Values of K_{iso} for both forms are available from the data of Figure 4. Values of K_e and K_k for NADPH, H_2folate, and TMP can be obtained from the equilibrium and kinetic data in Tables I and II. If we assume that the dissociation rate constants measured by competition (k_b) are those for the conversion of E_1L^* to E_1L, then values of the rate constant for the formation of E_1L^* (k_f) can be calculated. These calculated values are shown in Table V.

These results clearly indicate that the tight binding of the TMP relative to the substrate or coenzyme in the binary complex is due largely to the stability of the E_1L^* form of the complex. Williams et al. (1979, 1980) have used measurements of inhibition of the catalytic activity of the enzyme to demonstrate that a similar process is responsible for tight binding of inhibitors in the ternary complex. Hood & Roberts (1978) thought it likely that conformational differences between substrate and inhibitor binary complexes with the enzyme are responsible for a large part of their difference in binding energy. It seems likely that the E_1L to E_1L^* transition is also the manifestation of either a conformational readjustment of the complex or a positional change in the ligand.

Arguments similar to those used above may be applied to Scheme III, although we have insufficient data to make quantitative comparisons.

The results in Tables II and V also demonstrate that even if other steps occur between E_1L and the formation of E_1L^* the major factor governing the difference in affinity of the two isoenzymes for trimethoprim is the transition of E_1L^* to E_1L. This may reflect a difference in the energy of interaction of the inhibitor with the side chains of the protein since the leucine residue which is replaced forms part of the binding site for the methoxy groups of TMP. Alternatively, it may result from a difference in the energetics of an enzyme conformational change, although this seems less likely since a similar effect is not seen with dihydrofolate.

The formation of binary complexes between dihydrofolate reductase and the ligands described is thus at least a three-step process. The formation of E_1L takes place via a transitory intermediate, and a further step leads to the formation of E_1L^*. In the case of MTX, which binds strongly to both E_1 and E_2, parallel reaction pathways through E_1L and E_2L appear to lead to a final equilibrium between E_1L^* and E_2L^*.

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Mapping Adenosine Cyclic 3′,5′-Phosphate Binding Sites on Type I and Type II Adenosine Cyclic 3′,5′-Phosphate Dependent Protein Kinases Using Ribose Ring and Cyclic Phosphate Ring Analogues of Adenosine Cyclic 3′,5′-Phosphate†

Terry S. Yagura and Jon P. Miller*

ABSTRACT: A series of adenosine cyclic 3′,5′-phosphate (cAMP) derivatives containing modifications or substitutions in either the 2′, 3′, 4′, or 5′ position or the phosphate were examined for their abilities to activate type I isozymes of cAMP-dependent protein kinase (PK I) from rabbit or porcine skeletal muscle and type II isozymes of cAMP-dependent protein kinase (PK II) from bovine brain and heart. The studies revealed that the activation of both PK I and PK II isozymes requires a 2′-hydroxyl group in the ribo configuration, a 3′ oxygen in the ribo configuration, and a charged cyclic phosphate. The two isozymes appeared to differ in those portions of their respective cAMP-binding sites that are adjacent to the 4′ position of the ribose ring and the 3′ position, 5′ position, and phosphate portion of the cyclic phosphate ring.

The original proposal of Kuo & Greengard (1969) that the diverse actions of adenosine cyclic 3′,5′-phosphate (cAMP)[1] on eukaryotic physiology are all mediated through cAMP-dependent protein kinases is still the principal working hypothesis for the mechanism of action of cAMP (Rosen et al., 1977). Many cAMP analogues can efficiently activate various

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[1] Abbreviations used: cAMP, adenosine cyclic 3′,5′-phosphate; DEAE, diethylaminoethyl; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol.

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cAMP-dependent protein kinases (Meyer & Miller, 1974; Miller & Robins, 1976; Miller, 1977). Most of the published studies on the activation of protein kinases by cAMP analogues were completed before it became generally recognized that there are two isozymic forms of cAMP-dependent protein kinase, referred to as type I and type II (PK I and PK II, respectively) (Corbin et al., 1975a,b; Hoffmann et al., 1975).

The PK I and PK II isozymes appear to differ principally in their regulatory subunits (Hoffmann et al., 1975; Fleischer et al., 1976). If these differences between the regulatory subunits of the two isozymes extend to the respective cAMP-binding sites on these proteins, then certain analogues of cAMP may be more potent activators of PK I than of PK II, and vice versa. This hypothesis is supported by the results of Jones et al. (1973), who reported that 3-β-D-ribofuranosylimidazo[2,1-*i*]purine cyclic 3′,5′-phosphate and some 2-, 7-, or 8-substituted derivatives thereof were all more potent activators of a calf brain cAMP-dependent protein kinase (presumably a type II isozyme) than of a rabbit skeletal muscle cAMP-dependent protein kinase (presumably a type I isozyme). Further support for this hypothesis comes from our recent report (Yagura et al., 1980) that certain 2-substituted cAMP derivatives (e.g., 2-chloro-cAMP, 2-trifluoromethyl-cAMP, and 2-aza-cAMP) were significantly more potent activators of the rabbit skeletal muscle PK I than of the bovine heart PK II, whereas other 2-substituted cAMP derivatives (e.g., 2-*n*-butyl-cAMP and 2-styryl-cAMP) were significantly more potent activators of the bovine heart PK II than of the rabbit skeletal muscle PK I. In addition, a study of a group of 2-substituted 1,N^6-etheno-cAMP derivatives revealed that some of these analogues preferentially activated PK I isoenzymes but that others preferentially activated PK II isozymes (Miller et al., 1980b).

Although both PK I and PK II isozymes demonstrate significantly different cAMP-binding sites, PK I isozymes from different sources are quite similar in their response to various cAMP analogues, and, likewise, PK II isozymes from different sources respond similarly to a number of cAMP analogues (Yagura et al., 1980; Miller et al., 1980b; Yagura & Miller, 1980).

To further develop our understanding of the similarities and differences between the PK I and PK II cAMP-binding sites, we assembled a group of cAMP analogues with representative modifications in the ribose or the cyclic phosphate ring and examined their relative abilities to activate PK I and PK II isozymes.

