Adenosine Cyclic 3′,5′-Monophosphate Dependent Protein Kinase: Interaction of the Catalytic Subunit and Holoenzyme with *lin*-Benzoadenine Nucleotides†

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ABSTRACT: The interaction of *lin*-benzoadenosine di- and triphosphates with the catalytic subunit and type II holoenzymes of adenosine cyclic 3′,5′-monophosphate (cAMP) dependent protein kinase has been investigated by steady-state kinetics and fluorescence spectroscopy. *lin*-Benzo-ADP is a competitive inhibitor of the catalytic subunit with respect to ATP with a K_i (8.0 μM) similar to the K_i for ADP (9.0 μM). This value agrees well with the K_d (9.0 μM) determined by fluorescence polarization titration. Type II holoenzymes from bovine brain and skeletal muscle have K_d values for *lin*-benzo-ADP of 3.4 μM and 3.5 μM, respectively, and each binds approximately 2 mol/mol of R_2C_2 tetramer. Furthermore, fluorescence polarization studies indicate that both the catalytic subunit and type II holoenzyme bind *lin*-benzo-ADP rigidly, so that there is little or no rotation of the *lin*-benzoadenine portion of the molecule within the nucleotide binding site. *lin*-Benzo-ATP is a substrate for the phosphotransferase activities of protein kinase with peptides, water, or type II regulatory subunit as phosphoryl acceptors. With Leu-Arg-Arg-Ala-Ser-Leu-Gly as phosphoryl acceptor, the K_m for *lin*-benzo-ATP is 11.3 μM, and that for ATP is 11.9 μM. The V_{max} with *lin*-benzo-ATP is 20% of the V_{max} with ATP as the substrate [24.9 ± 1.8 μmol/(min·mg) vs. 5.0 ± 1.2 μmol/(min·mg)]. Thus *lin*-benzo-ATP is the best nucleotide substrate (besides ATP) for the catalytic subunit reported. 1,N^6-Etheno-ATP (ϵATP), on the other hand, is a poor substrate for the catalytic subunit with a K_m of 1.8 mM and a V_{max} that is 4% of the V_{max} for ATP, making it unsuitable as a fluorescence probe for cAMP-dependent protein kinase.

Adenosine cyclic 3′,5′-monophosphate dependent protein kinase (ATP:protein phosphotransferase; EC 2.7.1.37) catalyzes the phosphorylation of serine and threonine residues in proteins using MgATP as phosphoryl donor. The enzyme exists in the cell as an inactive tetramer composed of dissimilar regulatory (R) and catalytic (C) subunits and is activated by cAMP[1] as follows (Corbin et al., 1978):

$$R_2C_2 + 4cAMP \rightleftharpoons R_2(cAMP)_4 + 2C$$

Two classes of cAMP-dependent protein kinase exist in mammalian cells. These are referred to as types I and II on the basis of their order of elution from DEAE-cellulose (Corbin et al., 1975). The two forms differ primarily in their regulatory subunits (Beavo et al., 1975), which confer different properties to the respective holoenzymes. Although they are catalytically inactive toward exogenous protein substrates, both type I and type II holoenzymes interact with MgATP. Type II holoenzymes, for example, undergo an intramolecular autophosphorylation (Erlichman et al., 1974; Maeno et al., 1974; Rosen & Erlichman, 1975). The type I holoenzyme does not undergo autophosphorylation but possesses two high-affinity MgATP binding sites (K_d = 50 nM) per R_2C_2 tetramer (Beavo et al., 1975; Hofmann et al., 1975). Hoppe and co-workers have shown that the MgATP binding site is distinct from that of cAMP and have studied these sites using a series of ATP analogues that were modified in various portions of the molecule (Hoppe et al., 1977, 1978; Hoppe & Friest, 1979). Much less, however, is known about nucleotide binding to the type II holoenzymes due to the lack of a suitable probe.

Another method that has been used to study nucleotide binding sites is chemical modification. Affinity labeling with 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine (FSBA) revealed a lysine residue at the active site (Zoller & Taylor, 1979; Zoller et al., 1981). Modification with ethoxyformic anhydride suggested that a tyrosine residue is present in the MgATP binding site (Witt & Roskoski, 1975a). On the basis of modification with several reagents, it has been postulated that one or both of the enzymic cysteines are present at or near the active site (Sugden et al., 1976; Peters et al., 1977; Kupfer et al., 1979; Nelson & Taylor, 1981; Hartl & Roskoski, 1982).

