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Binding of Adenosine 3′,5′-Monophosphate Dependent Protein Kinase Regulatory Subunit to Immobilized Cyclic Nucleotide Derivatives†

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ABSTRACT: Several cyclic nucleotide derivatives with aminoalkyl side chains attached to the purine ring were synthesized and their interactions with adenosine 3′,5′-monophosphate (cAMP) dependent protein kinase were studied before and after immobilization to CNBr-activated Sepharose 4B. The soluble N^6-substituted derivatives were as effective as cAMP itself in activating protein kinase and were more effective than 8-substituted cAMP derivatives, whereas the 2-substituted cAMP derivatives and the cGMP derivatives were the least effective. All of the synthetic derivatives tested were poor substrates for beef heart phosphodiesterase being hydrolyzed at rates less than 2% for that of cAMP itself. Utilizing methodology developed to evaluate the affinity of protein kinase for immobilized cyclic nucleotides it was found that all of the immobilized cyclic nucleotides interacted with protein kinase in a biospecific manner as judged by the following criteria: (1) the immobilized cyclic nucleotides competed with cAMP for the binding sites on protein kinase; (2) the analogous spacer-arm did not compete; and (3) the effects of enzyme concentration, MgATP, and cleavage of the cyclic phosphate ring on the interactions of protein kinase with the immobilized cyclic nucleotides were the same as previously shown for free cAMP. In addition, the immobilized ligands were bound with the same order of effectiveness as the analogous soluble ligand. The observed K_a for the activation of 0.005 μM protein kinase by N^6-$H_2N(CH_2)_2$-cAMP was increased from 0.23 to 3 μM by the process of immobilization. This increase was unaffected by the coupling density and spacer-arm length. The observed K_b for 0.10 μM protein kinase binding to immobilized N^6-$H_2N(CH_2)_2$-cAMP was increased as the molecular sieving exclusion limit of the matrix used was decreased indicating that at least part of this decrease in apparent affinity upon immobilization is due to exclusion of the enzyme from a portion of the matrix and therefore from a fraction of the immobilized ligand molecules.

The enzyme, cAMP[1]-dependent protein kinase (EC 2.7.1.37), has been shown to exist as a tetramer having two different types of subunits, a catalytic subunit (C) which catalyzes the phosphorylation reaction, and a cAMP binding regulatory subunit (R) which inhibits the activity of the catalytic subunit in the absence of cAMP (Erlichman et al., 1973; Beavo et al., 1975). The mechanism for the activation of the enzyme involves its dissociation by cAMP as shown below:

$$R_2C_2 \text{ (inactive)} + 2\text{cAMP} \rightleftharpoons 2C \text{ (active)} + R_2(\text{cAMP})_2 \quad (1)$$

Several studies on the purification of the regulatory subunit by affinity chromatography using a variety of immobilized cAMP derivatives have been carried out (Wilchek et al., 1971; Cuatrecasas, 1972; Tesser et al., 1974; Jergil et al., 1974; Ramseyer et al., 1974; Dills et al., 1975a,b; Rieke et al., 1975). In these studies it was observed that the protein kinase was dissociated by the immobilized cyclic nucleotides but in only a few cases were successful elutions of the bound regulatory subunit described (Ramseyer et al., 1974; Dills et al., 1975a,b; Rieke et al., 1975). In order to develop better methodology for the purification of this protein, as well as to gain a better understanding of certain quantitative aspects of affinity chromatography in general, the binding of protein kinase to a variety of immobilized cyclic nucleotides was studied.

Experimental Section

Chemicals. Polyox was obtained from Union Carbide; 6-Cl-cPRMP, 8-Br-cAMP, and 8-Br-cGMP from Boehringer-Mannheim, [8-^{3}H]cAMP from Schwarz/Mann, 1,9-diaminononane from Chemical Procurement, Inc., and the other alkyl diamines and 2-aminoethanethiol hydrochloride from Aldrich. 2-Cl-cAMP was a gift from Dr. M. Helen Maguire of the University of Kansas Medical Center. CNBr-activated Sepharose 4B was obtained from Pharmacia and was swollen in 5 volumes of 1 mM HCl for 15 min before use. Sephadex G-10 and Sephadex G-150 from Pharmacia and Bio-Gel A-150 from Bio-Rad were activated with CNBr by the method

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[1] The abbreviations used are: cAMP, adenosine 3′,5′-monophosphate; cGMP, guanosine 3′,5′-monophosphate; cIMP, inosine 3′,5′-monophosphate; cPRMP, purine riboside 3′,5′-monophosphate; Mes, 2-(*N*-morpholino)ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N′*-tetracetic acid; DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)aminomethane.

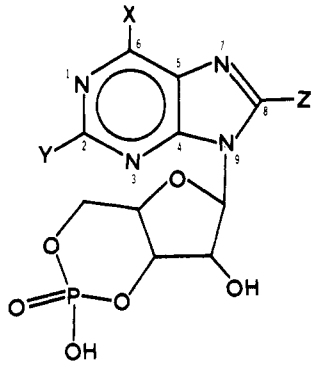

FIGURE 1: Structures of cyclic nucleotide derivatives discussed in this paper. The compounds with their corresponding substituents (X, Y, and Z, respectively) are as follows: cAMP, $-NH_2$, -H, -H; cIMP, -OH, -H, -H; cGMP, -OH, $-NH_2$, -H; 2-Cl-cAMP, $-NH_2$, -Cl, -H; 6-Cl-cPRMP, -Cl, -H, -H; 8-Br-cAMP, $-NH_2$, -H, -Br; 8-Br-cGMP, -OH, $-NH_2$, -Br; N^6-$H_2N(CH_2)_2$-cAMP, $-NH(CH_2)_2NH_2$, -H, -H; N^6-$H_2N(CH_2)_4$-cAMP, $-NH(CH_2)_4NH_2$, -H, -H; N^6-$H_2N(CH_2)_6$-cAMP, $-NH(CH_2)_6NH_2$, -H, -H; N^6-$H_2N(CH_2)_9$-cAMP, $-NH(CH_2)_9NH_2$, -H, -H; N^6-$H_2N(CH_2)_{12}$-cAMP, $-NH(CH_2)_{12}NH_2$, -H, -H; 8-$H_2N(CH_2)_2HN$-cAMP, $-NH_2$, -H, $-NH(CH_2)_2NH_2$; 2-H_2N-$(CH_2)_2HN$-cAMP, $-NH_2$, $-NH(CH_2)_2NH_2$, -H; 8-$H_2N(CH_2)_2HN$-cGMP, -OH, $-NH_2$, $-NH(CH_2)_2NH_2$; 8-$H_2N(CH_2)_2S$-cGMP, -OH, $-NH_2$, $-S(CH_2)_2NH_2$.