Materials and Methods

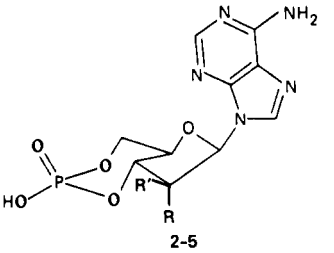
cAMP Analogues. The structures of the cAMP analogues are shown in Tables I–V. Previously reported methods were used to synthesize compounds **2** (Miller et al., 1976), **3** (Khwaja et al., 1972), **4** (Miller et al., 1973b), **12** (Shuman et al., 1973), **15** (Gulyaev et al., 1976), and **18** (Meyer et al., 1973a). Compound **5** was from Sigma Chemical Co. (St. Louis, MO). The rest of the compounds were generously supplied by the following investigators: **1**, A. Holý, Czechoslovak Academy of Sciences, Prague; **6** (Anisuzzaman et al., 1973), R. L. Whistler, Purdue University, West Lafayette, IN; **7** (Shealy & Clayton, 1973), Y. F. Shealy, Southern Research Institute, Birmingham, AL; **8** (Hubert-Habart & Goodman, 1969), G. R. Revankar, Brigham Young University, Provo, UT; **9**, **10** (Morr et al., 1974), and **13** (Murayama et al., 1970), B. Jastorff, Universität Bremen, West Germany; **11** and **14** (Jones et al., 1970), G. H. Jones, Syntex Research, Palo Alto, CA; **16** (Eckstein et al., 1974), F. Eckstein, Max-Planck-Institut für experimentalle Medizin, Göttingen, West Germany; **17** (Gohil et al., 1974), J. Nagyvary, Texas A & M University, College Station, TX. Compounds **17** and **18** were mixtures of axial and equatorial diastereomers.

cAMP-Dependent Protein Kinases. Rabbit skeletal muscle PK I was purified through the alumina C_γ step as described by Beavo et al. (1974) and exhibited a specific activity of 100–300 units/mg of protein, where 1 unit is that amount of enzyme which transfers 1 nmol of $H_2{}^{32}PO_4^-$ from [γ-^{32}P]ATP to histone per min at 30 °C. The method of Hoffmann et al. (1975) was carried through the alumina C_γ step to yield bovine heart PK II with a specific activity of 20–50 units/mg of protein. Bovine brain PK II was purified through the calcium phosphate gel step as described by Miyamoto et al. (1969) and exhibited a specific activity of 0.8–2.0 units/mg of protein. The method of Potter et al. (1978) was carried through the second DEAE-cellulose separation to yield porcine skeletal muscle PK I with a specific activity of 12–30 units/mg of protein. All the specific activities stated above were based on assays conducted in the presence of 5 μM cAMP, which represented an activation of 17–20-, 5–8-, 20–24-, and 7–12-fold for the rabbit skeletal muscle PK I, bovine heart PK II, bovine brain PK II, and porcine skeletal muscle PK I, respectively.

The protein kinase assays were performed by using the paper disk method previously described (Miller et al., 1973a,b). The assay for the kinase contained, in 0.1 mL, 5 μmol of sodium acetate (pH 6.0), 1 μmol of $MgCl_2$, 100 μg of histone) (Worthington HLY), 0.5 μmol of [γ-^{32}P]ATP (150000 cpm), 3.7 pmol of protein kinase holoenzyme, and various concentrations of the cAMP analogue being tested as an activator (1 nM–0.5 mM). The concentration of the holoenzyme was based on the cAMP-binding capacity of each enzyme preparation (Erlichman et al., 1973). The catalytic activity of the kinase was measured in the presence of a number of concentrations (at least seven) of the cyclic AMP analogue being tested as an activator, varied over at least a 100-fold concentration range. The amount of product formed was determined at several (at least three) time points (3–12 min) to ensure that linear reaction rates were being measured. The K_a for each analogue was determined from the x intercept (calculated from linear regression analysis) of a line described by a double-reciprocal plot of the above data (picomoles of phosphate transferred to histone)$^{-1}$ vs. [cyclic nucleotide]$^{-1}$ (Miller et al., 1973a, 1978). Only those apparent K_a values that resulted from lines with correlation coefficients ≥ 0.990 were considered acceptable. All data represent the results of triplicate determinations, which were reproducible within 15% of the value reported. K_a' = apparent K_a for cAMP/apparent K_a for the analogue, where K_a values for cAMP were 36, 41, 28, and 49 nM for the rabbit skeletal muscle, bovine heart, bovine brain, and porcine skeletal muscle, respectively. In those cases in which the highest concentration of the analogue (0.5 mM) yielded <50% of maximal activation of a protein kinase, the K_a' is reported as <0.0001. The K_a' values for **2**–**5** and **12** with the bovine brain PK II have previously been reported (Miller et al., 1973b; Shuman et al., 1973). The protein kinase specificity, K_a'(PK I)/K_a'(PK II) = [K_a'(rabbit muscle) + K_a'(porcine muscle)]/[K_a'(bovine heart) + K_a'-(bovine brain)].

The cAMP-dependent protein kinases were photoaffinity labeled with 5 μM 8-azido-[^{32}P]cAMP (2 Ci/mmol, ICN Radiochemicals) as described by Pomerantz et al. (1975) and then subjected to polyacrylamide gel electrophoresis on 1.5-mm 7.5% slab gels in the presence of 0.1% sodium dodecyl sulfate according to the method of Weber & Osborne (1969). The

Table I: Activation of PKI and PKII Isozymes by 2′-Derivatives of cAMP



2-5

Derivative				Protein Kinase Activation Ka′				Protein Kinase Specificity, Ka′(PKI)/Ka′(PKII)
				PKI		PKII		
No.	Name	-R	-R′	Rabbit Muscle	Porcine Muscle	Bovine Heart	Bovine Brain	
	cAMP	-OH	-H	1.0	1.0	1.0	1.0	1.0
2	2′-deoxy-cAMP	-H	-H	0.0070	0.0057	0.0048	0.0038	1.5
3	ara-cAMP	-H	-OH	0.0032	0.0021	0.0029	0.0013	1.3
4	2′-*O*-methyl-cAMP	$-OCH_3$	-H	0.0023	0.0029	0.0017	0.0023	1.3
5	2′-*O*-butyryl-cAMP	$-OCOnC_3H_7$	-H	0.0033	0.0041	0.0017	0.0030	1.6

^{32}P-labeled proteins were visualized by autoradiography, and the molecular weight standards (lactate dehydrogenase, malate dehydrogenase, carbonic anhydrase C, soybean trypin inhibitor, and cytochrome *c*) were visualized by staining with Coomassie blue. Each protein kinase was found to contain a single ^{32}P-labeled component. The mobilities of these components corresponded to molecular weights of 48 000, 55 000, 54 000, and 47 000 for the rabbit skeletal muscle PK I, bovine heart PK II, bovine brain PK II, and porcine skeletal muscle PK I, respectively, indicating that little or no proteolysis of the regulatory subunit had occurred during the preparation of the holoenzymes. In addition, each PK I preparation was thereby shown to be free of PK II and vice versa.

