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_b), when all *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_b and ω_r for both cGMP-dependent protein kinase and the catalytic subunit of cAMP-dependent enzyme (Table I) were in good agreement with theoretical values of r_b and ω_r calculated from Perrin's equations for *lin*-benzo-ADP rigidly bound to the respective enzymes. This indicates that *lin*-benzo-ADP binds rigidly to these protein kinases under the experimental conditions and that the observed rotation of *lin*-benzo-ADP bound to the respective enzyme is due to the rotation of the protein per se. Therefore, the value of r_b obtained from the dilution titrations can be used to calculate the K_d of *lin*-benzo-ADP for these enzymes from addition titrations as described under Materials and Methods.

The binding of *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 cGMP-dependent protein kinase in the presence and absence of [Ala[31]]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 *lin*-benzo-ADP by 2.3-fold. That result was consistent with our earlier observation using another peptide analogue, [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 cGMP-dependent protein kinase for the nucleotide.

Effect of Synthetic Peptide Analogues on Binding of lin-Benzo-ADP to cGMP-Dependent Protein Kinase. Series of

Table II: Effect of Peptide Analogues on Binding of *lin*-Benzo-ADP to cGMP-Dependent Protein Kinase[a]

no.	peptide	apparent K_m or K_i (μM)	V_{max} [μmol·(min·mg)$^{-1}$]	K_d (n)[b] −cGMP (μM)	K_d (n)[b] +cGMP (μM)
	none			7.8 (1.98)	10.3 (1.94)
1	Arg-Lys-Arg-Ser-Arg-Lys-Glu[c]	22[d]	4.4	7.1 (2.21)	9.4 (1.97)
2	Ac-Arg-Lys-Arg-Ser-Arg-Lys-Glu	47	13.4	7.4 (2.36)	8.1 (2.09)
3	Ala-Lys-Arg-Ser-Arg-Lys-Glu	204	0.2	6.9 (2.11)	9.7 (1.86)
4	Arg-Ala-Arg-Ser-Arg-Lys-Glu	64	<0.1	9.2 (1.78)	16.2 (1.54)
5	Arg-Lys-Ala-Ser-Arg-Lys-Glu	200	6.3	6.8 (1.83)	15.4 (1.92)
6	Arg-Lys-Arg-Ala-Arg-Lys-Glu	86 (K_i)		6.4 (2.06)	7.0 (2.25)
7	Arg-Lys-Arg-Thr-Arg-Lys-Glu	24	<0.1	7.1 (1.79)	11.1 (1.88)
8	Arg-Lys-Arg-Ser-Ala-Lys-Glu	105	0.4	7.3 (2.12)	8.5 (2.06)
9	Arg-Lys-Arg-Ser-Arg-Ala-Glu	29	20.0	7.8 (2.18)	22.4 (2.08)
10	Arg-Lys-Arg-Ser-Ala-Ala-Glu	267	6.6	7.8 (1.91)	10.8 (1.93)
11	Arg-Lys-Arg-Ser-Arg-Lys-Ala	26	0.9	7.0 (1.86)	12.2 (1.79)
12	Arg-Lys-Arg-Ser-Arg-Lys-Glu-Ser-Tyr-Ser-Val	10	21.4	6.4 (1.99)	10.8 (1.88)

[a] 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. [b] 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. [c] Peptide 1 (Glass & Krebs, 1979) corresponds to residues 29–35 in histone H2B which contain the major site of phosphorylation of this substrate catalyzed by cGMP-dependent protein kinase (Hashimoto et al., 1976). When an amino acid residue of the parent peptide has been substituted by a different residue, the latter is underlined. Ac, N^{α}-acetyl. [d] The kinetic 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 Kinase[a]

no.	peptide	[peptide] (μM)	K_d (n)[b] −cGMP (μM)	K_d (n)[b] +cGMP (μM)
	none		7.4 (2.03)	9.1 (2.06)
1	Arg-Lys-Arg-Ser-Arg-Lys-Glu[c]	100	ND	9.8 (1.88)
		1000	ND	10.1 (1.76)
4	Arg-Ala-Arg-Ser-Arg-Lys-Glu	350	ND	15.6 (1.41)
		1000	10.6 (1.84)	16.2 (1.55)
5	Arg-Lys-Ala-Ser-Arg-Lys-Glu	1000	7.9 (1.97)	18.4 (1.87)
9	Arg-Lys-Arg-Ser-Arg-Ala-Glu	150	ND	26.2 (1.74)
		1000	16.1 (1.86)	25.1 (1.81)
10	Arg-Lys-Arg-Ser-Ala-Ala-Glu	1000	15.3 (1.82)	22.1 (2.11)
13	Ac-Arg-Lys-Arg-Ser-Arg-Ala-Glu	150	ND	23.0 (1.94)
		1000	14.8 (1.79)	21.6 (1.84)
14	Arg-Lys-Arg-Ala-Arg-Ala-Glu	500	12.4 (1.91)	24.4 (1.78)
15	Ac-Arg-Lys-Arg-Ala-Arg-Ala-Glu	500	14.2 (1.89)	22.7 (2.08)

