A Kinetic Study of Interactions of (R_P)- and (S_P)-Adenosine Cyclic 3′,5′-Phosphorothioates with Type II Bovine Cardiac Muscle Adenosine Cyclic 3′,5′-Phosphate Dependent Protein Kinase†

Catherine A. O'Brian, Steven O. Roczniak, H. Neal Bramson, Janina Baraniak, Wojciech J. Stec, and E. T. Kaiser*

ABSTRACT: The stereoselectivity of the adenosine cyclic 3′,5′-phosphate (cAMP) binding sites on the regulatory subunit of the type II bovine cardiac muscle cAMP-dependent protein kinase was investigated by examining the interactions of (R_P)- and (S_P)-adenosine cyclic 3′,5′-phosphorothioates (cAMPS) with these sites. While activation of the holoenzyme and binding to the regulatory subunit of the type II kinase were observed for both of these diastereomers, there were significant differences between the interactions of the cAMPS isomers with the enzyme. In particular, the S_P isomer is more potent than the R_P species not only in the activation of reconstituted, as well as directly isolated, holoenzyme but also in the inhibition of [^{3}H]cAMP binding to the regulatory subunit. A marked preference for the binding of the S_P isomer to site 2 in the regulatory subunit exists. Hydrogen bonding of a functional group on the regulatory subunit with preferential orientation toward the exocyclic oxygen rather than the sulfur of the thiophosphoryl residue may be involved in the observed selectivity of cAMPS binding and activation. In addition to our findings on the stereoselectivity of the binding of cAMPS to cAMP-dependent protein kinase, we have established a method for the reconstitution of holoenzyme from the purified subunits without subjecting the regulatory protein to denaturing conditions.

The cAMP-dependent protein kinase of bovine cardiac muscle is thought to occur in two inactive, tetrameric forms, each containing two identical catalytic subunits and two type I or two type II regulatory subunits (Corbin et al., 1978; Erlichmann et al., 1973; Bechtel & Beavo, 1974; Beavo et al., 1974). Autophosphorylation sites exist in the type II but not in the type I regulatory dimer (Corbin et al., 1974, 1975). The binding of cAMP[1] to the regulatory subunits is believed to cause the holoenzyme to dissociate into two active catalytic subunits and a dimer of regulatory subunits (Corbin et al., 1978). It has been determined that each type II regulatory monomer has two nonidentical cAMP binding sites (Rannels & Corbin, 1980), and the stoichiometry of binding sufficient for activation appears to be two cAMP molecules per holoenzyme (Kerlavage & Taylor, 1982).

In recent years the use of nucleoside phosphorothioates containing chiral phosphorus centers has provided information regarding the stereochemistry of binding and catalysis in the enzymatic reactions involving the corresponding nucleotides (Eckstein, 1979). The phosphorothioate analogues of cAMP, (R_P)- and (S_P)-cAMPS, which are diastereomeric forms of cAMPS due to their different chirality at phosphorus, have potential use in elucidating the stereochemistry of the binding interactions of cAMP with the regulatory subunits of cAMP-dependent protein kinases. These cAMPS diastereomers can be obtained in pure form by stereospecific synthesis (Baraniak et al., 1979). In the procedure employed, cAMP is converted to a mixture of phosphoroanilidate diastereomers, which are separated by preparative TLC (Baraniak et al., 1979; Zielinksi & Stec, 1977; Lesnikowski et al., 1978). Each phosphoroanilidate diastereomer is metalated and then converted into the corresponding phosphorothioate diastereomer by stereospecific reaction with CS_2, which occurs with retention of configuration (Baraniak et al., 1979; Stec et al., 1976; Lesiak & Stec, 1978).

The purified S_P isomer of cAMPS has been used in determining that its hydrolysis catalyzed by bovine heart cyclic phosphodiesterase occurs with inversion of configuration at phosphorus (Burgers et al., 1979). In addition, (S_P)-cAMPS has been shown to stimulate bovine cardiac muscle protein kinase (Eckstein et al., 1974),[2] and various ring-substituted analogues of cAMP were found to bind to the type I regulatory subunit from rabbit muscle with a preference for the binding of the S_P isomers (Jastorff et al., 1979). In the present paper we have investigated the stereoselectivity of the cAMP binding sites on the regulatory subunit of the type II kinase from bovine cardiac muscle by the examination of the interactions of (R_P)- and (S_P)-adenosine cyclic 3′,5′-phosphorothioates with these sites. Both of these diastereomers activate the holoenzyme and bind to the regulatory subunit of the type II kinase.