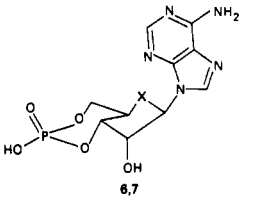
The protein kinase preparations were examined for their ability to undergo autophosphorylation when incubated under the standard assays conditions in the presence of 5 μM cAMP but lacking histone. When the assay was conducted in the presence of 0.1 mg of holoenzyme preparation, the bovine heart PK II and bovine brain PK II incorporated 1.7 mol and 1.9 mol of phosphate, respectively, per mol of holoenzyme within 1 min, with no increase in phosphate incorporation after 1 h, demonstrating that both PK II isozymes were primarily in the dephospho form as isolated. The PK I isozymes incorporated no measurable ^{32}P under the identical conditions after 1 h of incubation.

Each protein kinase preparation was examined for the presence of cyclic nucleotide phosphodiesterase in a reaction mixture containing (in 0.5 mL) 25 μmol of Tris-HCl, pH 7.5, 5 μmol of $MgCl_2$, 1 μmol of 2-mercaptoethanol, either 80 pmol of [8-^{3}H]cAMP (350 000 cpm), 2 μmol of [8-^{3}H]cAMP (420 000 cpm), or 160 pmol of [8-^{3}H]cGMP, and 20 μg–2 mg of protein kinase protein. After incubation for 1 h at 30 °C, the mixture was treated with 5′-nucleotidase and Dowex-1, and the radioactivity in the nucleoside fraction was determined as previously described (Miller et al., 1973a,b). None of the preparations used in these studies contained any measurable cyclic nucleotide phosphodiesterase activity when assayed under these conditions.

Results

Each cAMP analogue was examined for its ability to activate PK I isozymes from rabbit and porcine muscle and PK II isozymes from bovine brain and heart. The relative potency of each analogue as an activator of each kinase was quantitated by determining K_a' values.

Table II: Activation of PKI and PKII Isozymes by 4′-Derivatives of cAMP



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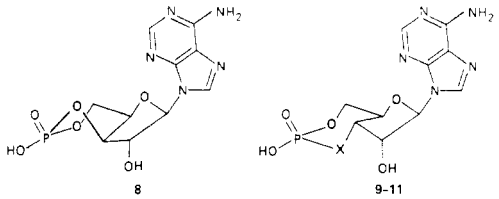
Derivative			Protein Kinase Activation, Ka′				Protein Kinase Specificity, Ka′(PKI)/Ka′(PKII)
			PKI		PKII		
No.	Name	-X-	Rabbit Muscle	Porcine Muscle	Bovine Heart	Bovine Brain	
	cAMP	-O-	1.0	1.0	1.0	1.0	1.0
6	4′-thio-cAMP	-S-	0.65	0.51	0.43	0.51	1.2
7	4′-methylene-cAMP	$-C(H_2)-$	0.032	0.019	0.17	0.24	0.12

Activation of PK I and PK II Isozymes by Ribose Ring Analogues of cAMP. The K_a' values were determined for cAMP analogues with modifications in the 2′ and 4′ positions of the ribose ring. In addition, L-cAMP (**1**) was examined; it produced no measurable activation of either PK I or PK II isozymes at concentrations 10^4 times the K_a values of cAMP ($K_a' < 0.0001$).

The K_a' values for cAMP analogues with three types of modifications in the 2′ position of the ribose ring are shown in Table I. The modifications were (a) removal of the 2′ oxygen to yield 2′-deoxy-cAMP (**2**), (b) alteration of the ribo configuration to yield ara-cAMP (**3**), and (c) substitution of the 2′ oxygen to yield 2′-*O*-methyl-cAMP (**4**) and 2′-*O*-butyryl-cAMP (**5**). All three types of 2′ modifications resulted in cAMP analogues exhibiting K_a values for either PK I or PK II isozymes that were 100 to 600 times those for cAMP. In addition, none of the 2′-modified cAMP analogues (**2**–**5**) demonstrated any preferential activation of either PK I or PK II isozymes.

The effects on protein kinase activation of 4′ modifications were examined by determining the K_a' values of cAMP analogues with either a sulfur or a methylene moiety replacing the oxygen atom in the D-ribofuranosyl ring, yielding 4′-thio-cAMP (**6**) and the carbocyclic analogue of cAMP 4′-

Table III: Activation of PKI and PKII Isozymes by 3′-Derivatives of cAMP



8 9-11

Derivative			Protein Kinase Activation, K_a'				Protein Kinase Specificity, $\frac{K_a'(PKI)}{K_a'(PKII)}$
			PKI		PKII		
No.	Name	-X-	Rabbit Muscle	Porcine Muscle	Bovine Heart	Bovine Brain	
	cAMP	-O-	1.0	1.0	1.0	1.0	1.0
8	xylo-cAMP		<0.0001	<0.0001	<0.0001	<0.0001	
9	3′-thio-cAMP	-S-	0.010	0.022	0.0048	0.0061	2.9
10	3′-amido-cAMP	-N(H)-	0.0039	0.0050	0.00043	0.00027	13.0
11	3′-methylene-cAMP	$-C(H_2)-$	<0.0001	<0.0001	<0.0001	<0.0001	

methylene-cAMP (**7**), respectively (Table II). Compound **6** was nearly as active as cAMP as an activator of either PK I or PK II isozymes. In contrast, **7** was considerably less active than **6** with both PK I and PK II but was almost 10-fold more potent as an activator of PK II than of PK I.

