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Mapping of the Two Intrachain Cyclic Nucleotide Binding Sites of Adenosine Cyclic 3′,5′-Phosphate Dependent Protein Kinase I†

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ABSTRACT: A series of cyclic nucleotide analogues were examined for their abilities to inhibit the binding of adenosine cyclic 3′,5′-phosphate (cAMP) to each of the two types of cAMP binding sites (A and B) on type I cAMP-dependent protein kinase (cAKI). Sixty-seven different analogues were examined that either contained substituents in the 1-, 2-, 6- (or N^6), and/or 8-positions or that included heterocyclic ring systems other than purine. The two sites were found to be similar in that both bound the syn conformation of cAMP and neither formed hydrogen bonds with the adenine ring of cAMP. Moreover, analogues were found that bound preferentially to either site A or site B. Analogues containing bulky, hydrophobic N^6-substituents were the most site A specific; for example, N^6-(1-methyl-3-phenyl-*n*-propyl)-cAMP was nearly 80-fold more potent as an inhibitor of cAMP binding to site A than to site B. Site B specificity resulted from the presence of either electron-withdrawing substituents in the 2-position or electron-donating substituents in the 8-position; for example, 2-chloro-cAMP and 8-amino-cAMP were approximately 10- and 20-fold, respectively, more potent as inhibitors of cAMP binding to site B than to site A.

The cAMP-dependent protein kinases exist in two isozyme forms: cAKI[1] and cAKII (Corbin et al., 1975). The two isozymes have virtually identical catalytic subunits but dissimilar regulatory subunits (Hofmann et al., 1975). In the absence of cAMP, both isozymes exist as inactive tetramers made up of two catalytic and two regulatory subunits (Hofmann et al., 1975; Rubin & Rosen, 1975). The binding of four molecules of cAMP to the regulatory subunit dimer results in the release of two active catalytic subunit monomers (Corbin et al., 1978).

For both isozymes, the two intrachain binding sites on the regulatory subunit monomers have been shown to be nonidentical based on the rate of [^{3}H]cAMP exchange (Døskeland, 1978; Rannels & Corbin, 1980) and the rate of association with [^{3}H]cAMP (Øgreid & Døskeland, 1981a). In qualitative studies, derivatives of cAMP containing 2- or 8-substituents preferentially inhibited binding to the slowly exchanging B site, whereas the rapidly exchanging A site interacted preferentially with 6-substituted cyclic nucleotide derivatives (Corbin et al., 1982).[2] No studies quantitating the relative affinities of cAMP analogues for sites A and B have appeared.

Site B specific derivatives stimulate the binding of [^{3}H]-cIMP to site A of the holoenzyme, suggesting that interactions between the two sites take place during cAMP activation of the protein kinases (Rannels & Corbin, 1981). Kinetic data have led to the suggestion that binding of cAMP to site B allows the subsequent binding of cAMP to site A (Døskeland & Øgreid, 1981; Øgreid & Døskeland, 1981b). An important, unanswered mechanistic question is whether activation requires binding to only one site or to both sites.

In the present work on cAKI, the K_I values for sites A and B have been determined for a series of cyclic nucleotide derivatives. These data have allowed us to begin mapping the

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[1] Abbreviations: cAKI, cAMP-dependent protein kinase, isozyme form I; cAKII, cAMP-dependent protein kinase, isozyme form II; RI, the regulatory moiety of cAKI; RII, the regulatory moiety of cAKII; C, the catalytic subunit of cAKI or cAKII; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; K_D, the apparent equilibrium dissociation constant for the interaction between [^{3}H]cAMP and RI; K_I, the apparent equilibrium inhibition constant.

[2] In the papers by Rannels & Corbin (1980) and Corbin et al. (1982), the site (A) showing rapid exchange of bound [^{3}H]cAMP is termed site 2, and the site (B) showing slower exchange is termed site 1.

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two sites and to determine the significant differences between them. In addition, these data are useful for designing new analogues with potentially greater specificity for either site A or site B. Highly site specific derivatives should prove most useful in functional studies of sites A and B.

Materials and Methods

Chemicals. [2,8-$^3H_2$]cAMP (36 Ci/mmol) was obtained from the Radiochemical Centre, Amersham, U.K. Nucleotides cAMP, cIMP, cGMP, N^6-monobutyryl-cAMP, 8-Br-cAMP and 8-azido-cAMP, stated by the manufacturer to be from 97 to 99% pure, were obtained from Sigma Chemical Co., St. Louis, MO. The synthesis of 8-amino-3-(β-D-ribofuranosyl)-*s*-triazolo[4,3-*a*]pyrazine cyclic 3′,5′-phosphate will be described in a subsequent publication (T. Huynh-Dinh, J. Igolen, P. Sturm, E. Reist, R. Suva, S. Døskeland, and J. Miller, unpublished results). The other cyclic nucleotide analogues were either synthesized as described previously (Boswell et al., 1973; Muneyama et al., 1971; Meyer et al., 1972, 1973, 1974, 1975a,b; Miller et al., 1973, 1978, 1980a,b, 1981; Yagura et al., 1980a), received as gifts from other investigators, or synthesized as described below. Compounds **30**, **31**, and **43** were gifts from Dr. G. Weimann, Boeheringer Mannheim GmbH, Mannheim, FDR (Michal et al., 1974). Analogues **33–38** and **40** of the general structure N^6-ROCHN-cAMP were generously supplied by Dr. G. B. Chheda, Roswell Park Memorial Institute, Buffalo, NY (Hong et al., 1975). The 8-seleno derivatives **12–15** were provided by Dr. S.-H. Chu, Brown University, Providence, RI (Chu et al., 1975). Compound **20** was a gift from Dr. Jisaburo Ueyanagi, Takeda Chemical Industris, Ltd., Osaka, Japan (Marumoto et al., 1979).