of March et al. (1974). Immobilized N^6-$H_2N(CH_2)_6$-AMP was prepared by the method of Guilford et al. (1972). Casein was prepared by the method of Reimann et al. (1971). [γ-^{32}P]ATP was prepared by the method of Glynn and Chappell (1964) as modified by Walsh et al. (1971).

Enzymes. Beef heart phosphodiesterase was obtained from Boehringer-Mannheim, *E. coli* and beef liver alkaline phosphatases from Sigma, and immobilized *E. coli* alkaline phosphatase from Worthington. Rabbit muscle protein kinase was purified through the first DEAE column step (Beavo et al., 1974b). Peak I from this preparation, which contains the majority of the activity in rabbit skeletal muscle, was used for these studies and is referred to throughout the paper as protein kinase.

Chemical Methods. Thin-layer chromatography was performed on Brinkman cellulose F plates using either 2-propanol–concentrated NH_4OH–H_2O, 7:1:2 (solvent A), or 1-butanol–acetic acid–H_2O, 5:2:3 (solvent B). Nucleotides were visualized with ultraviolet light and primary amines with ninhydrin. Products were concentrated with a Buchler rotary evaporator under reduced pressure at a water bath temperature of 40 °C. Ultraviolet spectra were determined on a Cary Model 14 recording spectrophotometer. Spectra of immobilized derivatives were obtained from 0.5% Polyox gel suspensions (Larsson and Mosbach, 1971). Ribose was determined by the orcinol method (Ashwell, 1957) and primary amines by the reaction with trinitrobenzenesulfonic acid (Satake et al., 1960).

Phosphate was determined by the Malachite Green method (Itaya and Ui, 1966) as modified by Stull.[2] Samples containing between 0.5 and 10 nmol of phosphate in 100 μl of 1 N HCl were mixed with 200 μl of 0.2% Malachite Green–2.7% ammonium molybdate in 1 N HCl. Five microliters of 1.5% aqueous Tween 20 were added, the solution was mixed, and the absorbance was read at 660 nm. When samples were ashed, the procedure of Ames (1966) was used.

Enzyme Assays. Protein kinase assays were performed by the filter paper method of Reimann et al. (1971) using casein as substrate. The reaction was started by the addition of [γ-^{32}P]ATP after a 5-min preincubation of the enzyme with the appropriate concentration of cAMP or derivative. The concentrations of cyclic nucleotide derivative needed to give half-maximal activation of protein kinase under the described assay conditions (referred to as "K_a")[3] were determined by visual inspection of dose–response curves obtained. This method was used because of the nonlinearity of plots in other methods for determining this parameter (see Swillens et al., 1974; Ogez and Segal, 1976). The same procedure was adapted to determine the K_a for immobilized N^6-$H_2N(CH_2)_2$-cAMP; in this case the reaction mixture was shaken gently during the incubation period.

Phosphodiesterase assays were performed by a modification of the method of Butcher and Sutherland (1962). Incubation mixtures contained 50 mM Tris-HCl (pH 7.2), 2.5 mM $MgCl_2$, 25 μg/ml of *E. coli* alkaline phosphatase, 1 mM of either free or immobilized cyclic nucleotide analogue, and appropriate amounts of phosphodiesterase. At designated times, 25-μl aliquots were withdrawn and added to 25 μl of cold 20% trichloracetic acid. After mixing, the samples were centrifuged in a clinical centrifuge and 25-μl aliquots removed and added to 75 μl of 1.33 M HCl. Phosphate assays were then performed as described above.

Reaction mixtures for the alkaline phosphatase assays contained 25 mM Tris-HCl (pH 7.5), 2.5 mM magnesium acetate, 0.4 μmol/ml of either soluble or immobilized N^6-$H_2N(CH_2)_6$-AMP, and appropriate amounts of alkaline phosphatase. At designated times, 25-μl aliquots were removed and added to 25 μl of 20% trichloracetic acid. After mixing and centrifugation, 25 μl of the supernatant was added to 75 μl of 1.33 N HCl and assayed for phosphate by the method described earlier.

Results

Synthesis of Cyclic Nucleotide Derivatives. The various N^6-ω-aminoalkyl-cAMP derivatives (Figure 1) were synthesized by a modification of the method of Guilford et al. (1972) used for making N^6-$H_2N(CH_2)_6$-AMP. For a typical synthesis, 25 mg (0.067 mmol) of 6-Cl-cPRMP was added to a solution of 4.35 mmol of the appropriate 1,ω-diaminoalkane in 5 ml of water.[4] After refluxing 2 h, the reaction mixture was cooled and applied to a Dowex-1X2 acetate column (1.5 × 20 cm) prewashed with distilled water, which was then washed sequentially with 25 ml of distilled water, 25 ml of 0.01 M NH_4Cl, and 25 ml of distilled water. The nucleotide was eluted with a linear gradient of 0–0.5 N acetic acid (4 column volumes). A single peak containing material that had a λ_{max} at 265 nm was obtained; this was pooled and concentrated. The desired products were obtained in yields of 70–90%.

The following cyclic nucleotide analogues, 8-$H_2N(CH_2)$-HN-cAMP, 2-$H_2N(CH_2)_2HN$-cAMP, and 8-$H_2N(CH_2)_2$-HN-cGMP (Figure 1) were synthesized by the above procedure using 8-Br-cAMP, 2-Cl-cAMP, or 8-Br-cGMP as appropriate and the 1,ω-diaminoalkane of the desired chain length. For these derivatives the reaction mixture was refluxed

[2] Stull, J. T., personal communication.