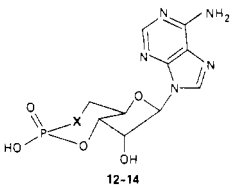
Activation of PK I and PK II Isozymes by Cyclic Phosphate Ring Analogues of cAMP. The K_a' values for cAMP analogues with modifications in the 3′ and 5′ positions and in the phosphate moiety of the cyclic phosphate ring are shown in Tables III–V, respectively.

cAMP analogues with two types of 3′ modifications were examined. These modifications were (a) alteration of the ribo configuration to yield xylo-cAMP (**8**) and (b) replacement of the 3′ oxygen by either a sulfur, amido, or methylene moiety to give 3′-thio-cAMP (**9**), 3′-amido-cAMP (**10**), or 3′-methylene-cAMP (**11**), respectively. Xylo-cAMP (**8**) produced no measurable activation of either PK I or PK II isozymes at concentrations 10^4 times the K_a values for cAMP. Compounds **9-11** demonstrate the same order of activity with both PK I and PK II isozymes: cAMP > **9** > **10** > **11**. The protein kinase specificity revealed that **9** had approximately the same relative potency with PK I and PK II, whereas **10** was more than 10-fold more potent as a PK I activator than as a PK II activator.

The 5′-modified cAMP analogues were analogous to the 3′-modified cAMP analogues in that the 5′ oxygen was replaced by either a sulfur, amido, or methylene moiety to yield 5′-thio-cAMP (**12**), 5′-amido-cAMP (**13**), or 5′-methylene-cAMP (**14**), respectively (Table IV). For both the PK I and PK II isozymes, the order of activity of these three analogues relative to cAMP was cAMP > **12** > **13** > **14**. The protein kinase specificity of the 5′-modified analogues revealed that **12** was 5 times more potent as an activator of PK II than of PK I and that **13** was 14 times more potent as an activator of PK I than of PK II. Compound **14**, although a very weak activator, was an equally potent activator of PK I and PK II.

Three additional types of analogues with modifications of the cyclic phosphate ring were examined. These modifications were (a) replacement of the phosphate, (b) replacement of the exocyclic oxygen, and (c) replacement or esterification of the hydroxyl (Table V). The only example of cyclic phosphate replacement is the adenosine cyclic 3′,5′-sulfate (**15**), which was inactive as an activator of either PK I or PK II. Replacement of the exocyclic oxygen by sulfur yielded cAMP phosphorothioate (**16**), which provided significant activation of both isozymes but was a more potent activator of PK II than of PK I. Esterification of cAMP gave the diastereomers of

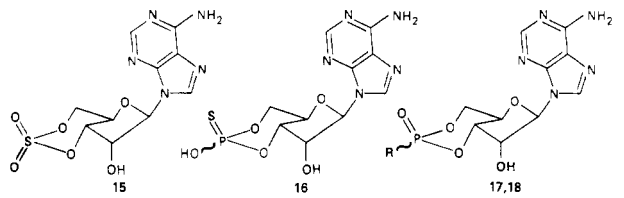
Table IV: Activation of PKI and PKII Isozymes by 5′-Derivatives of cAMP



12-14

Derivative			Protein Kinase Activation, K_a'				Protein Kinase Specificity, $\frac{K_a'(PKI)}{K_a'(PKII)}$
			PKI		PKII		
No.	Name	-X-	Rabbit Muscle	Porcine Muscle	Bovine Heart	Bovine Brain	
	cAMP	-O-	1.0	1.0	1.0	1.0	1.0
12	5′-thio-cAMP	-S-	0.10	0.14	0.66	0.51	0.21
13	5′-amido-cAMP	-N(H)-	0.023	0.017	0.0016	0.0023	10.0
14	5′-methylene-cAMP	$-C(H_2)-$	0.00087	0.00069	0.00057	0.00072	1.2

Table V: Activation of PKI and PKII Isozymes by Phosphate Derivatives of cAMP



15 16 17,18

Derivative			Protein Kinase Activation, K_a'				Protein Kinase Specificity, $\frac{K_a'(PKI)}{K_a'(PKII)}$
			PKI		PKII		
No.	Name	-R	Rabbit Muscle	Porcine Muscle	Bovine Heart	Bovine Brain	
	cAMP	-OH	1.0	1.0	1.0	1.0	1.0
15	Adenosine cyclic 3′,5′-sulfate		<0.0001	<0.0001	<0.0001	<0.0001	
16	cAMP phosphorothioate		0.094	0.077	0.46	0.57	0.17
17	cAMP ethyl ester	$-OC_2H_5$	<0.0001	<0.0001	<0.0001	<0.0001	
18	cAMP dimethyl-phosphoramidate	$-N(CH_3)_2$	<0.0001	<0.0001	<0.0001	<0.0001	

cAMP ethyl ester (**17**), and replacement of the hydroxyl gave the diastereomers of cAMP dimethylphosphoramidate (**18**); both of these analogues were unable to activate either PK I or PK II isozymes.

Discussion

It has been established that the activation of protein kinases by cAMP proceeds via binding of cAMP to the regulatory subunit of the inactive holoenzyme, to yield an active catalytic subunit (Langan, 1973; Walsh & Cooper, 1979). Under the conditions used here, where the substrates (MgATP and histone) are saturating and the substrates by themselves do not significantly modify the equilibrium of the binding of cAMP to the holoenzyme, the rate of histone phosphorylation should be proportional to the concentration of active catalytic subunit. Therefore, activation of the protein kinases by the analogues will be discussed in terms of the factors influencing binding to the holoenzyme.

Because the K_a for cAMP is dependent on holoenzyme concentration (Soderling & Park, 1974; Swillens et al., 1974), each K_a for cAMP and the analogues was determined at the same concentration of holoenzyme (37 nM). Under these conditions, the K_a values for cAMP with all four protein kinases were quite comparable; they ranged from 28 to 49 nM. Therefore, the K_a' values were compared directly without further normalization of the data. Recent results have shown that 2 mol of cAMP can bind to 1 mol of type I or type II regulatory subunit monomer (Corbin et al., 1978; Weber & Hilz, 1979) and that the cAMP binding sites of the type II

regulatory subunit are nonidentical (LaPorte et al., 1980). It is not known whether 1 or 2 mol of cAMP need to be bound per mol of regulatory subunit monomer in order to activate the homoenzymes. If both sites are involved there may be interactions between them of either a positive or negative nature. It is possible that some of the cAMP analogues reported here may be binding to only one of the two sites on either PK I or PK II or that the binding of an analogue to one site affects the binding to the second site.