Syntheses. All reactions required an anhydrous environment. All evaporations were conducted under diminished pressure at <40 °C unless stated otherwise. Thin-layer chromatography (TLC) utilized either system A, Merck cellulose F plates developed with isopropyl alcohol–concentrated ammonium hydroxide–water (7:1:2 v/v), system B, silica GF plates developed with methanol, or system C, silica GF plates developed with $CHCl_3$–MeOH (7:3 v/v). Visualization on all plates was by ultraviolet (UV) light. ^1H NMR spectra were obtained on a Varian EM 390 instrument; sugar protons, other than the anomeric proton, are not reported. Mass spectra were determined on an LKB Model 9000 mass spectrometer at 70 eV. UV spectra were obtained on a Perkin-Elmer 552 instrument. Total absorbance, expressed as AU_λ, is obtained by multiplying the measured absorbance at the indicated wavelength by the dilution factor required to obtain an on-scale reading and by the total volume of the solution. Diethylaminoethylcellulose used for chromatography was Whatman DE-52. Charcoal used for desalting was Barnebey-Cheney UU 1064 prepared as described by Zadrazil (1973).

N^6-Octyladenosine Cyclic 3′,5′-Monophosphate (**27**). To 6-chloropurine 9-β-D-ribofuranoside cyclic 3′,5′-monophosphate (0.170 g, 0.49 mmol; Michal et al., 1974) in 15 mL of EtOH at 70 °C was added octylamine (0.189 g, 1.46 mmol). After 2 h at 70 °C, the reaction was evaporated to dryness and the residue was partitioned between H_2O and Et_2O. The Et_2O extracts were evaporated to dryness. The residue was shaken with 10 mL of H_2O and treated with concentrated HCl to pH 2. The acidic H_2O was decanted from an oily deposit. The residue was triturated with an ethanol–ether solution to produce a crystalline solid, which was collected by suction filtration. Recrystallization from EtOH yielded 38 mg of white powder (18%): UV λ_{max} (ε) pH 1, 263 nm (18 900), pH 7, 268 (17 600), pH 11, 268 (17 400); mass spectrum, $(Me_3Si)_2$ derivative, *m/e* 585, 276, 310; TLC, system A, R_f 0.68. Anal. Calcd for $C_{18}H_{28}N_5O_6P \cdot 0.5H_2O$: C, 47.99; H, 6.48; N, 15.55. Found: C, 48.10; H, 6.34; N, 15.41.

N^6-(1,3,5-Estratrien-3-yl)adenosine Cyclic 3′,5′-Monophosphate (**29**). A solution of 6-chloropurine 9-β-D-ribofuranoside cyclic 3′,5′-monophosphate (0.101 g, 0.29 mmol; Michal et al., 1974) and 3-amino-1,3,5-estratriene (0.271 mg, 0.93 mmol; compound supplied by Dr. Joseph I. DeGraw, SRI International) in EtOH (5 mL) was refluxed for 22.5 h. The reaction was evaporated to dryness and the residue was triturated in Et_2O to yield a solid (187 mg) that was contaminated with the starting steroid, as determined by TLC, system C (R_f 0.18 for the desired product and 0.95 for the starting steroid). An EtOH solution of the crude solid was chromatographed on 10 mL of Dowex 50 X2 (H^+, 100–200 mesh) by eluting with 0.05 M HCl in EtOH–H_2O (5:1 v/v). The appropriate fractions, as determined by TLC and UV spectra, were combined and evaporated to dryness. The pale amber glass was redissolved in EtOH (1 mL) and the product was precipitated with Et_2O (30 mL). Gravity filtration yielded 99 mg of off-white solid (58%): UV λ_{max} (ε) (MeOH) 299 nm (24 900), pH 1, 279 (14 000), pH 7, 313 (21 800), pH 11, 314 (24 200); mass spectrum, $(Me_3Si)_2$ derivative, *m/e* 711, 696, 402, 373. Anal. Calcd for $C_{28}H_{34}N_5O_6P \cdot HCl$: C, 55.67; H, 5.84; N, 11.60. Found: C, 55.82; H, 5.86; N, 11.23.

4-Amino-7-(β-D-ribofuranosyl)-7H-pyrrolo[2,3-d]pyrimidine 5′-(Trichloromethyl Phosphonate) [*7-Deazaadenosine 5′-(Trichloromethyl Phosphonate)*]. To a mixture of 7-deazaadenosine (0.828 g, 3.11 mmol; compound supplied by Dr. Paul O'Connell, Upjohn Co.) in triethyl phosphate (32.5 mL) at 0 °C was added a solution of trichloromethyl phosphoryl dichloride (6.89 g, 23.2 mmol; Kennard & Hamilton, 1963; Makabe et al., 1978) in triethyl phosphate (23 mL). The reaction was monitored by TLC, system B. After 4 days at 3 °C, the reaction was poured into 900 mL of ice and the pH was adjusted to 7.5 with 1 M NaOH. The solution was extracted with Et_2O. The aqueous solution was briefly evacuated to remove residual Et_2O and the pH was adjusted to 3.0. This solution was desalted by stirring with 40 g of charcoal, filtering, and washing with H_2O to a constant conductivity measurement. The adsorbed nucleotide was eluted with EtOH–H_2O–NH_4OH (10:10:1 v/v). Evaporation of the eluant yielded a residue containing triethyl phosphate and the desired product. Addition of Et_2O yielded a white solid homogeneous on TLC, system B, R_f 0.80 (7-deazaadenosine, R_f 0.66). The solid was dissolved in H_2O (80 mL) and the pH was adjusted to 2.5 with 1 M HCl. After the solution was allowed to stand at room temperature for 2 days, white crystals were deposited (273 mg): UV λ_{max} (ε) pH 1, 270–272 nm (11 100), 227 (23 400), pH 7, 270 (11 800), pH 11, 270 (11 900). Anal. Calcd for $C_{12}H_{14}Cl_3N_4O_6P$: C, 32.20; H, 3.15; N, 12.52. Found: C, 31.89; H, 3.37; N, 12.20.

Evaporation of the mother liquor and trituration of the residue in EtOH yielded a solid (852 mg) contaminated with inorganic salts. A slurry of this solid in H_2O was stirred 1.5 h and suction-filtered to yield 579 mg of additional product. Chromatography of the filtrate on 50 mL of Dowex 50 X8 (H^+, 100–200 mesh) and elution with H_2O yielded another 98 mg of product (71%, total yield).