Yet another method for studying nucleotide binding sites involves the use of fluorescent nucleotide analogues, which allow rapid and sensitive measurement of protein–ligand interactions. *lin*-Benzoadenine nucleotides (Figure 1) are fluorescent "stretched out" analogues of adenine nucleotides in which a benzene ring (actually four carbons) has been inserted between the pyrimidine and imidazole rings, resulting in a 2.4-Å widening of the purine ring system (Leonard et al., 1975, 1976; Scopes et al., 1977). These compounds have been used as dimensional probes of the nucleotide sites of several enzymes, including adenosine deaminase (Leonard et al., 1976), mitochondrial ATP synthetase and adenine nucleotide carrier (Kauffman et al., 1978), and a group of phosphotransferases including pyruvate kinase, phosphofructokinase, phosphoglycerate kinase, and hexokinase (Scopes et al., 1977). Furthermore, Schmidt and co-workers (Schmidt et al., 1978) showed that *lin*-benzoadenosine cyclic 3′,5′-monophosphate

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[1] Abbreviations: cAMP, adenosine cyclic 3′,5′-monophosphate; Mops, 3-(*N*-morpholino)propanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; Ser-peptide, Leu-Arg-Arg-Ala-Ser-Leu-Gly; dansyl-Ser-peptide, N^{α}-dansyl-Leu-Arg-Ala-Ser-Leu-Gly; ϵATP, 1,N^6-etheno-ATP; HPLC, high-performance liquid chromatography; NBD-Cl, 7-chloro-4-nitro-2,1,3-benzoxadiazole; DEAE, diethylaminoethyl; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

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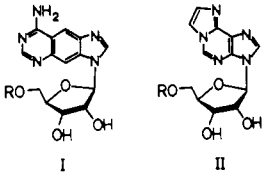

I II

FIGURE 1: Structures of (I) *lin*-benzoadenine and (II) 1,N^6-etheno-adenine nucleotides. R refers to the diphosphate or triphosphate substituents.

activates protein kinase and *lin*-benzoadenosine is an inhibitor of the enzyme.

We have examined the interaction of the fluorescent adenine nucleotide analogues *lin*-benzo-ADP, *lin*-benzo-ATP, and 1,N^6-etheno-ATP (ϵATP) with free catalytic subunit and type II holoenzymes from bovine skeletal muscle and brain. We find that *lin*-benzoadenosine di- and triphosphate, but not ϵATP, interact strongly with the catalytic subunit. *lin*-Benzo-ATP is a good substrate for the phosphotransferase reaction with a K_m similar to that of ATP and a V_{max} that is 20% that of ATP but ϵATP binds poorly and has a low V_{max} relative to that of *lin*-benzo-ATP making it unsuitable as a fluorescent probe of cAMP-dependent protein kinase.

Materials and Methods

Protein Kinase. Catalytic and regulatory subunits from bovine brain were purified as described previously (Hartl & Roskoski, 1982). Subunits were prepared from bovine skeletal muscle by the same methodology. Holoenzymes were reconstituted by incubating regulatory subunit with a 2-fold molar excess of catalytic subunit at 4 °C for 2 h followed by gel filtration on a 2.5 cm × 70 cm column of Sephadex G-100, 40 mesh (flow rate 10 mL/h), to resolve the holoenzyme from the catalytic subunit.

Assays. Phosphotransferase activity with *lin*-benzo-ATP as a substrate was measured by two different procedures. The first method is a modification of the HPLC assay developed by Kemp (1980). Incubation mixtures (0.2 mL) contained 50 mM Mops (pH 7.0), 10 mM $MgSO_4$, 100 μM dansyl-Ser-peptide, and 1 mM ATP, ϵATP, or *lin*-benzo-ATP. The reaction was initiated by adding catalytic subunit (final concentration of 10 μg/mL). Portions (20 μL) were removed at various times and added to 60 μL of ice-cold methanol. The samples were centrifuged at 13700*g* for 5 min to remove insoluble material. Portions (20 μL) were chromatographed on a reverse-phase column (Alltech, μBondapak C_{18}, 3.4 mm × 30 cm) under isocratic elution with 40 mM sodium phosphate (pH 7.0)–methanol (2:3 v/v) at a flow rate of 1.5 mL/min. A variable-wavelength UV monitor set at 210 nm was used to detect the products.

The second assay used was the phosphocellulose adsorption method of Witt & Roskoski (1975b) as modified (Hartl & Roskoski, 1982; Cook et al., 1982) with *lin*-benzo-[γ-^{32}P]ATP as phosphoryl donor and Ser-peptide as phosphoryl acceptor. *lin*-Benzo-[γ-^{32}P]ATP was synthesized by the method of Schendel & Wells (1973) with the following modifications. *lin*-Benzo-ADP was used in place of ADP; the reaction was carried out for 3 h instead of 15 min; and DEAE-cellulose (Whatman DE-52) was used in place of DEAE-Sephadex in the purification step. The assay using radiolabeled *lin*-benzo-ATP, which was more rapid and more sensitive than the HPLC method, was used to determine the kinetic parameters of the catalytic subunit with *lin*-benzo-ATP as substrate.