The enzyme preparations used in these studies were not homogeneous, but the degree of purification is such that in each case, the PK I isozyme preparations are free of detectable type II regulatory subunit and the PK II isozyme preparations are free of detectable type I regulatory subunit. In addition, all four preparations were free of detectable cyclic nucleotide phosphodiesterase activity, and the molecular weights of their regulatory subunit suggest that they have not undergone any significant proteolysis. Some of the analogues that demonstrated significant differences in K_a' values between PK I and PK II (**7**, **10**, **12**, **13**, and **16**) were examined for their ability to activate preparations of each of the four protein kinases that had been carried through at least one additional step of purification: through the Sepharose 6B step for rabbit skeletal muscle PK I and bovine heart PK II, through the Cellex-P–cellulose step for bovine brain PK II, and through the Sephacryl I step for porcine muscle PK I. The K_a values for both cAMP and the analogues obtained by using these more purified PK I and PK II preparations were essentially the same as those reported here.

Histone itself can activate (dissociate) cAMP-dependent protein kinases (Miyamoto et al., 1973), but the differences between K_a' values with PK I and PK II are probably not due to different susceptibilities of the two isozyme types to the effects of histone on the K_a for cAMP or for the analogues. We examined some of the analogs (**7**, **10**, **12**, **13**, and **16**) as activators of the PK I and PK II isozymes by using protamine as substrate in place of histone and found that the K_a values for cAMP and these analogues were all ~4-fold greater than the corresponding K_a values obtained by using histone as substrate.

Comparison of the K_a' values with rabbit muscle PK I and with porcine muscle PK I reveals that each analogue demonstrated comparable activity with the two PK I isozymes. These results further support our proposal that all or most type I isozymes have very similar cAMP-binding sites (Yagura et al., 1980; Yagura & Miller, 1980). Likewise, each analogue yielded quite similar K_a' values with bovine heart PK II and bovine brain PK II. This finding complements our previous results that led us to suggest that all or most type II isozymes have very similar cAMP-binding sites (Miller et al., 1980b; Yagura & Miller, 1980). These conclusions are tentative because only two PK I and two PK II isozymes were studied. Some of the analogs (**3**, **6**, **8**, **12**, and **18**) were also examined as activators of both PK I and PK II isozymes from rat liver and mouse epidermis and of a PK II isozyme from procine muscle; in each case, the analogues yielded K_a' values that were comparable to those for the PK I and PK II isozymes reported here (M. B. Scholten, A. Beck, and J. P. Miller, unpublished data). Because of the similarity of the two PK I isozymes and, likewise, of the two PK II isozymes, the ratio of the average K_a' value for PK I to the average K_a' value for PK II, K_a'(PK I)/K_a'(PK II), was used as a measure of the extent to which each analogue preferentially activated either PK I or PK II.

The lack of activity of L-cAMP (**1**) suggests that the proper juxtaposition of the purine and ribose–cyclic phosphate moieties is critical to the binding of cAMP to PK I and PK II. A similar conclusion was drawn from a study of the protein kinase activation by 5′-deoxy-5′-thioinosine cyclic 3′,5′-phosphorothioate, a cAMP analogue with both purine and cyclic phosphate ring modifications (Shuman et al., 1973). This doubly modified analogue was only a very poor PK II activator, whereas both singly modified parent compounds (inosine cyclic 3′,5′-phosphate and 5′-deoxy-5′-thioadenosine cyclic 3′,5′-phosphorothioate) demonstrated activity comparable to that of cAMP.

The very poor ability of 2′ derivatives of cAMP (**2**–**5**) to activate PK I isozymes mirrors our previous report of the low activity of these derivatives as PK II activators (Miller et al., 1973b). Our previous generalization that PK II requires an unsubstituted 2′-hydroxyl group in the ribo configuration can now be extended to include PK I isozymes.

An analogue of cAMP in which the ribofuranosyl ring had been opened between the 2′ and 3′ carbons (compound **16**, see Figure 1 for structure) was unable to compete with cAMP for binding to bovine muscle cAMP-dependent protein kinase (K. H. Scheit, personal communication). This suggests that protein kinase requires an intact ribofuranosyl ring in addition to the 2′-*ribo*-hydroxyl group.

The relative activities of the 4′ derivatives revealed both similarities and differences between the PK I and PK II isozymes. Replacement of the 4′ oxygen by sulfur was tolerated well by both PK I and PK II, but replacement by a methylene moiety was tolerated better by PK II than by PK I. These results suggest that the two isozymes differ in those portions of their respective cAMP-binding sites that are adjacent to the 4′ oxygen of cAMP. PK I may be more dependent than PK II on a hydrogen bond to the 4′ oxygen. Alternatively, PK I may be less tolerant than PK II to the steric effects of the 4′ protons of **7** or to changes in the conformational structure of the ribose–cyclic phosphate ring system resulting from the replacement of the 4′ oxygen of cAMP with a methylene moiety.

The lack of activity of xylo-cAMP (**8**) suggests that the configurations of the ribose and cyclic phosphate rings are critical for the activation of both PK I and PK II isozymes. The results are consistent with those of Drummond & Powell (1970), who reported that xylo-cAMP (**8**) was without activity as an activator of phosphorylase *b* kinase.