4-Amino-7-(β-D-ribofuranosyl)-7H-pyrrolo[2,3-d]pyrimidine Cyclic 3′,5′-Monophosphate (*7-Deazaadenosine Cyclic 3′,5′-Monophosphate*) (**59**). This compound was previously synthesized in a six-step procedure from 7-deazaadenosine (Hanze, 1968). We cyclized 7-deazaadenosine 5′-(trichloromethyl phosphonate) (0.050 g, 0.11 mmol) by treating its

mixture in dry DMF (3 mL) with a solution of potassium *tert*-butoxide (0.302 g, 2,7 mmol) in *t*-BuOH (2.7 mL) and DMF (2.7 mL) at room temperature (Makabe et al., 1978). The reaction was terminated after 3.5 h by pouring into ice water (20 mL). The solution was neutralized with 1 N HCl and lyophilized. The residual white solid formed a slurry with H_2O (25 mL) and was centrifuged. The residue was solubilized in 2 mL of H_2O with 1 drop of 4 M NH_4OH. Treatment of the solution with 1 M HCl to pH 2.3 (1 drop) did not effect crystallization. The solution was lyophilized to yield 12.5 mg of white solid: UV λ_{max} (ϵ) pH 1, 228 nm (22700), 270 (11000), pH 7, 270 (12000), pH 11, 270 (12000). [Hanze (1968) reported UV λ_{max} (ϵ) (0.01 N HCl) 226 nm (20800) and 270 (9470) for the sesquihydrate.] TLC, system A (identical with an authentic sample received as a gift from Dr. Paul O'Connell), R_f 0.53; ^{1}H NMR (Me_2SO-d_6 + D_2O) δ 6.13 (s, 1 H), 6.70 (d, 1 H), 8.16 (s, 1 H). The solution obtained from centrifugation, pH 7–8, was successfully desalted on Bio-Gel P-2 (100–200 mesh, 25 mm × 1000 mm column) by eluting with H_2O to yield another 19 mg of product (73%, total yield).

7-Amino-3-(β-D-ribofuranosyl)-3H-1,2,3-triazolo[4,5-d]-pyrimidine Cyclic 3′,5′-Monophosphate (8-Azaadenosine Cyclic 3′,5′-Monophosphate) (**60**). This compound was synthesized from 8-azaadenosine (Vega Biochemicals, Tucson, AZ) as previously described (Miller et al., 1978).

4-Amino-1-(β-D-ribofuranosyl)-1H-imidazo[4,5-c]pyridine Cyclic 3′,5′-Monophosphate (3-Deazaadenosine Cyclic 3′,5′-Monophosphate) (**58**). This compound was previously prepared by cyclization of 3-deazaadenosine 5′-(*p*-nitrophenyl phosphate) (Mizuno et al., 1975). Preparation and cyclization of 3-deazaadenosine 5′-(trichloromethyl phosphonate) gave improved yields with less difficulty. The phosphonate was prepared from 3-deazaadenosine (0.396 g, 1.49 mmol; Dr. John Montgomery, Southern Research Institute), as described for the 7-deaza analogue. The reaction took 24–48 h and was monitored by TLC, system A (R_f 0.66 for the phosphonate and R_f 0.52 for 3-deazaadenosine). A 40% crude yield was obtained after desalting on 13.8 g of charcoal: mass spectrum, $(Me_3Si)_4$ derivative, *m/e* 734; UV λ_{max} (ϵ) pH 1, 261 nm (12400), pH 7, 263 (12400), pH 11, 265 (12400); ^{1}H NMR (D_2O) δ 5.95 (d, 1 H, 7.25 (d, 1 H), 7.55 (br m, 1 H), 8.55 (asym d, 1 H). The phosphonate (0.277 g, 0.60 mmol) was cyclized as described for the synthesis of tubercidin cyclic 3′,5′-monophosphate. The reaction was terminated after 3 h and desalted on 25 g of charcoal as previously described. The nucleotide-containing eluant was concentrated to 15 mL (4500 AU_{260}) and chromatographed on DEAE cellulose (15 mm × 350 mm column), eluting with a 4-L linear gradient of water and 0.4 M triethylammonium bicarbonate. The appropriate fractions were concentrated and rechromatographed on 25 mL of Dowex 1 X2 (Cl^-, 100–200 mesh), eluting with 0.001 M HCl. The hydrochloric acid eluant was lyophilized and the residual solid was triturated in EtOH to yield 38 mg of white solid (17%): TLC, system A, R_f 0.43; ^{1}H NMR (Me_2SO-d_6 + D_2O) δ 6.07 (s, 1 H), 7.25 (d, 1 H), 7.71 (d, 1 H), 8.59 (s, 1 H); mass spectrum, $(Me_3Si)_3$ derivative, *m/e* 544; UV λ_{max} (ϵ) pH 1, 262 nm (10700), pH 7, 263 (10400), pH 11, 265 (10500). Anal. Calcd for $C_{11}H_{13}N_4O_6P{\cdot}H_2O{\cdot}HCl$: C, 35.45; H, 4.22; N, 14.64. Found: C, 35.34; H, 4.32; N, 14.87.

Protein Kinase I (cAKI). This was prepared from rabbit skeletal muscle as described by Døskeland (1978) except that the hydroxylapatite step was replaced by DEAE-Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden) chromatography. The free regulatory moiety (RI) of cAKI was prepared from cAKI by a modification (Døskeland & Kvinnsland, 1980) of the urea dissociation method of Schwechheimer & Hofmann (1977). The solution of urea contained 25 mM methylamine.

Assay for Inhibition of [^{3}H]cAMP Binding. The incubations (0.3 mL) were for 30–60 min at 37 °C in 15 mM Hepes–NaOH, pH 7.0, with 0.15 M KCl, 5 mM EDTA, 20 mM 2-mercaptoethanol, 2 mg/mL bovine serum albumin, and 2 mg/mL heat-stable protein fraction (Døskeland & Haga, 1978). When 8-azido-cAMP was tested, the concentration of 2-mercaptoethanol was 0.2 mM. EDTA, mercaptoethanol, and added proteins were required to protect RI against denaturation (Døskeland et al., 1977; Døskeland & Haga, 1978). The concentration of RI was 3–6 nM (with respect to cAMP binding sites) and the concentration of [^{3}H]cAMP was 60 nM. Each analogue was tested at 10 different concentrations, covering a 512-fold concentration range.