Polarization Titrations. Titrations were performed in an SLM 4800 spectrofluorometer interfaced with a Hewlett-Packard HP9825A calculator. Excitation was at 334 nm and emitted light was isolated with Schott KV 389 filters. Polarization (P_{obsd}) was calculated with a program supplied by SLM Instruments, Inc. Titrations were carried out at 23 °C in 50 mM Mops–100 mM NaCl, pH 7.0. Two types of titrations were carried out in order to determine binding constants.

(*a*) *Dilution Titrations.* *lin*-Benzo-ADP was added to a solution of protein kinase to a concentration of 0.1–6 μM, and its polarization was measured (P_f). In the absence of Mg^{2+}, this polarization was the same in the presence or absence of protein kinase, indicating that little or no binding occurs in the absence of metal under these conditions. $MgSO_4$ was then added to a final concentration of 10 mM and the polarization again recorded. The protein concentration was then varied (at constant nucleotide concentration) by repeatedly diluting the solution in the cuvette with a solution containing concentrations of magnesium and nucleotide equal to those in the cuvette. $1/P_{obsd}$ was plotted vs. 1/[protein kinase], and the value of P_b (i.e., the polarization value when all *lin*-benzo-ADP is bound) was obtained from the ordinate intercept, i.e., at infinite protein kinase concentration.

The theoretical polarization of *lin*-benzo-ADP rigidly bound to protein kinase was calculated with Perrin's equation (Perrin, 1926):

$$\frac{1}{P} - \frac{1}{3} = \left(\frac{1}{P_0} - \frac{1}{3}\right)\left(1 + \frac{RT\tau}{\eta V_0}\right)$$

where P_0 is the intrinsic polarization of the fluorophore, R is the universal gas constant, T is the absolute temperature, η is the viscosity, τ is the lifetime of the excited state (τ = 4.2 ns; VanDerLijn et al., 1978a), and V_0 is the molecular volume of the fluorescent unit (V = 0.739 cm^3/g; Sugden et al., 1976).

(*b*) *Addition Titrations.* After determination of P_f as described above, $MgSO_4$ was added and the polarization recorded. The nucleotide concentration was then varied, and P_{obsd} was measured at each nucleotide concentration. The concentration of bound nucleotide at each point was calculated from the following:

$$B = \frac{P_{obsd} - P_f}{P_b - P_f}[\textit{lin}\text{-benzo-ADP}]_t$$

where B is the concentration of *lin*-benzo-ADP bound to enzyme, $[\textit{lin}\text{-benzo-ADP}]_t$ is the total concentration of *lin*-benzo-ADP the cuvette, and P_b (determined from the dilution titration), P_f, and P_{obsd} are as described above. Once the values for bound *lin*-benzo-ADP are known, the data can be plotted as a Scatchard plot (bound/free vs. bound), the dissociation constant determined from the slope of the line ($K_d = -1/\text{slope}$), and the number of binding sites can be determined from the abscissa intercept.

The average angle of rotation (ω) of *lin*-benzo-ADP rigidly bound to catalytic subunit was calculated from the following equation (Perrin, 1929):

$$\frac{1}{P_b} - \frac{1}{3} = \left(\frac{1}{P_0} - \frac{1}{3}\right)\left(\frac{2}{3\cos^2\omega - 1}\right)$$

where P_0 is the intrinsic polarization of the fluorophore. The value of P_0 for *lin*-benzo-ADP was determined by measuring fluorescence polarization in solutions of various viscosities, where the viscosity (η) was varied with sucrose or glycerol; $1/P$ was plotted vs. T/η, and $1/P_0$ was obtained from the ordinate intercept where $\eta \rightarrow \infty$. The value of P_0 was calculated to be 0.398 for *lin*-benzo-ADP at 334 nm. All experimental values represent the means and standard errors of at least three independent determinations.

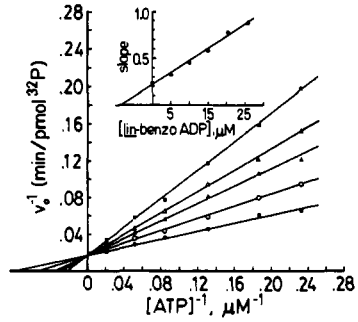

FIGURE 2: Competitive inhibition of catalytic subunit activity by *lin*-benzo-ADP. Catalytic subunit (10 ng) was incubated at 30 °C in a 0.2-mL volume that contained 50 mM Mops (pH 7.0), 10 mM $MgSO_4$, 0.25 mg/mL bovine serum albumin, 0.15 mM Ser-peptide, [γ-^{32}P]ATP concentrations from 4.2 to 50 μM (1200 cpm/pmol), and *lin*-benzo-ADP concentrations of 0 (●), 5.0 (○), 9.9 (▲), 14.9 (△), 20.1 (■), and 25 μM (not shown). (Inset) Slope replot of the data. The abscissa intercept corresponds to a K_i of 8 μM.