Both the 3′ oxygen and the 5′ oxygen appear to be important for the activation of PK I and PK II isozymes. However, PK I and PK II differ significantly in the effects of replacement of the 3′ and 5′ oxygen with a sulfur (compounds **9** and **12**), amido (compounds **10** and **13**), or methylene (compounds **11** and **14**) moiety. For both isozymes, a 5′ sulfur is well tolerated, and **12** is 5 times more potent as a PK II activator than as a PK I activator. In contrast, a 3′ sulfur is not well tolerated, and **9** is 3 times more potent as a PK I activator than as a PK II activator. Because oxygen and sulfur have different electron-density distributions, the lowered potency of **9** compared to cAMP may be due to the decreased strength of a hydrogen bond between the 3′ atom and that portion of the cAMP-binding site adjacent to the 3′ oxygen of cAMP. If a hydrogen bond is involved in the interaction between the 3′ oxygen of cAMP and the protein kinases, then the weakening of this putative hydrogen bond is more detrimental to the binding of cAMP to PK II than to the binding of cAMP to PK I. The 5′ oxygen appears to be less important than the 3′ oxygen in the binding of cAMP to PK I or PK II. In addition, weakening of a putative hydrogen bond between the 5′ oxygen of cAMP and the protein kinases is more detrimental to the binding of

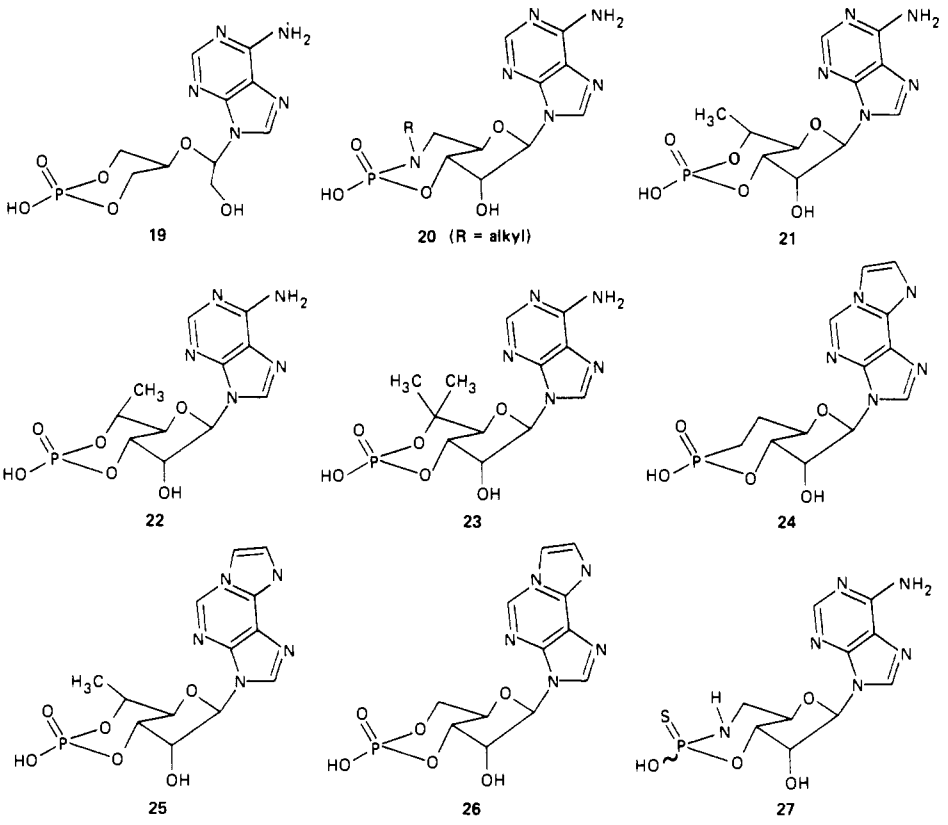


FIGURE 1: Structures of cAMP analogues.

cAMP to PK I than to the binding of cAMP to PK II.

The 3′-amido-cAMP (**10**) and the 5′-amido-cAMP (**13**) were considerably less active with PK I and PK II than were **9** and **12**, respectively. Similarly, the 3′-methylene-cAMP (**11**) and 5′-methylene'cAMP (**14**) were significantly less potent than **10** and **13**, respectively, with both isozymes. In addition, **10** and **13** preferentially activated PK I compared to PK II. K_a' values in agreement with those in Tables II and IV have been reported for **10** and **13** with rabbit muscle PK I and bovine heart PK II (Panitz et al., 1975) and for **11** and **14** with bovine heart PK II and bovine brain PK II (Kuo & Greengard, 1970). Moreover, **9**, **10**, and **13** were examined for their relative ability to compete with [^{3}H]cAMP for binding to rabbit muscle PK I (Jastorff et al., 1979), and the apparent K_i values thus obtained were in the same range as the K_a values reported here for these compounds.

The weak activity of **10**, **11**, **13**, and **14** may be due to (a) electronic differences, between either the amido or methylene moieties and oxygen, that prevent binding to the 3′ and 5′ position, (b) steric hindrances to binding due to the presence of one (for **10** or **13**) or two (for **11** and **14**) protons in the 3′ or 5′ position of the analogues, or (c) alterations in the conformational structure of the cyclic phosphate ring. As recently pointed out by Jastorff et al. (1979), on the basis of the data of Hong et al. (1976), the lone-pair electrons of the 3′ and 5′ nitrogens in **10** and **13**, respectively, are not available for hydrogen bonding because of their involvement in a hydrid interaction with the phosphorous orbital. The weak activity of **10** and **13** may therefore be due to the inability of these analogues to form hydrogen bonds between the 3′ or 5′ atom, respectively, of the cyclic nucleotide and the protein kinases. The difference between **13** and **14** in protein kinase specificity suggests that the weak activity of these analogues may be due to factors in addition to the inability to form a hydrogen bond.

X-ray studies show that **14** has conformational characteristics very similar to those of cAMP (Sundaralingam & Abola, 1972). Therefore, the detrimental effects of the 5′-amido or 5′-methylene substitutions may be due to a combination of electronic and steric differences between these analogues and cAMP. Jastorff & Bär (1973) examined 5′-amido-cAMP (**13**) and some 5′-(*N*-alkylamino)-cAMP derivatives (**20**, see Figure 1) as activators of a bovine skeletal muscle cAMP-dependent protein kinase and found that only **13** was an activator. Even the 5′-*N*-methylamino modification was not tolerated, suggesting that the low activity of **13** may be due in part to the steric effects of the proton in the 5′ position.