In a series of control experiments it was shown that the inhibition by 20 representative analogues could be completely overcome by increasing the concentration of [^{3}H]cAMP. The degree of inhibition was the same whether incubations were started by the addition of analogue, [^{3}H]cAMP, or a mixture of the two. Furthermore, the amount of [^{3}H]cAMP bound in the presence of the analogues was similar after 30, 45, and 60 min of incubation. Therefore, the inhibition was reversible and competitive, and an equilibrium was obtained within 30 min at 37 °C.

Determination of [^{3}H]cAMP Bound to Sites A and B. This was essentially as described by Øgreid & Døskeland (1981b). Briefly, an aliquot of the incubation mixture (0.25 mL) was mixed with 2.25 mL of ice-cold buffer (15 mM Hepes–NaOH, pH 7.0, 3. M NaCl, 5 mM EDTA, 20 mM 2-mercaptoethanol, 0.4 mg/mL serum albumin, 0.1 mg/mL heat-stable protein fraction, and 0.5 mM unlabeled cAMP). Samples (0.5 mL) of this mixture were withdrawn immediately and after 6, 18, and 24 h for determination of bound [^{3}H]cAMP by the $(NH_4)_2SO_4$ precipitation method (Døskeland et al., 1977). In this mixture (3.2 M NaCl at 0 °C), [^{3}H]cAMP bound to site A and site B exchange with unlabeled cAMP, with half-times of 1 and >24 h, respectively (Døskeland, 1978). The first sample removed will therefore give a measure of the total amount (sites A plus B) of [^{3}H]cAMP bound to RI, whereas the other three samples will contain chiefly [^{3}H]cAMP bound to site B. Plotting the logarithm of the values found for bound [^{3}H]cAMP after 6, 18, and 24 h of exchange vs. time and extrapolating to zero time provide an estimation of the amount of [^{3}H]cAMP bound to site B at the moment the incubation was stopped. The amount of [^{3}H]cAMP associated with site A was next calculated as total [^{3}H]cAMP bound (sites A plus B) minus [^{3}H]cAMP bound to site B.

Calculation of Kinetic Constants. The K_D for cAMP was calculated from double-reciprocal plots of the binding of [^{3}H]cAMP in the absence of analogues. The data for site B resulted in a straight line (K_D = 1.2 nM). A curved line (compatible with positive cooperativity)[3] was obtained for site A. The K_D for site A could thus not be determined, and the concentration of free [^{3}H]cAMP required to half-maximally saturate site A (1.7 nM) was used as an estimate of K_D.

The K_I values of the analogues were calculated by using a rearrangement of the Michaelis–Menten equation for competitive inhibition.

[3] We have recently found that the basis for the apparent positive cooperativity for [^{3}H]cAMP binding to site A is that the dissociation of [^{3}H]cAMP from one of the two A sites is 3–4 times more rapid when the other A site is vacant than when it is occupied by cAMP.

$$\frac{R_t}{Cx} = \frac{K_D}{[^3H]cAMP} + 1 + \frac{K_D i}{K_I[[^3H]cAMP]}$$

where Cx is the amount of [^{3}H]cAMP bound, R_t is the total concentration of binding site (A or B), [^{3}H]cAMP is the concentration of free labeled cAMP, i is the concentration of free analogue, and K_D is the apparent equilibrium dissociation constant for the interaction between [^{3}H]cAMP and site A or B of RI. A plot of R_t/Cx vs. i (Figure 1) will intersect the abscissa at 1 when [^{3}H]cAMP is $\gg K_D$ and have a slope of

$$\frac{1}{K_I}\frac{K_D}{[[^3H]cAMP]}$$

For all of the analogues reported here, i was varied at a constant total concentration (60 nM) of [^{3}H]cAMP. The concentration of free competitor, $i = i_t - (R_0 - Cx)$, where i_t is the total concentration of competitor. It should be noted that R was more than 95% saturated in the absence of i. The ratio of the slopes obtained for (unlabeled) cAMP and analogue will be K_I(cAMP)/K_I(analogue), which is a quantitative measure of the affinity of an analogue (relative to cAMP) for a binding site. All analogues were tested at least 3 times, and the standard error of the mean of K_I(cAMP)/K_I(analogue) was less than 20% of the value reported.

Results

Determination of the Affinity of Cyclic AMP Analogues for the Binding Sites of RI. The analogues were not available in a labeled form. Thus, their affinity for sites A and B of RI had to be deduced from their ability to compete with [^{3}H]cAMP for binding rather than from direct measurement of the amount of analogue bound.

There are interactions between the binding sites of RI and RII (Døskeland & Øgreid, 1981; Øgreid & Døskeland, 1981a). Therefore, if an analogue replaces [^{3}H]cAMP bound to one of the four sites of RI, the binding characteristics of the homologous site or the heterologous sites may be altered. If the remaining sites change their affinity for [^{3}H]cAMP and for the analogue to a similar extent and the binding sites are saturated, the degree of [^{3}H]cAMP displacement as a function of the free analogue concentration (i) will be unaltered and the plot of R_t/Cx vs. i and the determination of K_I(cAMP)/K_I(analogue) will not be affected. Only if the affinity for [^{3}H]cAMP and the analogue is altered disproportionately (i.e., the binding site specificity is changed) will the plot of R_t/Cx vs. i deviate from a straight line. Since the plots of R_t/Cx vs. i were straight lines for all analogues studied, we consider it unlikely that replacement of bound [^{3}H]cAMP by a cAMP analogue produces a major alteration of the specificity of the remaining sites.

A final point of concern was that the specificity of the cAMP binding sites of RI might have been altered during preparation. The binding site specificity for RI produced by urea dissociation of highly purified cAKI and cAKI rapidly prepared in the presence of protease inhibitor was compared and found to be similar for the analogues tested (Figure 1). Furthermore, the preparation of RI used in the present study reassociated stoichiometrically with the catalytic subunit of protein kinase and behaved on molecular sieve chromatography as expected for the RI dimer (data not shown).