[3] As discussed by Soderling and Park (1974), Swillens et al. (1974), and Ogez and Segal (1976), any term expressing a total concentration of ligand that produces a half-maximal effect on a dissociating system like protein kinase will be a function of enzyme concentration. The terms "K_a" and "K_b" are used in this manuscript for the concentrations of cyclic nucleotides giving half-maximal activation of protein kinase and binding to protein kinase, respectively; in all cases the enzyme concentrations are specified.

[4] The dihydrochloride form of 1,12-diaminodecane (Guilford et al., 1972) was used for the synthesis of N^6-$H_2N(CH_2)_{12}$-cAMP.

TABLE I: Spectral and Chromatographic Properties of Soluble Cyclic Nucleotide Derivatives.

Cyclic Nucleotide	λ_{max} (nm) ($\epsilon \times 10^{-3}$)[a]		Rel Mobility [R_f(deriv)/R_f(cAMP)][b]	
	pH 1	pH 11	Solvent A	Solvent B
N^6-$H_2N(CH_2)_2$-cAMP	262 (16.2)	267 (16.3)	1.04	0.98
N^6-$H_2N(CH_2)_4$-cAMP	263 (16.3)	267 (16.3)	1.10	1.13
N^6-$H_2N(CH_2)_6$-cAMP	262 (16.3)	267 (16.2)	1.19	1.76
N^6-$H_2N(CH_2)_9$-cAMP	263 (16.3)	269 (16.2)	1.59	2.21
N^6-$H_2N(CH_2)_{12}$-cAMP	263 (16.4)	268 (16.2)	1.62	2.57
8-$H_2N(CH_2)_2HN$-cAMP	275 (16.6)	276 (18.5)	0.80	0.76
2-$H_2N(CH_2)_2HN$-cAMP	253 (13.5) 295 (10.2)	258 (12.6) 285 (8.8)	0.61	0.92
8-$H_2N(CH_2)_2HN$-cGMP	256 (17.0) 290 (9.5)	262 (15.3)	0.66	0.57
8-$H_2N(CH_2)_2S$-cGMP	278 (13.1)	277 (14.3)	0.77	0.57

[a] Concentrations of nucleotide determined by phosphate assays. [b] Performed on Brinkman cellulose F plates; see Experimental Section.

TABLE II: Protein Kinase and Phosphodiesterase Kinetic Constants for the Soluble Cyclic Nucleotide Derivatives.[a]

Cyclic Nucleotide	Protein Kinase Reaction[b]		Phosphodiesterase Reaction[c]
	K_a (μM)	Rel V_{max}	Rel Rate (%)
cAMP	0.34 ± 0.15 (26)	1.00	100
cGMP	24 ± 16 (9)	1.01 ± 0.14	52 ± 8 (3)
cIMP	4.0 ± 1.9 (5)	0.91 ± 0.18	29 ± 14 (3)
N^6-$H_2N(CH_2)_2$-cAMP	0.23 ± 0.10 (5)	1.01 ± 0.14	0.20 ± 0.08 (22)
N^6-$H_2N(CH_2)_4$-cAMP	0.47 ± 0.26 (6)	1.02 ± 0.07	0.06 ± 0.02 (10)
N^6-$H_2N(CH_2)_6$-cAMP	0.17 ± 0.08 (6)	0.98 ± 0.14	0.05 ± 0.02 (8)
N^6-$H_2N(CH_2)_9$-cAMP	0.19 ± 0.12 (6)	1.02 ± 0.13	0.40 ± 0.04 (4)
N^6-$H_2N(CH_2)_{12}$-cAMP	0.29 ± 0.09 (6)	0.97 ± 0.11	0.05 ± 0.03 (5)
8-$H_2N(CH_2)_2HN$-cAMP	1.01 ± 0.27 (4)	1.14 ± 0.18	0.13 ± 0.05 (9)
2-$H_2N(CH_2)_2HN$-cAMP	47 ± 24 (6)	0.93 ± 0.13	1.75 ± 0.39 (4)
8-$H_2N(CH_2)_2HN$-cGMP	144 ± 27 (6)	1.06 ± 0.13	0.01 ± 0.01 (3)
8-$H_2N(CH_2)_2S$-cGMP	36.3 ± 16.5 (4)	1.04 ± 0.15	0.04 ± 0.03 (3)

[a] Average values are shown together with their standard deviations. The number of determinations are in parentheses. [b] Enzyme concentration, 0.005 μM. [c] Substrate concentration, 1 mM.

for 24 h to obtain a complete reaction. Yields in these preparations ranged from 50 to 90%.

8-$H_2N(CH_2)_2S$-cGMP (Figure 1) was prepared by a modification of the method of Tesser et al. (1972) for the preparation of the analogous cAMP derivative. The reaction mixtures contained 25 mg of 8-Br-cGMP, 100 mg of 2-aminoethanethiol hydrochloride, and 1.25 g of sodium methoxide in 5 ml of anhydrous methanol. The reaction mixture was refluxed for 6 h. Ten milliliters of distilled water was added and the methanol evaporated. The pH of the reaction was adjusted to 10.5 and the product isolated as described above. Yields ranged from 50 to 90%.

Characterization of Cyclic Nucleotide Derivatives. All derivatives were found to be free of contaminating nucleotides and ninhydrin positive material, as judged by thin-layer chromatography in solvents A and B; their relative mobilities are shown in Table I. The ultraviolet spectra (Table I) were similar to other 2-, 6-, and 8-substituted cyclic nucleotide derivatives that have been studied (Muneyama et al., 1971; Meyer et al., 1972; Miller et al., 1973; Jastorff and Freist, 1974). Each of these compounds contained ribose, phosphate, and primary amine in ratios of 1:1:1, consistent with the proposed structures of the derivatives.