The 5′-substituted cAMP derivatives shown in Figure 1—5′-*allo*-methyl-cAMP (**21**), 5′-*talo*-methyl-cAMP (**22**), and 5′,5′-dimethyl-cAMP (**23**) (Ranganathan et al., 1974)—exhibited K_a' values with bovine brain PK II of 0.6, 0.05, and 0.009, respectively (Murthy, 1972). The significantly greater tolerance of the protein kinase for the equatorial methyl group of **21** than for the axial methyl group of **22** suggests that the bovine brain PK II has strict steric requirements in that area of its cAMP-binding site adjacent to the 5′ carbon of cAMP.

The PK I and PK II isozymes differ in their capacity to tolerate the steric and/or electronic effects of the 5′-thio and 5′-amido moieties. The PK II isozymes are more tolerant than the PK I isozymes to the effects of substituting a sulfur for the 5′ oxygen. If the difference is due to a greater sensitivity of PK I isozymes to only the electronic or only the steric effects of a 5′ sulfur, then it is difficult to understand why 5′-amido-cAMP (**13**) is 10-fold more potent as an activator of PK I than of PK II and why 5′-methylene-cAMP (**14**) is of equal potency with the two isozymes. These differences may result from divergent electronic and steric requirements of the two isozymes. The preferential activation of PK II by **12** may be a result of the greater tolerance of PK II for an electronic alteration of the 5′ oxygen (Shuman et al., 1973), whereas the preferential activation of PK I by **13** may be due to the greater tolerance of PK I for steric or conformational changes produced by the amido moiety (Panitz et al., 1975). The greater

tolerance of PK I to the steric or conformational alterations present in **13** and **14** is apparently not consistent with the report of Jones et al. (1973), who found that 5′-methylene-1,N^6-etheno-cAMP (**24**) and 5′-*allo*-methyl-1,N^6-etheno-cAMP (**25**) were 10 times and 70 times, respectively, more potent activators of bovine brain PK II than of rabbit muscle PK I (see Figure 1 for structures). These latter results should be interpreted with caution because 1,N^6-etheno-cAMP (**26**, Figure 1) itself preferentially activates PK II (Jones et al., 1973; Miller et al., 1980b) and because the addition of a 1,N^6-etheno moiety to certain cAMP analogues has been found to dramatically and unpredictably alter the PK I/PK II isozyme specificity of the analogues (Miller et al., 1980b).

The lack of activity of adenosine cyclic 3′,5′-sulfate (**15**) and of the diastereomers of cAMP ethyl ester (**17**) and cAMP dimethylphosphoramidate (**18**) indicates that both PK I and PK II require a changed phosphate for binding, a conclusion reached by Severin et al. (1975), who noted that **15** had a very low affinity for porcine brain cAMP-dependent protein kinase. Other alkyl (methyl and propyl) and a number of benzyl triesters of cAMP have also been shown to lack significant activity as activators of bovine brain PK II or bovine heart PK II (Gillen & Nagyvary, 1976; Engels & Schlaeger, 1977).

cAMP phosphorothioate (**16**), in which the exocyclic oxygen of the cyclic phosphate ring is replaced by sulfur, was 6 times more potent as a PK II activator than as a PK I activator. This analogue was half as active as cAMP with the PK II isozymes. This finding confirms the report of Eckstein et al. (1974), who examined **16** as an activator of bovine heart PK II. Because **16** is a mixture of its two diastereomers (Eckstein et al., 1974), it is possible that only one of them is able to activate the protein kinases. Jastorff & Bär (1973) separated the diastereomers of 5′-amido-cAMP phosphorothioate (**27**, Figure 1) and found that one was 4 times more potent than the other (the absolute configuration of the diastereomers was not determined) as an activator of bovine muscle cAMP-dependent protein kinase; this suggests that one of the diastereomers of **16** may be more potent than the other as a kinase activator.

Jastorff et al. (1979) has presented a model for the chemical interactions of cAMP with the regulatory subunit of rabbit skeletal muscle PK I based on the ability of ribose ring and cyclic phosphate ring analogues of cAMP to compete with [^{3}H]cAMP for binding to the holoenzyme. These investigators propose that the ribose–cyclic phosphate ring system is bound to the kinase by means of interactions with the 2′-hydroxyl group, the 3′ oxygen, the 5′ oxygen, and the negative charge. The results presented here are consistent with this model and allow its extension to the porcine skeletal muscle PK I, to the bovine brain PK II, and to the bovine heart PK II. As discussed above, the data presented here show that there are significant differences between PK I and PK II with respect to their abilities to be activated by certain cAMP analogues with modifications in the 3′ and 5′ positions and in the cyclic phosphate. Specifically, the 5′ oxygen is relatively more important for the activation of PK I, and the 3′ oxygen is relatively more important for the activation of PK II. Moreover, Jastorff et al. (1979) has suggested that the charged group on the rabbit skeletal muscle PK I is directed toward one of the two exocyclic oxygens on the cyclic phosphate ring. The results presented here suggest that this may not be the case for the PK II isozymes. To support this conclusion, we have examined the individual diastereomers (kindly supplied by Dr. W. J. Stec) of cAMP phosphorothioate (**16**) and found that the S_p and R_p diastereomers exhibited K_a' values of 0.18 and 0.012, respectively, with the rabbit skeletal muscle PK I (J. P. Miller and W. J. Stec, unpublished results). In contrast, these two analogues give K_a' values of 0.39 and 0.55, respectively, with the bovine brain PK II (J. P. Miller and W. J. Stec, unpublished results). These results suggest that the charged group on the bovine brain PK II is located between the two exocyclic oxygens on the cyclic phosphate ring and that the charged group on the rabbit muscle PK I is directed toward the equatorial oxygen. Our data extend those of Jastorff et al. (1979) by demonstrating that PK I is significantly more sensitive than is PK II to the effects of replacing the 4′ oxygen with a methylene moiety. These results suggest that a hydrogen bond to the 4′ oxygen may be involved in interaction between cAMP and PK I but that such an interaction is apparently unnecessary for interaction between cAMP and PK II.