Binding of Cyclic Nucleotide Analogues Bearing Substituents on the Adenine Ring. (1) 2-Substituted Analogues. The inhibition constants of 2-substituted cyclic AMP analogues are shown in Table I. Binding to site A is reduced relative to that of cAMP for all substituents. Analogues with linear

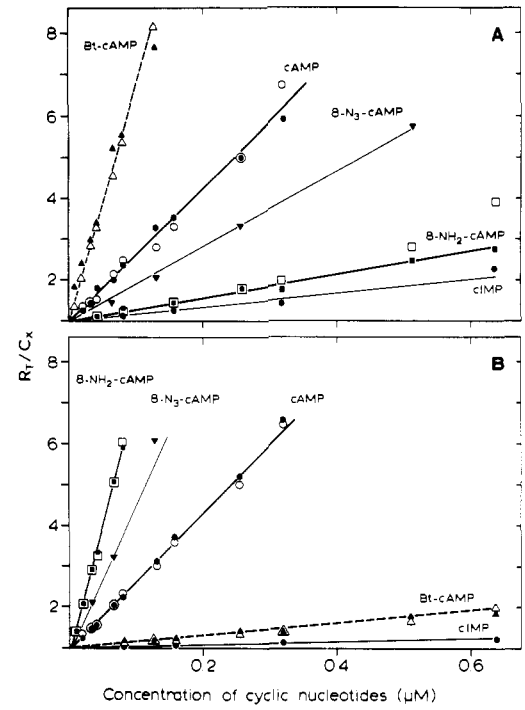


FIGURE 1: Plot of R_t/Cx vs. the concentration of some cAMP analogues. The binding protein was either RI (closed symbols) obtained by urea dissociation of highly purified cAKI or cAKI (open symbols) purified by the rapid procedure described by Døskeland (1978) with the modification that benzamidine (40 mM) rather than phenylmethanesulfonyl fluoride was present in the buffers to minimize proteolysis. The incubations contained 60 mM [^{3}H]cAMP and the concentrations of cyclic nucleotides indicated on the abscissa. The incubation conditions and the method for separate determination of the amount of [^{3}H]cAMP bound (Cx) to sites A and B were as described under Materials and Methods. The maximum binding capacities (R_t) of sites A and B were determined in parallel experiments where RI and cAKI were incubated in the presence of 30, 60, 120, and 240 nM [^{3}H]cAMP. The analogues tested were N^6-monobutyryl-cAMP [Bt-cAMP (▲, △)], 8-azido-cAMP [8-N_3-cAMP (▽)], 8-NH_2-cAMP (□, ■), and cIMP (●). Unlabeled cAMP (○, ●) was also tested. The data for sites A and B are shown in the upper and lower part of the figure, respectively.

Table I: Site Selectivity of 2-Substituted cAMP Analogues

compd no.	2-substituent	K_I(cAMP)/K_I(analogue) site A	K_I(cAMP)/K_I(analogue) site B	specificity, B/A
1	-H (cAMP)	1.0	1.0	1.0
2	-nC_4H_9	0.26	0.72	2.8
3	-iC_4H_9	0.071	0.13	1.8
4	-nC_8H_{17}	0.28	0.75	2.7
5	-C_6H_5	0.058	0.064	1.1
6	-Cl	0.24	3.0	13
7	-SnC_3H_7	0.054	0.15	2.8
8	-NH_2	0.043	0.35	8.1
9	-$N(CH_3)_2$	0.0069	0.028	4.1

substituents (**2**, **4**) have higher affinities than those with substituents branched at the point of attachment to the adenine ring (**3**, **5**). This is consistent with the "linear hydrophobic slot" binding site model discussed by Yagura et al. (1980b). In addition, the electronic character of the substituent influences binding: those analogues with substituents that donate electrons bind less well (**8**, **9**).

The pattern of binding to site B is similar to that obtained for site A. All of the analogues bind to site B with a higher affinity than that seen at site A, but the degree of reduction of affinity due to branching is more pronounced. Electron-donating substituents also inhibit binding of analogues to site

Table II: Site Selectivity of 8-Substituted cAMP Analogues

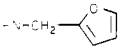
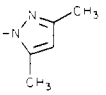
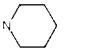
compd no.	8-substituent	K_I(cAMP)/K_I(analogue) site A	site B	specificity A/B	B/A
10	-Br	1.3	1.0	1.3	
11	$-SCH_2COO^-Na^+$	0.025	0.038		1.5
12	=Se	0.56	0.18	3.1	
13	$-SeCH_3$	0.85	1.2		1.4
14	$-SeC_2H_5$	1.1	1.4		1.3
15	$-SeCH_2C_6H_5$	0.91	0.45	2.0	
16	$-NHCH_3$	0.21	3.5		17
17	$-NH(CH_2)_2OH$	0.11	1.4		13
18		0.061	1.7		28
19	$-NHCH_2C_6H_5$	0.057	0.85	15	
20		0.044	0.0047	9.2	
21	$-N_3$	0.55	2.2		4.0

Table III: Site Selectivity of N^6-Substituted cAMP Analogues

compd no.	6-substituent	K_I(cAMP)/K_I(analogue) site A	site B	specificity, A/B
22	-NHOH	1.0	0.39	2.7
23	$-N(CH_3)_2$	1.2	0.38	3.1
24	$-N(CH_2CH_3)_2$	2.7	0.31	7.3
25		1.3	0.22	6.2
26	$-NHOCH_2C_6H_5$	0.41	0.087	3.9
27	$-NHnC_8H_{17}$	1.0	0.063	16
28	$-NHCH_2C_6H_5$	0.79	0.21	3.9
29	-NH(estratrienyl)	0.28	0.035	8.0
30	$-NHCH(CH_3)CH_2C_6H_5$	2.3	0.056	41
31	$-NHCH(CH_3)CH_2CH_2C_6H_5$	2.9	0.037	78
32	$-NHC(O)nC_3H_7$	3.6	0.093	38
33	$-NHC(O)NHnC_3H_7$	0.77	0.21	3.8
34	$-NHC(O)NHCH(CH_3)_2$	0.53	0.072	7.3
35	$-NHC(O)NHC(CH_3)_3$	0.48	0.068	7.1
36	$-NHC(O)NHCH_2CHCH_2$	0.62	0.092	6.7
37	$-NHC(O)NHCH_2CH_2CH(CH_3)_2$	0.98	0.20	5.0
38	$-NHC(O)NHnC_8H_{17}$	0.59	0.094	6.2
39	$-NHC(O)NHC_6H_5$	2.5	0.21	12
40	$-NHC(O)NHC_6H_4oCl$	1.8	0.16	11

B, whereas electron withdrawal (**6**) enhances binding. Since electron withdrawal enhances binding to only site B, analogues with these substituents exhibit a considerable site B preference.