Experimental Procedures

Materials. 8-[(6-Aminohexyl)amino]adenosine cyclic 3′,5′-phosphoric acid–Agarose, cAMP, and cGMP were purchased from Sigma, while [^{3}H]cAMP was obtained from Amersham. (R_P)- and (S_P)-adenosine cyclic 3′,5′-phosphorothioates were prepared as previously described (Baraniak et al., 1979). Proteinase K was purchased from Boehringer Mannheim. Peptide **1**, Leu-Arg-Arg-(*o*-NO_2)-

† From the Departments of Biochemistry (C.A.O., S.O.R., and E.T.K.) and Chemistry (H.N.B. and E.T.K.), The University of Chicago, Chicago, Illinois 60637, and the Centre of Molecular and Macromolecular Studies, Polish Academy of Sciences, 90-362 Lodz, Boczna 5, Poland (J.B. and W.J.S.). *Received January 14, 1982; revised manuscript received June 1, 1982.* The support of this research by National Institutes of Health Grant GM-19037 and by NIH Training Grant 5T32 GM-07183 is gratefully acknowledged.

* Address correspondence to this author after Sept 1, 1982, at The Rockefeller University, New York, New York 10012.

[1] Abbreviations: ATP, adenosine 5′-triphosphate; BSA, bovine serum albumin; cAMP, adenosine cyclic 3′,5′-phosphate; cAMPS, adenosine cyclic 3′,5′-phosphorothioate; cGMP, guanosine cyclic 3′,5′-phosphate; DEAE, diethylaminoethyl; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Mops, 3-(*N*-morpholino)propanesulfonic acid; NaDodSO$_4$, sodium dodecyl sulfate; TLC, thin-layer chromatography; Tris, tris(hydroxymethyl)-aminomethane.

[2] The cAMPS described in the paper by Eckstein et al. (1974) is now known to be (S_P)-cAMPS (F. Eckstein, personal communication).

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Tyr-Ser-Leu-Gly, used for spectrophotometric assays of protein kinase, was synthesized as reported by Bramson et al. (1980). All other materials were the highest quality commerically available.

Purification of Catalytic and Regulatory Subunits. The catalytic subunit of cAMP-dependent protein kinase from bovine cardiac muscle was prepared as reported by Bramson et al. (1982). The regulatory subunit was purified by the method of Corbin et al. (1978). After gel filtration both the catalytic and regulatory subunits appeared to be homogeneous by $NaDodSO_4$–polyacrylamide gel electrophoresis on Laemmli slab gels (Laemmli, 1970). The respective yields of catalytic and regulatory subunits from 6.47 kg of bovine cardiac muscle were 39 and 50 mg. Protein concentrations were determined by the Coomassie procedure (Sedmark & Grossberg, 1977).

Preparation of cAMP-Free Regulatory Protein. Regulatory protein that was essentially free of cAMP and contained bound cGMP was prepared by eluting the regulatory protein from the cAMP affinity column with 50 mM cGMP in phosphate buffer and dialyzing 2 mg of protein (2 mg/mL) against 1-L volumes of 150 mM phosphate buffer with 1 mM EDTA and 1 mM DTT at pH 7.5 in four 12-h intervals. The resulting protein bound 1.7 ± 0.2 cAMP molecules per subunit, as measured by filter assays and equilibrium dialysis, and inhibited the catalytic subunit 94%, when the catalytic subunit and regulatory subunit had equimolar concentrations.

The amount of exchangeable cGMP in the protein sample after dialysis was determined by incubating the protein with [^{3}H]cGMP (133 cpm/pmol) for 4 h and following the removal of radioactivity from the sample during dialysis. The concentrations of cAMP and cGMP were determined by using the New England Nuclear cAMP and cGMP radioimmunoassay kits according to the provided instructions. The dialyzed regulatory protein, 0.95 mg/mL, was incubated with 0.024 mg/mL proteinase K in phosphate buffer at pH 7.5 and 37 °C for 2 h. The addition of another aliquot of proteinase K made the sample 0.91 mg/mL in regulatory protein and 0.045 mg/mL in proteinase K. The digestion was terminated 2 h later by making the solution 0.5 mM in PMSF. Free cAMP and cGMP in the digested sample were determined by radioimmunoassays.