Table I: Binding Constants of *lin*-Benzo-ADP to Catalytic Subunit and Type II Holoenzyme[a]

enzyme	K_d (μM)	$\bar{n}$ [b]	K_i (μM)
catalytic subunit[c]	8.95 ± 1.48	0.98 ± 0.20	8.0 ± 2.1
type II holoenzyme (brain)	3.35 ± 1.08	2.2 ± 0.1	
type II holoenzyme (skeletal muscle)	3.51 ± 1.11	1.8 ± 0.3	

[a] K_d values and number of binding sites per enzyme ($\bar{n}$) were determined from Scatchard plots of data obtained from fluorescence-polarization titrations. K_i was determined kinetically as described in the legend to Figure 2. [b] $\bar{n}$, number of binding sites per catalytic subunit or holoenzyme. [c] Catalytic subunits from brain and skeletal muscle yielded the same values (ten determinations).

Results

Interaction of lin-Benzo-ADP with Catalytic Subunit. It has been shown (Schmidt et al., 1978) that *lin*-benzoadenosine is an inhibitor of protein kinase. If *lin*-benzo-ADP interacts at the active site, then it would be expected to be a competitive inhibitor with respect to MgATP. Catalytic subunit was therefore incubated with varied ATP concentrations at a fixed, saturating Ser-peptide concentration (150 μM; Cook et al., 1982) in the presence of various fixed concentrations of *lin*-benzo-ADP. As shown in Figure 2, Mg-*lin*-benzo-ADP is a competitive inhibitor with respect to MgATP. A slope vs. *lin*-benzo-ADP replot (Figure 2, inset) is linear and yields a K_i for *lin*-benzo-ADP of 8.0 ± 2.1 μM. This corresponds well with the K_i for ADP of 9.0 ± 2.1 μM (Table I). Thus *lin*-benzo-ADP, which is 2.4 Å wider in the adenine moiety, binds to the active site of the catalytic subunit as well as the natural reaction product, ADP.

Measurement of K_d by Fluorescence-Polarization Titration. For measurement of the interaction of *lin*-benzo-ADP with the catalytic subunit and holoenzymes, fluorometric titrations were performed. *lin*-Benzo-ADP was used instead of *lin*-benzo-ATP because protein kinase possesses intrinsic ATPase activity (Armstrong et al., 1979) for which *lin*-benzo-ATP is a substrate (not shown). There is no change in the fluorescence emission spectrum of *lin*-benzo-ADP in the presence of catalytic subunit (not shown). Binding to the enzyme, however, is accompanied by an increase in polarization (Figure 3). The binding of *lin*-benzo-ADP to free catalytic subunit and type II holoenzymes from skeletal muscle and brain was measured by fluorescence-polarization titration (Figure 3) as described under Materials and Methods. The results are summarized

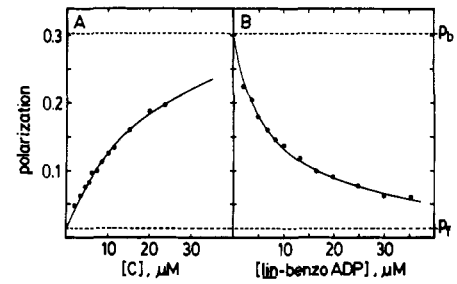

FIGURE 3: Polarization titrations of catalytic subunit with *lin*-benzo-ADP. Measurements were performed at 23 °C in 50 mM Mops (pH 7.0), 0.1 M NaCl, and 10 mM $MgSO_4$. (Panel A) Dilution titration. Catalytic subunit was varied at a constant (5 μM) *lin*-benzo-ADP concentration in the presence of 10 mM $MgSO_4$. (Panel B) Addition titration. *lin*-Benzo-ADP was varied at a constant (6 μM) catalytic subunit concentration by addition of aliquots of a concentrated *lin*-benzo-ADP solution.

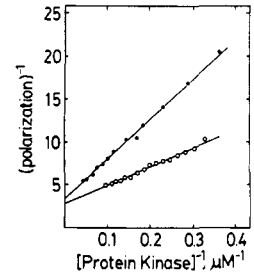

FIGURE 4: Determination of P_b for catalytic subunit and type II holoenzyme. Dilution titrations were performed with catalytic subunit (●) and type II holoenzyme (○) as described in the legend to Figure 3A and under Materials and Methods. From the ordinate intercepts, P_b values of 0.298 and 0.334 were obtained for catalytic subunit and holoenzyme, respectively.