(*2*) *8-Substituted Analogues.* Generally, the binding to either site was little affected by introducing -Br or substituents with Se as the first atom in the 8-position of cAMP (Table II). Exceptions to this were cAMP carrying a charged substituent (**11**), which had a low affinity for both sites A and B, and 8-Se-cAMP (**12**), which had a lowered affinity for site B. A striking result was that the introduction of an electron-donating $-NH_2$ or secondary amine in the 8-position lowered the affinity for site A, whereas the affinity for site B was increased or unaltered. These combined effects resulted in a large site B preference for 8-amino analogues. 8-Azido-cAMP (**21**) also showed a clear site B preference.[4]

[4] We have recently studied the equilibrium binding of 8-azido-[^{3}H]-cAMP to sites A and B of RI. The apparent K_D for binding to site B was 0.6 nM, and site A was half-maximally saturated at 4 nM 8-azido-[^{3}H]cAMP. The ratio K_D([^{3}H]cAMP)/K_D(8-azido-[^{3}H]cAMP) is thus close to the ratio K_I([^{3}H]cAMP)/K_I(8-azido-[^{3}H]cAMP) given in Table II.

Table IV: Site Selectivity of 1- and 6-Substituted Cyclic Nucleotide Analogues

compd	substituent(s)	K_I(cAMP)/K_I(analogue) site A	site B	specificity, A/B
41	6-H	0.73	0.34	2.1
42	6=O	0.10	0.027	3.9
43	$6-OnC_8H_{17}$	0.16	0.062	2.6
44	6=S	0.14	0.22	0.62
45	$6-SCH_2C_6H_5$	0.39	0.12	3.2
46	$1-CH_3$, $8-SCH_2C_6H_5$	0.37	0.082	4.5
47	$1-CH_3$, 6=O	0.85	0.30	2.8
48	1→O	0.52	0.13	4.0
49	1→O, 8-Br	0.32	0.099	3.2
50	1→O, 6-OH	0.038	0.020	1.9

Table V: Site Selectivity of 1,N^6-Etheno Cyclic Nucleotide Analogues

compd no.	substituent(s)	K_I(cAMP)/K_I(analogue) site A	site B	specificity A/B	B/A
51	1,N^6-etheno	0.32	0.11	2.9	
52	1,N^6-etheno, 2-aza	0.35	0.18	2.0	
53	1,N^6-etheno, 2-nC_4H_9	0.13	0.36		2.8
54	1,N^6-etheno, 2-C_6H_5	0.0076	0.070		9.2
55	β-C_6H_5, 1,N^6-etheno	0.42	0.17	2.5	
56	β-C_6H_5, 1,N^2-etheno (cGMP)	0.014	0.022		1.6

(*3*) *6-Substituted Analogues.* Site A accommodated all substituents on the 6-amino group (Table III). Very large groups, such as N^6-estratrienyl (**29**), and groups with branching distal to the amino group (**34**, **35**) showed a slight reduction in binding. Compounds with branching close to the 6-amino group, as well as N^6,N^6-dialkyl analogues, showed enhanced binding to this site, suggesting a hydrophobic interaction of the substituent with the enzyme.

In contrast, binding to site B is reduced by the addition of any substituent on the 6-amino group—from 3-fold for small substituents to almost 30-fold as the size of the substituent increases. In general, larger and branched groups show the lowest level of binding. The substituents that give the greatest inhibition of binding to site B are those that enhance binding to site A, producing analogues with a large degree of site A specificity.

Modifications of the 6-amino group that would prevent proton donation to a hydrogen bond (**23**–**25**) do not show any significant effect on binding affinity compared with monosubstituted compounds of similar sizes (**22**, **28**). Loss of a hydrogen bond contributing 10 kJ/mol should reduce binding affinity 35-fold.

Binding of Analogues with Alterations in the Adenine Ring. Replacement of the 6-amino group with oxygen (cIMP, **42**) or sulfur (**44**) causes a large reduction in binding to both sites (Table IV). The affinities of the resulting analogues are lower than that of 9-β-D-ribofuranosylpurine cyclic 3′,5′-phosphate (**41**), in which the 6-amino group is replaced by hydrogen, indicating that oxygen and sulfur in this position have an inhibitory effect on binding. This effect is partially alleviated by alkylation of the oxygen (**43**) or sulfur (**45**), which fixes the molecules in the enol tautomer. The affinity of **43** for site B is the same as that of **27**, which has a nitrogen in the 6-position. Furthermore, methylation of N^1 of cIMP (**47**), which stabilizes the keto tautomer, also increases the binding affinity

Table VI: Site Selectivity of Adenine Ring-Modified Cyclic Nucleotide Analogues

compd no.	compd	K_I(cAMP)/K_I(analogue) site A	site B	specificity A/B	B/A
57	2-aza-cAMP	0.81	0.27	3.0	
58	3-deaza-cAMP	0.031	0.19		6.1
59	7-deaza-cAMP	1.3	0.88	1.5	
60	8-aza-cAMP	0.69	1.0		1.4
61	1-β-D-ribofuranosyl-benzimidazole cyclic 3′,5′-phosphate	0.058	0.38		6.6
62	8-amino-3-(β-D-ribofuranosyl)-*s*-triazolo[4,3-*a*]pyrazine	0.012	0.036		3.0

of molecules with a 6-oxygen. Neither N^1 nor O^6 alkylation of cIMP changes the pK_a of the cyclic IMP molecule (Wolfenden, 1969). In contrast, alkylation of N^1 of cAMP raises its pK_a to 8.15 (Wolfenden, 1969), which may explain the inhibitory effect of this modification (**46**).

Oxidation of N^1 of cAMP (**48**) reduces binding to both sites, particularly site B. The N^1-oxide and 6-keto seem to be independent modifications since their effects on affinity to either site are additive.

The 1,N^6-etheno modification (**51**) reduces binding to both sites (Table V); as with other modifications to this portion of the molecule, site B is more sensitive. Adding a phenyl group to the etheno bridge (**55**) has no further effect. Addition of a 1,N^2-etheno bridge to cGMP (**56**) actually increases the binding of this compound to both sites compared with cGMP (**68**).