Activation of cAMP-Dependent Protein Kinase by Cyclic Nucleotide Derivatives. The protein kinase activity assay (Reimann et al., 1971) was used to study abilities of the cyclic nucleotide derivatives to activate the enzyme. Table II shows the K_a values for various cyclic nucleotide derivatives determined at a holoenzyme concentration of 0.005 μM (based on cAMP binding capacity assuming the stoichiometry depicted in eq 1). It can be seen that N^6-substituted cAMP derivatives had K_a's that did not differ significantly from that for cAMP itself, while derivatives of cAMP substituted in the 2- and 8-positions had K_a's significantly higher than that for cAMP. 8-$H_2N(CH_2)_2S$-cGMP had a K_a similar to that for cGMP itself, while the 8-$H_2N(CH_2)_2HN$-cGMP value was significantly higher. The V_{max} values for the various derivatives did not differ significantly from that for cAMP. This result is what would be expected assuming that the derivatives activate protein kinase by the same mechanism shown in eq 1. These results are similar to those of other investigators in experiments with 2-, 6-, and 8-substituted cyclic nucleotide derivatives (for reviews, see Meyer and Miller, 1974; Simon et al., 1973).

Hydrolysis of Cyclic Nucleotide Derivatives by Beef Heart Phosphodiesterase. The susceptibility of the cyclic nucleotide derivative to hydrolysis by beef heart phosphodiesterase was evaluated (Table II). The data show that all of the derivatives tested were hydrolyzed at a rate less than 2% of that for cAMP. Studies in other laboratories have shown similar results with other 2-, 6-, and 8-substituted cyclic nucleotide derivatives (for

reviews, see Meyer and Miller, 1974; Simon et al., 1973).

Immobilization of Cyclic Nucleotide Derivatives. Immobilized cyclic nucleotides were prepared as previously described (Dills et al., 1975b). The washed gel (CNBr-activated Sepharose 4B unless otherwise indicated) was suspended in an equal volume of 100 mM sodium bicarbonate buffer, pH 9.5, in the presence of the cyclic nucleotide at concentrations ranging from 0.2 to 4.0 mM. After shaking for 2 h, the gel was filtered, washed with distilled water, and added to an equal volume of 1 M ethanolamine hydrochloride (pH 9.5). After shaking overnight the gel was filtered and washed exhaustively with distilled water. Coupling densities[5] were determined by phosphate analysis. Yields of immobilized derivative were essentially quantitative except for these prepared with matrices other than Sepharose 4B. To prevent hydrolytic cleavage of the immobilized nucleotide from the matrix (Tesser et al., 1974), the immobilized derivatives were stored in 10 mM citrate buffer at pH 5.0. Immobilized cyclic nucleotides were characterized by their ultraviolet spectra in 0.5% Polyox (Larsson and Mosbach, 1971); in each case λ_{max} values similar to those for the soluble derivatives were obtained.

Assay for the Binding of Immobilized Derivatives to Protein Kinase. The interaction of the protein kinase with the immobilized cyclic nucleotide derivatives was studied by measuring the amount of residual cAMP binding capacity of the enzyme preparation after treatment with these compounds.[6] For these experiments either 0.005 or 0.10 μM protein kinase holoenzyme (based on cAMP binding capacity assuming the stoichiometry shown in eq 1) was incubated at 23 °C in 33 mM Mes buffer (pH 6.5), 5 mM magnesium acetate, 0.3 mM EGTA, and varying concentrations of immobilized nucleotide. Aliquots of the suspension were removed, cooled to 0 °C, and added to 2 ml of cold 20 mM potassium phosphate buffer (pH 6.9), 4 mM magnesium acetate, and (unless indicated otherwise) 0.4 μM [8-^{3}H]cAMP (300–700 cpm/pmol). After mixing, the suspension was filtered and washed as described previously (Beavo et al., 1974b). Figure 2 shows the time course of the regulatory subunit binding to several concentrations of immobilized N^6-$H_2N(CH_2)_2$-cAMP at 23 °C as measured by this method. The rates of binding were dependent on immobilized nucleotide concentration. In all cases binding had reached plateau values by 30 min and was stable for up to 120 min. Assuming that all of the cAMP binding sites are available to free [8-^{3}H]-cAMP, it seems likely that both cAMP binding sites of the regulatory subunit dimer (see eq 1) are bound to the immobilized derivative. If this were not the case, the samples treated with high concentrations of immobilized nucleotide would have reached plateau values after only half of the cAMP binding capacity was bound. From curves obtained in experiments conducted with 30-min incubation times and relatively low protein kinase concentrations, it was possible to determine the concentration of immobilized cyclic nucleotide needed to remove one-half of the binding capacity from solution; this concentration was referred to as a "K_b" for the immobilized derivative.[3]

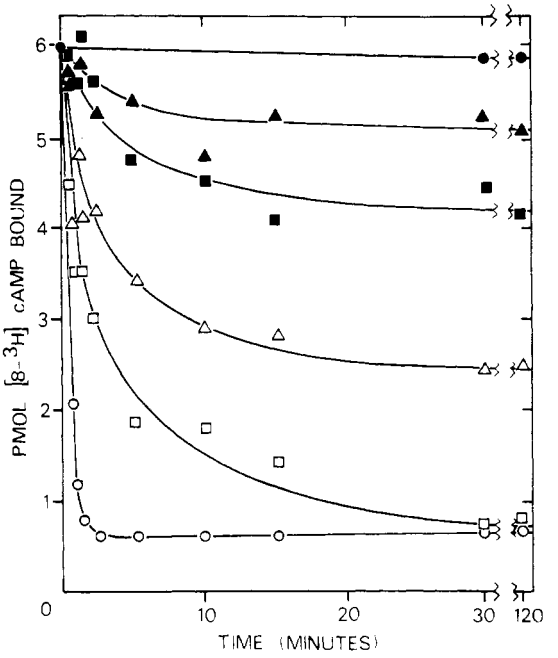

FIGURE 2: Time course of the binding of protein kinase regulatory subunit to immobilized N^6-$H_2N(CH_2)_2$-cAMP. Reaction mixture contained 33 mM Mes (pH 6.9), 5 mM magnesium acetate, 0.3 mM EGTA, 0.10 μM protein kinase holoenzyme, and 33 μM (○), 11 μM (□), 3.7 μM (△), 1.2 μM (■), 0.41 μM (▲), or no (●) immobilized N^6-$H_2N(CH_2)_2$-cAMP. Reactions were started by the addition of enzyme. After given times at 23 °C aliquots (30 μl) were withdrawn and assayed at 0 °C for the binding of [8-^{3}H]cAMP by the Millipore filter method as described in the text.