Table II: Fluorescence Polarization of Enzyme-Bound *lin*-Benzo-ADP

	theoretical[a]		obsd[b]	
enzyme	P_b	ω	P_b	ω
catalytic subunit (skeletal muscle)	0.297	26°	0.301 ± 0.010	25°
type II holoenzyme (skeletal muscle)	0.369	14°	0.334 ± 0.017	20°

[a] P_b and ω were calculated from Perrin's equations as described under Materials and Methods. [b] Values for P_b are from polarization titrations (Figure 3). The value for average angle of rotation (ω) was calculated with this value and P_o for *lin*-benzo-ADP of 0.40 as described under Materials and Methods.

in Table I. The K_d of 9.0 ± 1.5 μM for the free catalytic subunit determined by this method agrees well with the kinetically determined K_i of 8.0 ± 2.1 μM. Type II holoenzymes from skeletal muscle and brain had K_d values of 3.5 μM and 3.4 μM, respectively, and both bound 2 mol of *lin*-benzo-ADP/mol of holoenzyme. These results confirm and extend those of Granot and co-workers (Granot et all., 1980), who determined, using nuclear magnetic resonance, that type II holoenzyme binds ADP and MnADP with higher affinity than does free catalytic subunit. Thus the presence of type II regulatory subunit from skeletal muscle or brain results in an increase in affinity of the enzyme for adenine or *lin*-benzo-adenine nucleotides.

We determined the polarization of *lin*-benzo-ADP bound to catalytic subunit and type II holoenzyme by extrapolating a plot of P^{-1} vs. [protein kinase]$^{-1}$ to zero (Figure 4). The values of P_b for catalytic subunit and type II holoenzyme were 0.301 and 0.334, respectively. These values correlated well with P_b values calculated for rigidly bound *lin*-benzo-ADP with

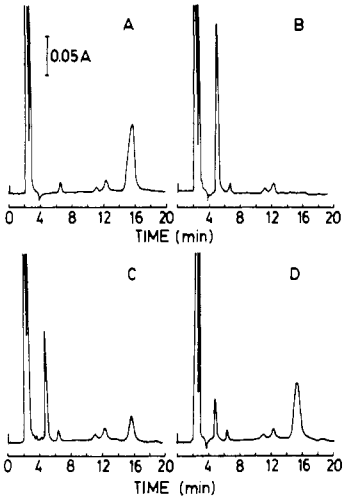

FIGURE 5: HPLC assay of catalytic subunit using ATP, *lin*-benzo-ATP, and ϵATP. Catalytic subunit (1.5 μg) was incubated at 30 °C in 0.2 mL containing 50 mM Mops, 10 mM $MgSO_4$, 0.1 mM dansyl-Ser-peptide, and 1 mM ATP, *lin*-benzo-ATP, or ϵATP. Portions (20 μL) were removed at various times and added to 60 μL of ice-cold methanol. HPLC (20-μL aliquots) was run as described under Materials and Methods. (Panel A) Before adding enzyme; (panel B) after 4 min with 1 mM ATP; (panel C) after 4 min with 1 mM *lin*-benzo-ATP; (panel D) after 4 min with ϵATP. Dansyl-Ser-peptide elutes at 15.5 min and the phosphorylated peptide elutes at 5 min. The large peaks at 2–3 min represent nucleotides, and the small peaks at 6.3, 11.2, and 12.3 min are impurities in the dansyl-Ser-peptide.

the Perrin equation (Table II). Furthermore, our polarization data allowed us to calculate the average angle of rotation of bound *lin*-benzo-ADP during the lifetime of the excited state. The values of 25 and 20° obtained for the catalytic subunit and holoenzyme, respectively, are very close to the theoretical values of 26 and 14° calculated from the Perrin equation. Thus, most or all of the observed rotation is due to rotation of the protein inself. This indicates that both the catalytic subunit and type II holoenzyme bind *lin*-benzo-ADP rigidly, so that there is little or no rotation within the adenine binding site.