The affinities of analogues with modifications of the purine ring system are shown in Table VI. Substitution of carbon for N^3 causes a substantial drop in affinity to site A. [This modification also causes the pK of **58** to rise to 7.0 from 3.8 for cAMP (Miller et al., 1978).] A similar loss in affinity is seen with compound **61**, which lacks N^1, N^3, and the 6-amino group. Site B also discriminates against analogues with carbon at the 3-position but to a lesser extent than does site A. In addition, 2-aza-cAMP (**57**) shows reduced affinity for site B. Both 2-aza and 3-deaza have a reduced electron density at the 3-position of the adenine ring (Miller et al., 1978).

Binding of Disubstituted Cyclic AMP Analogues. Since a goal of this research is the design of site A and site B specific cAMP analogues, several analogues with two separate modifications were tested to determine whether the effects would be additive. Table VII shows the affinities of these analogues to both sites, along with the affinities calculated by multiplying the K_I(cAMP)/K_I(analogue) ratios of the monosubstituted parent compounds. The most striking result is the disparity between expected and actual affinity of those molecules carrying the 2-*n*-butyl substituent as one of their modifications (compounds **63**, **64**, and **66**). The extent of this disparity is sufficient to reverse the specificity of the molecules. The lack of predictability in activation constants of compounds carrying this group has been previously reported (Yagura & Miller, 1980). The other compounds examined show an acceptable correspondence between the predicted and measured affinities.

Discussion

The data presented here provide the first quantitative analysis of the binding of cAMP to both binding sites on the regulatory subunit of cAKI. These data allow us to conclude that the syn conformation of cAMP is bound to both sites and that neither site derives a significant amount of binding energy from hydrogen bonds to the adenine ring of cyclic AMP. The data also provide some insights into the nature of the binding interactions between the adenine ring and each site, as well as a description of the enzyme surface in regions adjacent to the adenine binding sites.

The cAMP molecule in solution exists as a mixture of syn and anti conformations, due to rotation around the glycosidic bond (Pullman & Saran, 1976; Fazakerly et al., 1977; Hayashi et al., 1979). Bulky substituents in the 8-position of the adenine ring hinder this rotation, producing molecules preferentially in the syn conformation (Ikehara et al., 1972). Since such modifications do not decrease the binding of cyclic AMP analogues to either site, the analogues—and hence cAMP—are probably bound in the syn conformation to both sites. Similar experiments have been performed by using cAKII (Rannels & Corbin, 1980), and this enzyme appears different from cAKI in that bulky substituents in the 8-position inhibit binding to site A. This suggests that either the anti conformation of cAMP binds to site A of cAKII or 8-substituents inhibit binding to this site for other reasons.

Potential sources of binding energy of the adenine ring to the regulatory subunit include hydrogen bonds. Evidence has already been presented that hydrogen bonding contributes to the binding of the ribose cyclic phosphate moiety to one of the sites (Jastorff et al., 1979; de Wit et al., 1982). These hydrogen bonds were found to provide 15–19 kJ/mol binding energy. On the adenine ring, nitrogen atoms at N^1, N^3, N^7, and the 6-amino group are all capable of participating in such interactions. If a hydrogen bond to the adenine ring were to contribute only 10 kJ/mol binding energy, analogues that are incapable of forming that bond should show a 35-fold reduction in binding. Analogues that cannot form hydrogen bonds at N^1, N^7, and the 6-amino group do not show significantly reduced affinities at either site. These results indicate that no significant amount of binding energy is obtained from hydrogen bonds to these positions. Analogues lacking a 3-nitrogen bind well to site B but show a 20–30-fold reduction in binding to site A, suggesting that the lone pair electrons on the 3-nitrogen may participate in a hydrogen bond to the enzyme. However, electron density calculations predict that 2-aza-cAMP should have a low electron density in the lone pair at the 3-position and thus would also have reduced hy-

Table VII: Site Selectivity of Various Disubstituted Cyclic Nucleotide Analogues

compd no.	substituent(s)	K_I(cAMP)/K_I(analogue) site A observed	site A expected	site B observed	site B expected	specificity A/B	B/A
63	2-nC_4H_9, 8-Br	2.2	0.34	0.23	0.72	9.8	
64	2-nC_4H_9, 8-$SCH_2C_6H_5$	0.49	0.22	0.14	0.72	3.5	
65	6=O, 8-NH_2	0.012	0.017	0.21	0.10		18
66	2-nC_4H_9, 6=O	0.028	0.026	0.11	0.019		4.0
67	2-NH_2, 6-$N(C_2H_5)_2$	0.099	0.12	0.17	0.11		1.7
68	2-NH_2, 6=O (cGMP)	0.0046	0.0043	0.014	0.0095		3.0

drogen bonding capability (Miller et al., 1978). Since 2-aza-cAMP binds to site A as well as cAMP, it is unlikely that the binding of cAMP to site A involves a hydrogen bond to the 3-nitrogen. Loss of the 3-nitrogen affects the electron distribution at other protions of the purine ring, including the pK of the heterocyclic ring system (Miller et al., 1978), and these changes may also affect binding to the enzyme.

Since hydrogen bonding does not contribute to the binding of the adenine ring to the enzyme, the binding energy must arise from either hydrophobic interactions or from π electron interactions between the protein and the adenine ring (otherwise known as stacking interactions or induced dipole interactions). The results presented here suggest that binding energy is probably derived from a combination of these effects. The contribution of π electron interactions was previously presented by LaPorte et al. (1980), who proposed that an active site tryptophan residue interacted with the adenine ring of cAMP at one of the binding sites. The same study showed that both sites quenched the fluorescence of 1,N^6-etheno-cAMP and hence have either aromatic or sulfur-containing amino acid side chains capable of interacting with the bound fluorophore. In addition to these amino acid side chains, it is possible for the aromatic adenine ring to interact with the amide linkages of the protein chain (Robinson & Jencks, 1965). The results presented in this paper show that a number of modifications that affect the resonance electron distribution of the purine ring have large effects on the binding affinity of the molecule to both sites. Modifications that would increase the resonance electron distribution in the C^6–N^1–C^2 portion of the molecule, such as 6-oxo (cIMP) or 2-dimethylamino, severely reduce affinity, whereas electron withdrawal in this region increases binding affinity. Since a probable function of the adenine ring binding sites is to discriminate between adenine and guanine, one might anticipate that the binding sites would be sensitive to differences in electronic structure in this portion of the molecule. In addition, electron donation at the 8-position causes significant changes in affinity to both sites. The strong dependence of binding upon electron distribution indicates that an electronic interaction between cAMP and cAKI allows the protein to discriminate between structurally similar nucleosides.