Reconstitution of Holoenzyme and Activation by Cyclic Nucleotide. Regulatory protein that contained bound cGMP was incubated at 37 °C for 4 h with the catalytic subunit at ratios of regulatory subunit to catalytic subunit of 2.0, 1.2, and 1.0. In each case, the total protein concentration was approximately 1 mg/mL, and the buffer was 50 mM Mops with 1 mM DTT and 1 mM EDTA at pH 7.0. The activity of the catalytic subunit was monitored on a Cary 219 spectrophotometer by the absorbance change at 430 nm due to the transfer of the γ-phosphoryl group of ATP to peptide **1**. The relative activities in the presence and absence of 50 μM cAMP were used to calculate the percentage of inactivation of the catalytic subunit by the regulatory subunit. The reaction mixtures contained 50 mM Mops, 1 mM DTT, 1 mM EDTA, 10 mM $MgSO_4$, 0.2 mg/mL BSA, 2 mM ATP, 50 μM peptide, 20–50 nM catalytic subunit, specified amounts of regulatory subunit, and either 50 μM or no cAMP at pH 7.0 and 30 °C. Reactions were initiated by the addition of peptide. The results obtained in all experiments involving the reconstituted holoenzyme were compared to those of control experiments using gel-filtered reconstituted holoenzyme and crude holoenzyme. A G-150 superfine column (0.9 × 50 cm) in 10 mM phosphate with 1 mM EDTA and 1 mM DTT at pH 6.8 was employed to separate reconstituted holoenzyme from cGMP and the free subunits of protein kinase. The gel-filtered reconstituted holoenzyme was assayed for both cAMP and cGMP by using radioimmunoassay kits by the method described in connection with the preparation of regulatory subunit. In order to obtain partially purified holoenzyme, we followed the procedure of Rubin et al. (1974) for the purification of bovine heart protein kinase through the elution of cAMP-dependent protein kinase activity from the DEAE-cellulose resin. This fraction is referred to as crude holoenzyme. On the basis of the stoichiometry of [^{3}H]cAMP binding to reconstituted holoenzyme, the concentration of crude holoenzyme was determined by [^{3}H]cAMP binding assays.

The activation of reconstituted holoenzyme was monitored by the absorbance change at 430 nm due to the phosphorylation of peptide **1**. The ratio of regulatory subunit to catalytic subunit was 2:1. Specified amounts of cyclic nucleotide were incubated with kinase under the conditions used in the studies of the inactivation of the catalytic subunit by the regulatory subunit. The reaction mixture was equilibrated for 20 min, and the reaction was initiated by the addition of peptide **1**. To determine whether or not the cyclic nucleotide affects phosphotransferase activity by direct interaction with the catalytic subunit, we carried out experiments under conditions identical with those used for activation studies, but in the absence of regulatory protein.

Measurements of Cyclic Nucleotide Binding to Regulatory Dimer. The phosphate buffer (50 mM phosphate) contained 1 mM EDTA and 1 mM DTT at pH 6.8. The incubation buffer was phosphate buffer with 0.5 mg/mL BSA and 2 M NaCl at pH 6.8. Mes buffer (25 mM) contained 0.01 M NaCl, 1 mM DTT, 1 mM EDTA, and 0.5 mg/mL BSA at pH 6.8.

The binding of cyclic nucleotide to the regulatory dimer was assayed by the Millipore filtration technique and by equilibrium dialysis. In all of the Millipore filter assays, a 25- or 50-μL aliquot was withdrawn from a reaction mixture at a specified time, diluted into 5 mL of ice-cold phosphate buffer, and filtered on a Millipore HA 0.45 μM filter. The filter was rinsed with 5 mL of ice-cold phosphate buffer added to the tube in which dilution had been made and then with an additional 10 mL of the same buffer. After being dried in an oven for at least 30 min, the filters were placed in 5 mL of Aquasol, and the radioactivity on the filters was counted in a Beckman LS-7000 scintillation counter.