lin-Benzo-ATP and ϵATP as Substrates for Catalytic Subunit. To determine if *lin*-benzo-ATP and ϵATP are substrates for the catalytic subunit, a method was required to follow formation of phosphorylated product. Kemp (1980) developed the use of the N^{α}-dansyl derivative of Leu-Arg-Arg-Ala-Ser-Leu-Gly (dansyl-Ser-peptide) as phosphoryl acceptor. The phosphorylated and unphosphorylated forms of the peptide were then separated by HPLC on a reverse-phase octadecasilane column. So that one could compare the abilities of ATP, *lin*-benzo-ATP, and ϵATP to act as phosphoryl donor in the protein kinase reaction, catalytic subunit was incubated with 0.1 mM dansyl-Ser-peptide and 1 mM concentrations of ATP, *lin*-benzo-ATP, or ϵATP. Aliquots were removed at various times and analyzed by HPLC. Figure 5 shows chromatographs of reaction mixtures before addition of catalytic subunit (panel A) and after 4 min of incubation of enzyme with ATP (panel B), *lin*-benzo-ATP (panel C), or ϵATP (panel D) as phosphoryl donor. In all cases, appearance of the phosphorylated form of the peptide (retention time 4.5 min) and concomitant disappearance of the dephospho form of the peptide (retention time 15 min) were observed. The time course of the reaction (Figure 6) shows that the reaction was essentially complete within 2–4 min with ATP as substrate and was 85% complete within 8 min with *lin*-benzo-ATP as phosphoryl donor. Under identical conditions, however, ϵATP was a much poorer substrate than either ATP or *lin*-benzo-ATP. Thus the effectiveness of nucleotides as phosphoryl

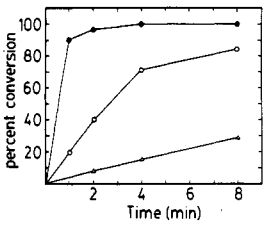

FIGURE 6: Time course of phosphorylation of dansyl-Ser-peptide by ATP, *lin*-benzo-ATP, and ϵATP. Dansyl-Ser-peptide was phosphorylated with ATP (●), *lin*-benzo-ATP (○), or ϵATP (△) as described in the legend to Figure 5. Percent conversion is (area under "phospho" peak)/(area under "phospho" peak + area under "dephospho" peak) × 100.

Table III: Kinetic Parameters for Catalytic Subunit with ATP, *lin*-Benzo-ATP, and ϵATP as Substrates

substrate	K_m (μM)	V_{max} [μmol/(min·mg)]	k_{cat} (min^{-1})
ATP[a]	11.9 ± 2.7	24.9 ± 1.8	996 ± 72
lin-benzo-ATP[a]	11.3 ± 3.1	5.0 ± 1.2	200 ± 48
ϵATP[b]	1800	1.1	44

[a] Determined by radioisotopic assay with Ser-peptide as phosphoryl acceptor as described under Materials and Methods. Reactions were performed at a fixed, saturating concentration of Ser-peptide (0.15 mM). Nucleotide concentrations were varied from 4 to 100 μM. K_m and V_{max} were determined from plots of v_0 vs. v_0/[nucleotide] according to Hofstee (1952). [b] Determined by HPLC assay with dansyl-Ser-peptide as phosphoryl acceptor as described under Materials and Methods. ϵATP concentrations were varied from 0.1 to 4 mM.

donors in the protein kinase reaction was ATP > *lin*-benzo-ATP ≫ ϵATP. For determination of whether the decreased effectiveness of the fluorescent nucleotides is due to a lower V_{max}, a higher K_m, or both, steady-state kinetic studies were performed with *lin*-benzo-ATP and ϵATP. The results are summarized in Table III. ϵATP has both a reduced V_{max} and a high K_m relative to those of ATP, which probably reflects poor binding to the catalytic subunit. *lin*-Benzo-ATP, on the other hand, has a K_m of 11.3 ± 3.1 μM, which corresponds well with the K_m for ATP of 11.9 ± 2.7 μM. However, the V_{max} with *lin*-benzo-ATP is 5.0 ± 1.2 μmol/(min·mg of protein), which is only 20% of the V_{max} with ATP as the substrate.

Autophosphorylation of Type II Holoenzymes with lin-Benzo-ATP. Type II holoenzymes undergo an intramolecular autophosphorylation reaction characterized by the incorporation of 1 mol of phosphate/mol of regulatory protomer (Erlichman et al., 1974; Maeno et al., 1974; Rosen & Erlichman, 1975; Rangel-Aldao & Rosen, 1976). We find that *lin*-benzo-ATP is a substrate for the autophosphorylation of type II holoenzyme from bovine skeletal muscle (Figure 7). In the absence of cAMP, the rate of phosphorylation is greater than or equal to the rate when ATP is the phosphoryl donor (Figure 7). However, when cAMP was present, ATP and *lin*-benzo-ATP behave quite differently. Whereas the rate of regulatory subunit phosphorylation by ATP is increased by cAMP, the rate of phosphorylation by *lin*-benzo-ATP is decreased in the presence of cAMP. This suggests that *lin*-benzo-ATP is a better sustrate for the intramolecular phosphorylation reaction of the holoenzyme than for the intermolecular phosphorylation of $R_2(cAMP)_4$, which occurs in the presence of cAMP (Rangel-Aldao & Rosen, 1976).