Hoppe et al. (1978) has presented a model for the interaction of cAMP with the rabbit skeletal muscle PK I based on the ability of adenine ring analogues of cAMP to compete with [^{3}H]cAMP binding to the holoenzyme. In this model the N^6 position does not interact with PK I and there is a hydrophobic region adjacent to the 2 position, conclusions consistent with our data on the bovine brain PK II (Boswell et al., 1973; Meyer et al., 1973b, 1975; Yagura et al., 1980; Miller et al., 1980a). In addition, we have shown that, in general, there is no significant effect of either modification (Miller et al., 1978) or substitution (Miller et al., 1973a,c, 1980a; Muneyama et al., 1974; Uno et al., 1976; Yagura & Miller, 1980) of the imidazole portion (7 and 8 positions) of the adenine ring system on the ability of the resulting analogues to activate bovine brain PK II.

Jastorff et al. (1979) has further refined this model for the rabbit muscle PK I by suggesting that the adenine ring system is bound in a "hydrophobic cleft" without any hydrogen bonding interactions between the cAMP binding site on the regulatory subunit and the ring nitrogens. Our results with PK II isozymes from bovine brain, bovine heart, and rat liver indicate that the lone-pair electrons of the nitrogen in the 3 position of the adenine ring system are involved in the binding of cAMP to the regulatory subunit of type II cAMP-dependent protein kinases (Miller et al., 1978). This conclusion is based largely on the poor ability of 2-aza-cAMP and of 3-deaza-cAMP to activate these three PK II isozymes. We have recently found that these two analogues are able to activate PK I isozymes from rabbit muscle and porcine muscle almost as efficiently as does cAMP (Yagura et al., 1980; Miller, 1981). In addition, the presence of electron-withdrawing substituents in the 2 position was found to be detrimental to the binding of the analogues to PK II but improved binding to PK I (Yagura et al., 1980). Taken together, these results allow us to extend the model of Jastorff et al. (1979) for the interaction of cAMP with PK I and to suggest that the binding of the adenine ring system is different for PK II. We suggest that the interaction of cAMP with PK I involves the formation of a charge-transfer complex between at least the pyrimidine portion of the adenine ring system and the cAMP binding site since a reduction in the electron density of the ring improves binding to the type I isozymes. In contrast, a vital interaction between PK II and cAMP involves the formation of a hydrogen bond between the 3 nitrogen and the cAMP binding site.

In summary, the PK I and PK II isozymes are similar in their relative abilities to be activated by analogues with modifications or substitutions in the 2′, 6, 7, and 8 positions and with modifications or substitutions that eliminate the charge on the cyclic phosphate, where the 2′-hydroxyl group

and charged phosphate are essential for the activation of both isozymes and where the 6-amino group and imidazole ring are apparently uninvolved in the activation. In contrast, the PK I and PK II isozymes differ in the relative abilities to be activated by analogues with modifications or substitutions in the 3′, 4′, 5′, and 3 positions and with modifications that alter the charge distribution on the cyclic phosphate, where the 4′ and 5′ oxygens and an unaltered charge distribution on the cyclic phosphate are more important for the activation of PK I than of PK II, and where the 3′ oxygen and 3 nitrogen are more important for the activation of PK II than of PK I.

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pH Properties and Chemical Mechanism of Action of 3-Hydroxy-3-methylglutaryl Coenzyme A Reductase†

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ABSTRACT: The pH variation of the kinetic parameters V and V/K for the oxidation of mevaldate by $NADP^+$ in the presence of CoA (reverse reaction) and for the reduction of mevaldate by NADPH in the presence or absence of CoA (forward reaction) for the reactions catalyzed by 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase was examined. In the reverse reaction a group, X, on the enzyme with a pK of 7.9 must by unprotonated for $NADP^+$ binding and catalysis. The presence of $NADP^+$ shifts this pK to a value below 6. The V/K profile for mevaldate shows that deprotonation of a group, Y, with a pK of 6.7 decreased the reaction rate by a factor of 27. In the forward reaction, the pK of the X group was about 6.9 except when CoA and mevaldate were both present, in which case it was shifted to 7.8. CoA decreased the K_ms for mevaldate about 10-fold without changing the V_{max} at the optimum protonation state. The catalytic group, X, was identified as a cationic acid, probably histidine. A catalytic mechanism is proposed in which the protonated form of histidine induces hydride transfer from the A side of NADPH by donating a proton to the carbonyl of HMG-CoA or to the aldehyde form of mevaldate. The role of the Y group, which from its pK of 6.7 and the chemistry involved may be a carboxyl group, is presumably to catalyze conversion of mevaldate thiohemiacetal formed in the reduction of HMG-CoA to CoA and the free aldehyde form of mevaldate. Mevaldate was shown by ^1H NMR to contain 44% hydrate in D_2O and 39% in H_2O. When an enzymatic method was used, it was also determined that only one stereoisomer of mevaldate is used by HMG-CoA reductase.

The enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA)[1] reductase catalyzes the two-step reduction of (4*R*,3*S*)-HMG-CoA to the isoprenoid precursor, (2*R*,3*R*)-mevalonate (Cornforth et al., 1974). This compound is a precursor in the synthesis of terpenes and steroids that are vital compounds in plants and animals. Because of the close correlation of mammalian HMG-CoA reductase activity with the rate of cholesterol biosynthesis, the physiological regulation of this enzyme in rat liver and human fibroblasts has been extensively studied [for reviews, see Rodwell et al. (1976) and Gibson & Ingebritsen (1978)]. Since the yeast enzyme presents some features similar to those of the mammalian enzyme (Kawaguchi, 1970; Hatanaka et al., 1970) and since its purification and physicochemical properties are well-defined (Qureshi et al., 1976a), the current study of the chemical mechanism of HMG-CoA reductase action was undertaken with this enzyme.

The kinetic mechanism and the probable rate-limiting step of the reaction catalyzed by yeast HMG-CoA reductase were determined with steady-state kinetics by Qureshi et al. (1976b) in the reverse and forward reactions by analyzing the two partial reductive steps:

$$\text{mevaldate} + NADP^+ + \text{CoA} \rightleftharpoons \text{HMG-CoA} + NADPH + H^+ \quad (1)$$

$$\text{mevaldate} + NADPH + H^+ \rightleftharpoons \text{mevalonate} + NADP^+ \quad (2)$$

Qualitative and quantitative initial velocity analysis of reactions

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[1] Abbreviations used: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; DMF, dimethylformamide; NMR, nuclear magnetic resonance; Me_4Si, tetramethylsilane.