[a] 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_m or K_i values. These kinetic constants are listed in Table II, except for peptides 13–15.[3] [b] 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. ND, not determined. [c] Same as footnote *c* in Table II.

histone H2B(29–35) peptides which varied in structure (Glass & Krebs, 1982) were used to investigate which amino acid residues were responsible for inducing the change in nucleotide affinity of cGMP-dependent protein kinase. These studies were performed in the presence and absence of saturating amounts of cGMP, since in the absence of cyclic nucleotide the holoenzyme is quite inactive in phosphotransferase activity toward peptide substrates. In the absence of both cGMP and peptide substrate, *lin*-benzo-ADP bound to the holoenzyme with a mean K_d of 7.6 μM (Tables II and III), identical with that reported in earlier experiments (Bhatnagar et al., 1985). In the absence of cGMP, none of the peptides at a concentration of 200 μM altered *lin*-benzo-ADP affinity; the mean K_d value with all peptides was 7.3 μM (Table II). The stoichiometry of binding of *lin*-benzo-ADP to the enzyme was 2.0 mol/mol of holoenzyme dimer.[2] Neither cGMP nor peptides altered this value.

When cGMP-dependent protein kinase was activated by cGMP in the absence of peptide, the K_d for *lin*-benzo-ADP increased only slightly (Tables II and III). In the presence of cGMP, addition of [Ala^{34}]histone H2B(29–35) (peptide 9) caused a large increase in the K_d value for *lin*-benzo-ADP (Table II). However, the parent peptide in the series, histone H2B(29–35), which has lysine instead of alanine at position 34, had no effect on nucleotide binding. N^{α}-Acetylation of the parent peptide (peptide 2) or addition of more residues to the COOH terminus (peptide 12) failed to alter nucleotide affinity. Substitution of alanine for arginine-29 or arginine-33 (peptides 3 and 8) also did not produce analogues that altered binding of *lin*-benzo-ADP. [Ala^{33},Ala^{34}]histone H2B(29–35), like the [Ala^{34}]peptide which also lacks lysine-34, did not affect nucleotide affinity at a concentration of 200 μM (Table II). Replacements of the phosphorylatable serine-32 with either alanine or threonine (peptides 6 and 7) or of the acidic residue with alanine (peptide 11) were also without effect. Replacing either lysine-30 or arginine-31, on the other hand, resulted in the only other peptides (peptides 4 and 5) that decreased the affinity of cGMP-dependent protein kinase for nucleotide. At this peptide concentration the decreases in nucleotide affinity caused by [Ala^{30}]-, [Ala^{31}]-, or [Ala^{34}]histone H2B(29–35)

[2] Peptide 4 consistently (n = 5) failed to give binding higher than 1.6 mol/mol of cGMP-dependent protein kinase holoenzyme in the presence of cGMP (Tables II and III). The reason for this is unknown. Peptide 4 did not show anomalous binding stoichiometry with the catalytic subunit of cAMP-dependent protein kinase (Table IV).

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

no.	peptide	[peptide] (μM)	apparent K_m or K_i (μM)	V_{max} [μmol·(min·mg)$^{-1}$]	K_d (μM) (n)[b]
	none				10.6 (1.11)
1	Arg-Lys-Arg-Ser-Arg-Lys-Glu[c]	200	113[e]	1.2	7.2 (1.16)
		500			9.1 (1.06)
4	Arg-Ala-Arg-Ser-Arg-Lys-Glu	1000	183	<0.1	12.3 (0.86)
9	Arg-Lys-Arg-Ser-Arg-Ala-Glu	500	73	17.3	7.6 (1.08)
13	Ac-Arg-Lys-Arg-Ser-Arg-Ala-Glu	500	ND	ND	7.8 (1.11)
16	Leu-Arg-Arg-Ala-Ser-Leu-Gly[d]	100	16	20.2	9.4 (1.41)
		1000			8.8 (1.26)
17	Leu-Arg-Arg-Ala-Ala-Leu-Gly	5000	376 (K_i)		7.4 (1.08)

[a] 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. [b] Same as footnote *b* in Table II. [c] Same as footnote *c* in Table II. [d] Peptide 16 corresponds to the sequence at the site of phosphorylation in L-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. [e] Kinetic constants are taken from Glass et al. (1981), Glass (1983), and Kemp et al. (1977). ND, not determined.