The validity of this method for measuring the binding of the immobilized cyclic nucleotide to the enzyme depends on the fact that no exchange of regulatory subunit occurs between the immobilized cyclic nucleotide and [^{3}H]-cAMP at the low temperature of the second incubation. As shown in Figure 3, there was no exchange of immobilized ligand with free cAMP for up to 2 h at 0 °C (open triangles and open squares). The data in Figure 3 also illustrate that protein kinase does not bind to immobilized spacer arms or an immobilized AMP derivative.

Effect of Enzyme Concentration on the Binding of Protein Kinase to Immobilized N^6-$H_2N(CH_2)_2$-cAMP. It has been shown that the concentration of ligand needed for half-maximal dissociation or activation of an enzyme as depicted in eq 1 will increase as the total enzyme concentration increases (Soderling and Park, 1974; Swillens et al., 1974; Ogez and Segal, 1976). Such an increase in the concentration of cAMP required for activation has been observed (Beavo et al., 1974b) and, when direct binding studies were carried out at higher enzyme concentrations, increased apparent binding constants have been observed (Hofmann et al., 1975). To substantiate the biospecificity of the binding of the regulatory subunit to the immobilized cyclic nucleotides, we studied this binding at two different enzyme concentrations. The left panel of Figure 4 shows that an increase of the enzyme concentration from 0.005 to 0.10 μM resulted in an increase in the observed K_b from 0.1 to 2 μM. This result supports the assumption that the immobilized nucleotides are interacting biospecifically with cAMP-dependent protein kinase by the same mechanism as shown in eq 1 for cAMP.

Effect of the Presence of MgATP on the Binding of Protein

[5] The coupling density of the gel is defined as micromoles of immobilized ligand per milliliters of packed gel.

[6] The amount of cAMP binding capacity removed from solution is assumed to be due to binding of the protein kinase regulatory subunit to the immobilized derivative by the same mechanism depicted in eq 1. Data supporting this assumption are presented in this paper and in other papers in which cAMP-dependent protein kinase regulatory subunit was separated from its catalytic subunit and purified using immobilized cyclic nucleotides (Ramseyer et al., 1974; Dills et al., 1975a,b). This removal of cAMP binding capacity from solution is described in this paper as "binding of regulatory subunit to immobilized cyclic nucleotides".

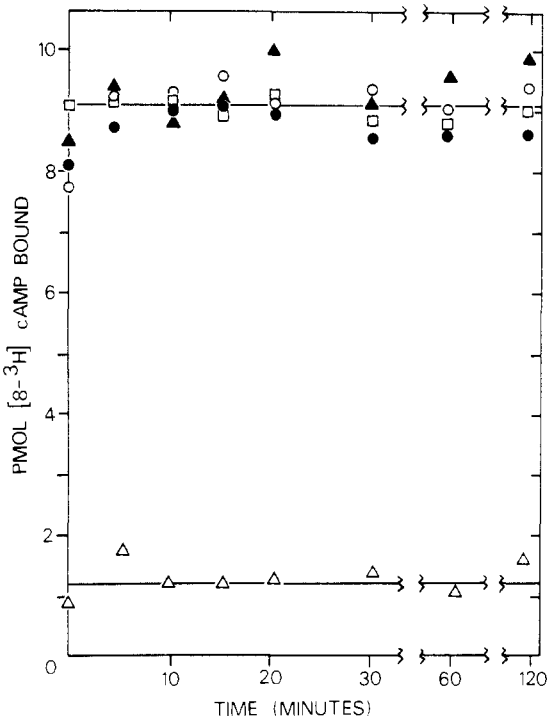

FIGURE 3: Competition between various immobilized ligands and [8-^{3}H]cAMP for cAMP-dependent protein kinase regulatory subunit. Preincubation mixtures at 23 °C contained (in 600 μl) 33 mM Mes (pH 6.9), 5 mM magnesium acetate, 0.3 mM EGTA, and 0.10 μM protein kinase holoenzyme in the presence of 0.04 mM (240 nmol) immobilized N^6-$H_2N(CH_2)_2$-cAMP (△), 0.4 mM immobilized N^6-$H_2N(CH_2)_6$-AMP (●), 0.4 mM immobilized 1,9-diaminomonane (▲), or no immobilized ligand (○,□). After 30 min at 23° 9.4 ml 20 mM phosphate buffer (pH 6.9) at 0° containing 4 mM Mg acetate and 0.4 μM [8-^{3}H]cAMP was added followed by mixing. In one case (□) 240 nmol of immobilized N^6-$H_2N(CH_2)_2$-cAMP was added after the treatment with [8-^{3}H]cAMP. The mixtures were maintained at 0°. At designated time points 750 μl aliquots were removed and bound [8-^{3}H]cAMP was determined.