de Wit et al. (1982) have argued that because binding affinity does not correlate well with the calculated polarizability of the nucleotide, stacking interactions do not make a significant contribution to the binding. This conclusion may be unjustified. The polarizability of a molecule is calculated along a single axis, but the actual polarizability is different along different axes (Jencks, 1969). Thus, the energy of the stacking interaction will depend on the geometry of the interacting molecules. The calculated polarizability may have little value in predicting protein–substrate interactions involving constrained orientations and partial overlap. These effects have been demonstrated in solution by Nakano & Igarashi (1970), who measured the association tendencies of 10 heteroaromatic compounds with adenine and with guanosine and found a different order of preference for each. Thus, structural features that affect the geometry of the interacting molecules in solution can override polarizability in determining degree of association. Mutai et al. (1975) found that the degree of internal association between coupled adenine and indole depends strongly on the attachment point ot the adenine, which affects the orientation of the two molecules in the complex.

The contribution of hydrophobic interactions to the binding is difficult to assess. The solubility of the bases used probably does not accurately reflect hydrophilicity. However, modifications that placed a charge on the adenine ring would certainly disrupt a hydrophobic bond. On the basis of studies by Wolfenden (1969), 1-methyl-8-benzylthio-cAMP (**46**) should have a pK of about 8.3, resulting in 95% of the molecules bearing a positive charge under the assay conditions. This compound binds well to site A, indicating that the presence of a positive charge does not disrupt binding. Binding to site B is reduced by a factor almost equal to the degree of protonation. Either the presence of a charge group disrupts binding or the changes in electronic structure resulting from N^1-methylation affect both the binding and pK. The enzyme surface adjacent to the adenine ring has nonpolar regions, as demonstrated by apparent hydrophobic interactions with analogues bearing nonpolar substituents on the 6-amino group, as well as some 2-substituents. The lack of binding of analogues bearing a charge in the 8-position suggests that this region also is nonpolar.

One of the objectives of this work is to design cyclic AMP analogues with a high degree of site specificity. Analysis of the data indicates that several types of modifications will produce the specificity desired. The most significant compounds yielding site A specificity are those in which a bulky hydrophobic substituent is attached to the 6-amino group. Other modifications, such as the N^1-oxide and 2-aza, produce only moderate degrees of site A specificity. However, these changes may be significant enough to warrant the synthesis of the doubly modified compounds because if the effects are additive, a compound with a specificity of >300 could be produced.

Site B specificity is conferred by modifications that withdraw electrons at C^2 (Cl, CF_3) and that donate electrons at C^8 (8-NHR). Thus, the combination of these modifications may produce molecules that are 300-fold more specific for site B than for site A.

Additivity is an important consideration in these arguments, and the results obtained from doubly modified compounds are mixed in this regard. The results suggest that those modifications producing mainly electronic effects are most likely to be additive, whereas substituents having steric or combined steric/hydrophobic effects are least reliable, especially in the 2-position. Clearly, more compounds need to be tested before firm conclusions can be drawn.

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Registry No. **1**, 60-92-4; **2**, 52940-93-9; **3**, 52940-94-0; **4**, 56505-93-2; **5**, 51554-25-7; **6**, 39023-65-9; **7**, 56505-98-7; **8**, 30630-06-9; **9**, 51554-34-8; **10**, 23583-48-4; **11**, 72549-37-2; **12**, 52019-21-3; **13**, 52019-22-4; **14**, 52109-39-4; **15**, 52109-40-7; **16**, 33823-18-6; **17**, 33823-17-5; **18**, 33818-12-1; **19**, 31319-90-1; **20**, 84433-42-1; **21**, 31966-52-6; **22**, 50884-70-3; **23**, 28048-42-2; **24**, 38183-17-4; **25**, 31319-84-3; **26**, 50939-37-2; **27**, 84433-43-2; **28**, 32115-08-5; **29**, 84433-44-3; **30**, 52301-28-7; **31**, 34152-83-5; **32**, 13117-60-7; **33**, 50802-08-9; **34**, 84433-45-4; **35**, 84433-46-5; **36**, 84433-47-6; **37**, 84433-48-7; **38**, 53928-14-6; **39**, 50655-14-6; **40**, 84433-49-8; **41**, 53303-84-7; **42**, 3545-76-4; **43**, 52483-93-9; **44**, 20096-88-2; **45**, 38183-22-1; **46**, 58418-43-2; **47**, 78033-39-3; **48**, 39023-61-5; **49**, 57872-68-1; **50**, 84433-50-1; **51**, 38806-37-0; **52**, 50663-90-6; **53**, 75925-29-0; **54**, 75925-30-3; **55**, 43157-45-5; **56**, 78080-27-0; **57**, 52989-05-6; **58**, 57024-61-0; **59**, 16719-36-1; **60**, 67190-31-2; **61**, 76461-19-3; **62**, 68797-11-5; **63**, 58418-36-3; **64**, 58418-40-9; **65**, 51239-35-1; **66**, 56506-03-7; **67**, 84433-51-2; **68**, 7665-99-8; N^6-monobutyryl-cAMP, 13117-60-7; 8-NH_2-cAMP, 30685-40-6; cIMP, 3545-76-4; 6-chloropurine 9-β-D-ribofuranoside cyclic 3′,5′-monophosphate, 31319-73-0; octylamine, 111-86-4; 3-amino-1,3,5-estratriene, 6702-04-1; 7-deazaadenosine, 69-33-0; 3-deazaadenosine, 6736-58-9; 7-deazaadenosine 5′-(trichloromethyl

phosphonate), 84433-52-3; 3-deazaadenosine 5′-(trichloromethyl phosphonate), 84433-53-4; protein kinase, 9026-43-1.

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