Table V: Effect of Peptide Analogues on Binding of ADP to Cyclic Nucleotide Dependent Protein Kinases[a]

no.	peptide	[peptide] (μM)	[cGMP] (μM)	K_d cGMP-dep protein kinase (μM)	K_d cAMP-dep protein kinase (μM)
	none			11.6	9.0
			20	12.8	
1	Arg-Lys-Arg-Ser-Arg-Lys-Glu	100	20	54	10.2
4	Arg-Ala-Arg-Ser-Arg-Lys-Glu	350	20	78	9.9
6	Arg-Lys-Arg-Ala-Arg-Lys-Glu	1000	20	34	9.9
9	Arg-Lys-Arg-Ser-Arg-Ala-Glu	150	20	106	9.0
14	Arg-Lys-Arg-Ala-Arg-Ala-Glu	500	20	126	10.1

[a] The dissociation constants (K_d's) of ADP for each cyclic nucleotide dependent protein kinase were determined by fluorescence anisotropy displacement titrations as described under Materials and Methods. The indicated concentrations of peptide analogues were present at approximately 5 (or greater) times that of their respective K_m or K_i values. These kinetic constants are listed in Table II. In the case of cGMP-dependent protein kinase, near-saturating concentrations of cGMP were present or absent as indicated.

were manifest only in the presence of cGMP.

The K_m 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 μM concentration of histone H2B(29–35) still failed to change the K_d for *lin*-benzo-ADP (Table III). Concentrations of 1000 μM of the Ala^{30}, Ala^{31}, or Ala^{34} analogues produced no further decreases in *lin*-benzo-ADP affinity (Table III) than did 200 μM peptide (Table II). A high concentration of [Ala^{33},Ala^{34}]histone H2B(29–35) maximally decreased nucleotide affinity (Table III), an effect not seen at 200 μM (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 Ala^{34} substitution and have kinetic constants similar to [Ala^{34}]histone H2B-(29–35),[3] also decreased the affinity for *lin*-benzo-ADP. High concentrations of all of the Ala^{34}-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.[4]

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_i 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. The K_d of ADP for the protein kinases was determined by fluorescence anisotropy displacement titrations in the presence of relatively high concentrations of several peptides. ADP binding to the catalytic subunit of cAMP-dependent protein kinase was not altered by the presence of any of the peptides (Table V). However, peptide-induced effects on ADP binding were seen with cGMP-dependent protein kinase in the presence of cGMP. The parent peptide histone H2B(29–35) and those with alanine replacements at positions 30, 32, and 34 significantly decreased the binding affinity of the cGMP-dependent enzyme for ADP. Peptides with an alanine substitution at position 34 (peptides 9 and 14) produced a 10-fold increase in the K_d value for ADP (Table V) while causing only 2–3-fold increases in K_d for *lin*-benzo-ADP. Thus, the clear differences between cGMP-dependent protein kinase and the catalytic subunit of the cAMP-dependent enzyme with respect to the effect of peptides on nucleotide binding affinity were also observed with ADP. These results further demonstrate the utility of *lin*-benzo-ADP in assessing nucleotide binding. Although histone H2B(29–35) failed to alter the affinity of

[3] T. M. Horton, V. L. Williams, and D. B. Glass, unpublished observations.

[4] D. B. Glass, unpublished observations.

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 substantially, 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 μM. 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 decreased the binding affinity for ADP, but most had no effect on the dissociation constant for *lin*-benzo-ADP. However, analogues 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 Ala^{34}-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_i values of the peptides and their ability to alter nucleotide affinity. Histone H2B(29–35) and [Ala^{34}]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 significantly decreased *lin*-benzo-ADP affinity and the former was without effect. [Ala^{34}]histone H2B(29–35) was chosen as a substrate for cGMP-dependent protein kinase in the 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-dependent 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 cGMP-dependent protein kinase retains its regulatory and catalytic domains in the same polypeptide chain, which makes the activated holoenzyme unlike the free catalytic subunit of the cAMP-dependent enzyme. Native histones are known to interact with a polyarginine binding site in the regulatory domain of the former enzyme to produce a time-dependent loss of activity (Walton & Gill, 1980, 1981). Histone H2B(29–35) 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 conclusion as to whether or not the effects of peptides on nucleotide 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 Ala^{34} analogue when *lin*-benzo-ADP binding is measured. This dissimilarity between 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 an ionic interaction with the β-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 γ-phosphate of ATP (Hashimoto et al., 1982; Puri et al., 1985b).

In the absence of cGMP, the peptides had no significant effect on nucleotide binding to cGMP-dependent protein kinase. Autophosphorylation of the enzyme, but not phosphorylation 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. Extremely high concentrations of [Ala^{34}]histone H2B(29–35) were able to decrease nucleotide affinity in the absence of cGMP. With millimolar concentrations of this peptide, the enzyme exhibits −cGMP/+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.[4] 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_d 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.

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