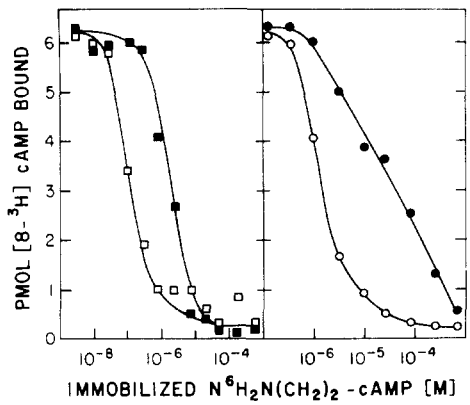

FIGURE 4: (Left panel) Binding of protein kinase to immobilized N^6-$H_2N(CH_2)_2$-cAMP at two enzyme concentrations. Reaction mixtures contained 33 mM Mes (pH 6.9), 5 mM magnesium acetate, 0.3 mM EGTA, either 0.10 μM (■) or 0.005 μM (□) protein kinase holoenzyme and designated concentrations of immobilized N^6-$H_2N(CH_2)_2$-cAMP. After 30 min at 23 °C aliquots of either 30 μl (■) or 600 μl (□) were removed, added to 4 μM [8-^{3}H]cAMP and assayed for the binding of [8-^{3}H]cAMP. (Right panel) Binding of protein kinase to immobilized N^6-$H_2N(CH_2)_2$-cAMP in the presence and absence of MgATP. Reaction mixtures contained 33 mM Mes (pH 6.9), 5 mM magnesium acetate, 0.3 nM EGTA, 0.10 mM protein kinase holoenzyme and designated concentrations of N^6-$H_2N(CH_2)_2$-cAMP in the presence (●) or absence (○) of 1 mM ATP. After 30 min at 23 °C, aliquots (30 μl) were removed, added to 4 μM [8-^{3}H]cAMP, and assayed for the binding of [8-^{3}H]cAMP.

Kinase to Immobilized N^6-$H_2N(CH_2)_2$-cAMP. The apparent affinity of the peak I isozyme of protein kinase for cAMP has been shown to be decreased by the presence of MgATP (Haddox et al., 1972; Beavo et al., 1974b; Hofmann et al., 1975). To further verify the biospecificity of the binding of protein kinase regulatory subunit to immobilized cyclic nucleotides, the effect of MgATP on the K_a for protein kinase binding to immobilized N^6-$H_2N(CH_2)_2$-cAMP was evaluated. The right panel of Figure 4 shows that the observed K_b was increased from 2 to 25 μM by the presence of 1 mM MgATP.

Factors That Influence the Apparent Affinity of Protein Kinase for Immobilized N^6-$H_2N(CH_2)_2$-cAMP. The abilities of soluble and immobilized N^6-$H_2N(CH_2)_2$-cAMP to activate protein kinase were compared using the protein kinase activity assay at a holoenzyme concentration of 0.005 μM. Under these conditions it was observed that the K_a was increased about tenfold to 3 μM by the process of immobilization. At least three explanations are possible for this decrease in apparent affinity: (1) The binding of the protein kinase regulatory subunit to an immobilized cyclic nucleotide might screen other neighboring immobilized ligands thereby preventing their interaction with other subunits; (2) binding of the protein might be sterically hindered by the matrix; or (3) some of the immobilized ligand molecules could be situated in a portion of the matrix from which the protein kinase is excluded.

To examine the first possibility we studied the binding of protein kinase to preparations of immobilized N^6-$H_2N(CH_2)_2$-cAMP with coupling densities[5] ranging from 0.15 to 3.54 μmol/ml. Assuming random coupling throughout the matrix, the lower coupling densities would have ligand molecules farther apart and therefore less likely to be screened by protein binding to neighboring ligands. We found that the K_b obtained at one coupling density did not differ significantly from those obtained at others. This result suggests that screening of neighboring ligand molecules was of small consequence during regulatory subunit binding.

TABLE III: Effect of Spacer-Arm Length on Binding of Protein Kinase Regulatory Subunit to Immobilized N^6-ω-Aminoalkyl-cAMP Derivatives.

Immobilized Nucleotide	Obsd K_b[a] (μM)
N^6-$H_2N(CH_2)_2$-cAMP	2.0
N^6-$H_2N(CH_2)_4$-cAMP	2.5
N^6-$H_2N(CH_2)_6$-cAMP	2.2
N^6-$H_2N(CH_2)_9$-cAMP	2.8
N^6-$H_2N(CH_2)_{12}$-cAMP	2.4

[a] Protein kinase holoenzyme concentration, 0.10 μM.

To evaluate the second possibility immobilized N^6-substituted cAMP derivatives with different chain lengths were prepared from a single batch of CNBr-activated Sepharose 4B and their binding to protein kinase was studied. Table III shows that the K_b values obtained for these derivatives did not differ significantly. This result implies that there was little steric hindrance which could be overcome by increasing the spacer-arm length (Cuatrecasas, 1970).

To test the third possibility, preparations of Sephadex G-10, Sephadex G-150, Sepharose 4B, and Bio-Gel A-150 were activated with CNBr by the method of March et al. (1974) and coupled as described earlier with N^6-$H_2N(CH_2)_2$-cAMP. The ability of each of these preparations to bind protein kinase regulatory subunit was evaluated. The data in Table IV show

TABLE IV: Effect of Matrix on Binding of Protein Kinase Regulatory Subunit to Immobilized N^6-$H_2N(CH_2)_2$-cAMP.

Matrix Used to Prepared Immobilized N^6-$H_2N(CH_2)_2$-cAMP[a]	Obsd K_b[b] (μM)
Sephadex G-10	11.0
Sephadex G-150	6.3
Sepharose 4B	2.8
Bio-Gel A-150	1.1

[a] Activated as described by March et al. (1974) and coupled as described in the text. [b] Protein kinase holoenzyme concentration, 0.10 μM.

TABLE V: Binding of Protein Kinase Regulatory Subunit to Several Immobilized Cyclic Nucleotides.

Immobilized Nucleotide	Obsd K_b[a] (μM)
N^6-$H_2N(CH_2)_2$-cAMP	2.5
8-$H_2N(CH_2)_2HN$-cAMP	4.4
2-$H_2N(CH_2)_2HN$-cAMP	18
8-$H_2N(CH_2)_2S$-cGMP	13
8-$H_2N(CH_2)_2HN$-cGMP	70

[a] Protein kinase holoenzyme concentration, 0.10 μM.