Binding of lin-Benzo-ADP to Chemically Modified Catalytic Subunit. Several chemical-modification reagents have been used to identify amino acid residues at or near the active

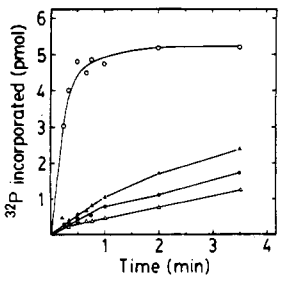

FIGURE 7: Autophosphorylation of type II holoenzyme with ATP and *lin*-benzo-ATP. Skeletal muscle R_2C_2 (15 nM) was incubated on ice in a reaction mixture (0.3 mL) containing 50 mM Mops (pH 7.0), 10 mM $MgSO_4$, 1 mg/mL bovine serum albumin, and 0.5 μM [γ-^{32}P]ATP (○, ●) or 0.5 μM *lin*-benzo-[γ-^{32}P]ATP (△, ▲) in the presence (○, △) and absence (●, ▲) of 5 μM cAMP. Portions (40 μL) were removed at the indicated times, spotted on Whatman 3MM paper strips, and immersed immediately in cold 10% trichloroacetic acid. The strips were washed twice in cold 5% trichloroacetic acid (10 mL/strip), once with water (10 mL/strip), twice with acetone (5 mL/strip), and once with petroleum ether (2 mL/strip). After the strips were air-dried, radioactivity was measured by liquid-scintillation spectrometry.

site of the catalytic subunit. These include ethoxyformic anhydride (Witt & Roskoski, 1975a), 5′-[*p*-(fluorosulfonyl)-benzoyl]adenosine (FSBA; Hixson & Krebs, 1979; Zoller & Taylor, 1979; Zoller et al., 1981), and NBD-Cl (Hartl & Roskoski, 1982). All three of these reagents have been postulated to modify residues at or near the nucleotide binding site of the catalytic subunit. So that one could determine if these compounds alter or inhibit nucleotide binding, catalytic subunit was reacted with the respective reagents, and *lin*-benzo-ADP binding to the modified enzyme was subsequently measured by fluorescence-polarization titration. FSBA, NBD-Cl, and ethoxyformic anhydride prevent *lin*-benzo-ADP from binding to the catalytic subunit (not shown). Thus, these compounds, which are thought to modify residues that are at or near the active site of the catalytic subunit, appear to act in part by inhibiting nucleotide binding.

Discussion

We have studied the interaction of *lin*-benzo-ADP, *lin*-benzo-ATP, and ϵATP with the catalytic subunit and type II holoenzymes of cAMP-dependent protein kinase. *lin*-Benzo-ADP, which is stretched out by 2.4 Å in its adenine moiety (Scopes et al., 1977), binds as well to the active site of the catalytic subunit as does the native nucleotide ADP. This is supported by two lines of evidence. First, *lin*-benzo-ADP is a linear competitive (Cleland, 1970) inhibitor with respect to MgATP (Figure 2) as is ADP. Second, the K_i determined from inhibition studies and the K_d determined from fluorescence-polarization studies are very close to the kinetically determined K_i for ADP (Table I). In addition, *lin*-benzo-ATP is a good substrate for the catalytic subunit, with a K_m equal to that of ATP and a V_{max} that is 20% of the V_{max} for ATP. A decreased V_{max} relative to ATP is characteristic of several enzymes including pyruvate kinase, 3-phosphoglycerate kinase, and acetate kinase (Leonard et al., 1978). However, protein kinase has the highest affinity for *lin*-benzoadenine nucleotides of any protein tested to date. Although the catalytic subunit will utilize other purine nucleotides such as GTP and ITP, they have K_m values of greater than 0.1 mM and V_{max} values that are around 10% of the V_{max} with ATP (Walsh & Krebs, 1973; Cook et al., 1982). Also, the present study shows that ϵATP, which contains an altered adenine ring, with N1 and N^6 binding prevented, is also a poor substrate for the catalytic subunit (Table III). Hoppe and co-workers (Hoppe et al., 1978) measured the binding of a series of ATP analogues to the catalytic subunit and found that alterations in the adenine portion of the molecule resulted in analogues with lower affinities for the nucleotide binding site. Modification of the amino group at position 6 or replacement of this group with an oxygen or hydrogen resulted in the largest decrease in affinity, indicating strict recognition of this position by the enzyme. Our present results are in complete accord with these findings. ϵATP, which is modified at N^6, binds poorly to the catalytic subunit, but *lin*-benzoadenine di- and triphosphates bind well to the nucleotide binding site. Our results indicate that although the adenine-binding portion of the nucleotide binding site has a high specificity for an unsubstituted amino group at position 6 or the equivalent (possibly as a hydrogen-bond donor), it is flexible enough to accommodate the bulky *lin*-benzoadenine moiety, which is 2.4 Å wider than adenine.