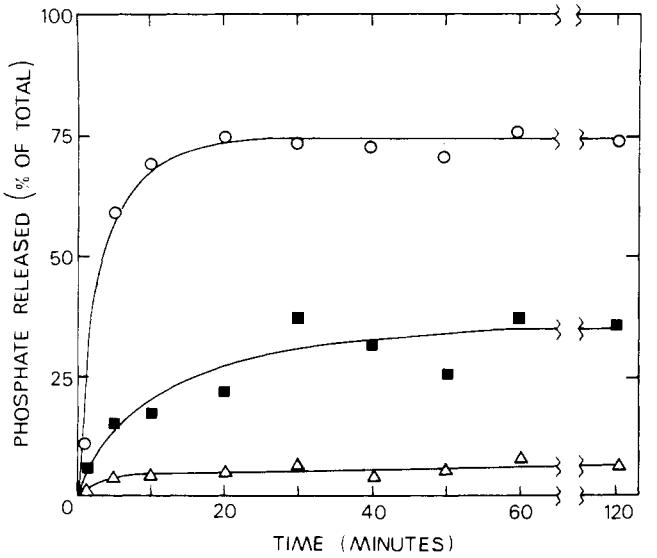

FIGURE 5: Hydrolysis of immobilized N^6-$H_2(CH_2)_6$-AMP by alkaline phosphatases. Reactions in 250 μl contained 50 mM Tris-HCl (pH 7.4), 25 mM magnesium acetate, 0.4 mM immobilized N^6-$H_2(CH_2)_6$-AMP, and 0.033 unit (based on soluble N^6-$H_2N(CH_2)_6$-AMP hydrolysis) of *E. coli* alkaline phosphatase (○), 0.024 unit beef liver alkaline phosphatase (■), or 0.014 unit of immobilized *E. coli* alkaline phosphatase (△). At designated time points, 20-μl aliquots were removed and assayed for phosphate as described in the text.

that immobilized N^6-$H_2N(CH_2)_2$-cAMP prepared using matrices with high molecular weight exclusion limits prior to CNBr activation had lower K_b's for protein kinase binding than the same derivative prepared from matrices with low exclusion limits, supporting the hypothesis that the protein cannot bind to some of the immobilized ligand molecules since it is excluded from portions of the gel available to the nucleotide.

If this last possibility (i.e., exclusion of the protein from a portion of the bound ligand) is a significant factor, one might also expect an enzyme with a large molecular weight to bind to a smaller amount of the immobilized ligand than one with a relatively small molecular weight. This is demonstrated by the results of an experiment (Figure 5) in which similar amounts of alkaline phosphatases of different molecular weights manifested different abilities to hydrolyze 0.4 mM immobilized N^6-$H_2N(CH_2)_6$-AMP. The enzyme from *E. coli* (molecular weight 80 000; Rothman and Byrne, 1963) removed phosphate rapidly for 20 min after which a plateau value representing about 70–75% of the total N^6-$H_2N(CH_2)_2$-AMP was reached. The rate obtained with the beef liver enzyme (molecular weight 150 000; Engström, 1964) appeared to plateau between 25 and 35% while less than 5% of the total phosphate was removed by the immobilized *E. coli* enzyme. In each case the initial and final values were verified by ashing the washed gel and measuring the remaining phosphate. The addition of 1 mM AMP to the reaction mixtures at 60 min resulted in a rapid release of phosphate demonstrating that the plateau values were not the result of enzyme inactivation or of differences in specific activity. In other experiments the same plateau values were reached with different concentrations of each of these enzymes (based on activity toward soluble N^6-$H_2N(CH_2)_2$-AMP). These data strongly support the idea that different proportions of immobilized ligand are available to the different size proteins. They therefore also suggest that part of the decrease in apparent affinity of protein kinase for N^6-$H_2N(CH_2)_2$-cAMP upon immobilization is due to limited availability of the coupled ligand.

Effect of Varying Positions of Spacer Arm on the Interaction of Protein Kinase and Immobilized Cyclic Nucleotide Derivatives. The binding of protein kinase to immobilized cyclic nucleotide with spacer arms attached at different positions on the purine ring was evaluated as shown in Table V. The immobilized derivatives bound the regulatory subunit in the same order of effectiveness (Table V) that the corresponding soluble derivatives activated protein kinase (Table II). This observation, together with the lack of effect of spacer-arm length on the K_b (Table III), suggests that the differences in the affinities of various immobilized cyclic nucleotides for regulatory subunit are due primarily to properties of the ligand itself and are not due to factors introduced by the coupling procedure.

Interaction of Immobilized Derivatives with Phosphodiesterase. Since hydrolysis of immobilized cyclic nucleotides would limit their usefulness in affinity chromatography, the ability of these derivatives to serve as substrates for beef heart phosphodiesterase was evaluated with the alkaline phosphatase coupled assay described earlier. No hydrolysis of the immobilized cyclic nucleotides was observed. This negative result was not due to failure of alkaline phosphatase used in the assay to hydrolyze the immobilized product of the phosphodiesterase reaction since under the same conditions *E. coli* alkaline phosphatase was able to liberate 70% of the total phosphate from immobilized N^6-$H_2N(CH_2)_6$-AMP within 10 min as shown in Figure 5.

Discussion

In this paper we present the results of a study on the binding affinity of cAMP-dependent protein kinase regulatory subunit to a number of immobilized cyclic nucleotide derivatives. This binding has been shown to be biospecific as judged by the following criteria: (1) the immobilized ligand and the analogous soluble ligand interact with the protein in a competitive manner; (2) this competitive interaction is not mimicked by the analogous spacer arm; and (3) the interaction with the im-

mobilized ligand is influenced by the same factors (enzyme concentration and the presence of MgATP in this case) that affect the interaction with the corresponding soluble ligand. The competition between the binding of cAMP and the immobilized ligand (Figure 2) fulfills the first criteria. The data in Figure 3, which show that this competition was not mimicked by immobilized spacer arms, fulfill the second. The third criteria was demonstrated in several experiments. Cleavage of the cyclic phosphate ring (e.g., substitution of AMP for cAMP) abolishes the ability of the protein to interact with both soluble (Walsh et al., 1968) and immobilized derivatives (Figure 2). The interactions of protein kinase with the immobilized derivatives was altered in a similar manner by the presence of MgATP or by changes in enzyme concentration (Figure 4) as the interaction of the enzyme with cAMP (Haddox et al., 1972; Beavo et al., 1974b; Hofmann et al., 1975). In addition chemical substitutions on the purine ring affected the interaction with the soluble (Table II) and immobilized derivatives (Tables III and V) in the same manner.

These studies also demonstrate that there is a reduction in the apparent affinity of protein kinase for N^6-$H_2N(CH_2)_2$-cAMP after immobilization. Several observations indicate that at least a part of this apparent reduction in affinity may be due to the exclusion of the protein from ligand molecules coupled to portions of the matrix inaccessible to the protein (Table IV and Figure 5). cAMP-dependent protein kinases have molecular weights of about 170 000 (Krebs et al., 1973; Erlichman et al., 1973), although gel filtration experiments give values of greater than 250 000 (Rubin et al., 1972) presumably due to a high degree of asymmetry (Erlichman et al., 1973). A protein of this size would be partially excluded from the matrix used for cyclic nucleotide immobilization and therefore from a fraction of immobilized ligand molecules. This result suggests that the choice of support matrix is an important consideration in the design of affinity columns particularly for the purification of high molecular weight proteins with relatively low affinities. It may also explain, at least in part, why coupling of ligand to large polymers prior to immobilization (e.g., Parikh et al., 1974; Wilchek and Miron, 1974) has been helpful in other systems.

The N^6-ω-aminoalkyl-cAMP derivatives, which bind the regulatory subunit most effectively (Table V), seem to be the best for the affinity chromatographic purification of this protein. It could be argued that a derivative with a lessened affinity might be more useful in that the protein would require less severe conditions for its elution. However, since the protein can be eluted from all the derivatives with 30 mM cAMP (Dills et al., 1975b, 1976), the immobilized ligands with higher affinities would seem to be better in that more rigorous washing conditions can be used to remove contaminating proteins.

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Binding of Chloromethyl Ketone Substrate Analogues to Crystalline Papain†

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ABSTRACT: Papain (EC 3.4.22.2) is a proteolytic enzyme, the three-dimensional structure of which has been determined by x-ray diffraction at 2.8 Å resolution (Drenth, J., Jansonius, J. N., Koekoek, R., Swen, H. M., and Wothers, B. G. (1968), *Nature (London) 218,* 929–932). The active site is a groove on the molecular surface in which the essential sulfhydryl group of cysteine-25 is situated next to the imidazole ring of histidine-159. The main object of this study was to determine by the difference-Fourier technique the binding mode for the substrate in the groove in order to explain the substrate specificity of the enzyme (P_2 should have a hydrophobic side chain (Berger and Schechter, 1970)) and to contribute to an elucidation of the catalytic mechanism. To this end, three chloromethyl ketone substrate analogues were reacted with the enzyme by covalent attachment to the sulfur atom of cysteine-25. The products crystallized isomorphously with the parent structure that is not the native, active enzyme but a mixture of oxidized papain (probably papain-SO_2^-) and papain with an extra cysteine attached to cysteine-25. Although this made the interpretation of the difference electron density maps less easy, it provided us with a clear picture of the way in which the acyl part of the substrate binds in the active site groove. The carbonyl oxygen of the P_1 residue is near two potential hydrogen-bond donating groups, the backbone NH of cysteine-25 and the NH_2 of glutamine-19. Valine residues 133 and 157 are responsible for the preference of papain in its substrate splitting. By removing the methylene group that covalently attaches the inhibitor molecules to the sulfur atom of cysteine-25 we obtained acceptable models for the acyl-enzyme structure and for the tetrahedral intermediate. The carbonyl oxygen of the P_1 residue, carrying a formal negative charge in the tetrahedral intermediate, is stabilized by formation of two hydrogen bonds with the backbone NH of cysteine-25 and the NH_2 group of glutamine-19. This situation resembles that suggested for the proteolytic serine enzymes (Henderson, R., Wright, C. S., Hess, G. P., and Blow, D. M. (1971), *Cold Spring Harbor Symp. Quant. Biol. 36,* 63–70; Robertus, J. D., Kraut, J., Alden, R. A., and Birktoft, J. J. (1972b), *Biochemistry 11,* 4293–4303). The nitrogen atom of the scissile peptide bond was found close to the imidazole ring of histidine-159, suggesting a role for this ring in protonating the N atom of the leaving group (Lowe, 1970). This proton transfer would be facilitated by a 30° rotation of the ring around the C^{β}–C^{γ} bond from an in-plane position with the sulfur atom to an in-plane position with the N atom. The possibility of this rotation is derived from a difference electron-density map for fully oxidized papain vs. the parent protein.

Papain (EC 3.4.22.2), a cysteine protease from the fruits of the papaya tree (Carica papaya), has been studied extensively and in a number of reviews (Glazer and Smith, 1971; Drenth et al., 1971) its biochemical and structural properties have been summarized. Although the position of the catalytic site on the enzyme surface is known, the exact interaction of the enzyme with its substrates has not yet been established. It has been generally agreed upon that the hydrolysis of a substrate by papain, like the serine proteases, proceeds via a covalent acyl-enzyme intermediate, according to the general equation:

SCHEME I

$$\text{acylation: } E + S \rightleftarrows ES \rightleftarrows ES^{\dagger}_{tetr} \rightarrow ES^{*} + P_1$$
$$\text{deacylation: } ES^{*} + H_2O \rightleftarrows ES^{\ddagger}_{tetr} \rightarrow E + P_2$$

In this scheme ES is the Michaelis complex, ES* is the acyl-enzyme formed through the Cys-25 thiol group, P_2 is the acid, and P_1 the alcohol or amine moiety of the hydrolyzed substrate. Both acylation and deacylation most likely proceed via a tetrahedral intermediate ($ES^{\dagger}_{tetr}$, $ES^{\ddagger}_{tetr}$ in Scheme I) in which the carbonyl carbon atom undergoing nucleophilic attack has four tetrahedrally arranged bonds. Whereas the evidence for acyl-enzyme formation in the papain-catalyzed hydrolysis pathway is abundant (Lowe, 1970; Smolarsky, 1974), no direct

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