The polarization data in Table II indicate that the *lin*-benzoadenine moiety is tightly bound by the catalytic subunit. This is supported by the work of Granot and co-workers (Granot et al., 1979), who studied the interaction of Co-$(NH_3)_4$ATP with the catalytic subunit by nuclear magnetic resonance. They found that the glycosidic conformational angle (χ), which is the angle between the C-1′–O bond of ribose and the plane of the adenine ring, changes from 44 (in the binary complex) to 84° upon binding to the catalytic subunit. This indicates a strong interaction between the enzyme and adenosine portion of Co$(NH_3)_4$ATP. This tight binding by protein kinase differs from the binding of *lin*-benzo-ATP to *Escherichia coli* aspartate transcarbamylase reported previously. *lin*-Benzo-ATP (an allosteric activator) and ϵATP (an allosteric inhibitor) are both bound in a manner such that there is much rotational freedom within the nucleotide binding site (Chien & Weber, 1973; VanDerLijn et al., 1978b).

The presence of type II regulatory subunit from bovine skeletal muscle or brain increases the affinity for *lin*-benzo-ADP approximately 3-fold (Table I). This is similar to the results obtained for MnADP binding to bovine heart type II holoenzyme (Granot et al., 1979) and MgADP binding to rabbit skeletal muscle type I holoenzyme (Hoppe et al., 1978). The bovine brain and skeletal muscle type II holoenzymes both bound approximately 2 mol of *lin*-benzo-ADP/mol of holoenzyme and had identical affinities for the nucleotide (Table I). There was no evidence for cooperativity between the sites. The type II regulatory subunits from bovine brain and cardiac muscle differ in immunochemical properties (Fleischer et al., 1976; Erlichman et al., 1980) and in their affinity for cAMP (Erlichman et al., 1980). The regulatory subunits from these sources also differ in their interaction with the catalytic subunits in the presence of cAMP (Hartl & Roskoski, 1982). In spite of these differences, the holoenzymes, which differ in their regulatory subunits but share a common catalytic subunit (Hartl & Roskoski, 1983), appear to interact identically with *lin*-benzo-ADP. As with the catalytic subunit, the *lin*-benzoadenosine moiety appears to be tightly bound by the type II holoenzymes (Table II) with little freedom to rotate within the adenine binding site.

lin-Benzo-ATP is a substrate for the autophosphorylation reaction (Figure 7). Surprisingly, dissociation of the holoenzyme by cAMP results in a decreased rate of phosphorylation as opposed to the increase observed when ATP is the phosphoryl donor. Rangel-Aldao & Rosen (1976) postulated that the autophosphorylation reaction is intramolecular in the absence of cAMP and intermolecular in the presence of cAMP. Thus the difference in rates of autophosphorylation may be

related to the higher affinity of the holoenzyme for *lin*-benzo-ATP; the nucleotide may be bound in a more favorable orientation for catalysis in the intramolecular reaction than in the intermolecular reaction.

Chemical modification studies have been performed in this and other laboratories in order to identify amino acid residues at or near the active site of the catalytic subunit. 5′-[*p*-(Fluorosulfonyl)benzoyl]adenosine, which modifies a lysine at the active site of the catalytic subunit (Zoller & Taylor, 1979; Zoller et al., 1981), blocks binding of *lin*-benzo-ADP. Furthermore, ethoxyformic anhydride, which has been postulated to modify a tyrosine residue in the MgATP binding site (Witt & Roskoski, 1975a), also blocks *lin*-benzo-ADP binding. It has been suggested on the basis of modification by iodoacetamide (Sugden et al., 1976), DTNB (Peters et al., 1977), *N*-tosyl-L-lysine chloromethyl ketone (Kupfer et al., 1979), iodoacetic acid (Nelson & Taylor, 1981), and NBD-Cl (Hartl & Roskoski, 1982) that one, and possibly both, of the cysteines in the catalytic subunit is at or near the active site. When the sulfhydryl groups are modified with NBD-Cl, the catalytic subunit loses its ability to bind *lin*-benzo-ADP. Thus NBD-Cl, FSBA, and ethoxyformic anhydride, which have been postulated to modify residues at or near the nucleotide binding site, do indeed block nucleotide binding to the catalytic subunit.

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Registry No. I (R = diphosphate), 61925-59-5; I (R = triphosphate), 61925-58-4; II (R = triphosphate), 37482-17-0; ADP, 58-64-0; ATP, 56-65-5; Leu-Arg-Arg-Ala-Ser-Leu-Gly, 65189-71-1; protein kinase, 9026-43-1.

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