Reaction mixtures for studies of the displacement of bound [^{3}H]cAMP from the regulatory protein consisted of 400 μL of 1 μM [^{3}H]cAMP in incubation buffer, specified amounts of cAMPS, 0.18–0.21 μM regulatory subunit, and sufficient incubation buffer such that the final volume was 500 μL. Measurements on the time courses of this process were initiated by the addition of cold cyclic nucleotide to the reaction mixture. The compositions of the reaction mixtures employed in observing the time course of the binding of [^{3}H]cAMP are described under Results. After incubation of the cyclic nucleotide containing solution at 25 °C for 5 min, regulatory dimer was added, producing a concentration of the regulatory subunit within the range of 10–30 nM. After a thorough mixing with a Vortex mixer, the solution was incubated at 25 °C. At various intervals 50-μL aliquots were withdrawn and assayed by the Millipore filtration technique.

Equilibrium dialysis was conducted in an apparatus with a 300-μL chamber bisected by a Fisher cellulose dialysis cell membrane. On one side of the membrane, 5.4 pmol of regulatory dimer or holoenzyme was added. The opposite side

contained incubation buffer with 450 pmol of [^{3}H]cAMP (6500 cpm/pmol) and with various quantities of the cAMPS diastereomers. After being shaken for 18 ± 2 h at room temperature, two 25-μL aliquots were taken from each side. Each aliquot was diluted into 525 μL of water and counted in 5 mL of Aquasol.

Analysis for Possible Epimerization of cAMPS Diastereomers Due to Their Interaction with Regulatory Dimer. The possibility that enzyme-catalyzed epimerization of the cAMPS diastereomers at the chiral phosphorus center might occur was tested by incubating 40 μM regulatory dimer and 800 μM cAMP, (R_P)-cAMPS, or (S_P)-cAMPS. After 30 or 40 h at room temperature, 10 μL of solution was spotted onto a precoated silica gel plate. The plate was chromatographed in the solvent system 2-propanol–concentrated aqueous ammonia–water (7:1:1). The R_f values for cAMP, (S_P)-cAMPS, and (R_P)-cAMPS were 0.37, 0.53, and 0.56, respectively.

Results

An investigation of the inactivation of the catalytic subunit by the regulatory subunit was facilitated by the removal of essentially all of the cAMP from the regulatory protein. The determination of the cyclic nucleotide concentration of the sample by radioimmunoassay required the liberation of bound cyclic nucleotide from the regulatory protein. The protein was digested by proteinase K, the serine protease that effectively digests native proteins (Ebeling et al., 1974). The destruction of the cAMP binding sites was ascertained by the complete loss of cAMP binding, as monitored by filter assays. No discrete bands were visible when 5 μg of digested protein was electrophoresed on 10% Laemmli slab gels (Laemmli, 1970). As measured by radioimmunoassays, there were 0.093 ± 0.005 equiv of cAMP and 1.4 ± 0.1 equiv of cGMP per regulatory subunit. Measurement of the amount of [^{3}H]cGMP bound to the dialyzed protein, 1.4 ± 0.2 equiv per regulatory subunit, agreed with the results of the radioimmunoassay.

The inactivations of the catalytic subunit by the regulatory protein were 94%, 97%, and 99%, at ratios of regulatory subunit to catalytic subunit of 1.0, 1.2, and 2.0, respectively. cAMP and cGMP were removed from the reconstituted holoenzyme by gel filtration, as demonstrated by radioimmunoassays for cAMP and cGMP. The limit of detection of the assays was approximately 0.005 cyclic nucleotide equiv per regulatory monomer. The gel-filtered reconstituted holoenzyme was 98% inactive in the absence of cyclic nucleotide. Crude holoenzyme was 99% inactive in the absence of cyclic nucleotide. In the absence of regulatory protein, the initial rates of phosphotransferase activities of the catalytic subunit were unaffected by the presence of 100 μM (R_P)-cAMPS, 100 μM (S_P)-cAMPS, and 100 μM cAMP. Thus, differences in the initial rates in the presence of the regulatory subunit appear to be due only to interactions between the cyclic nucleotides and the regulatory subunit.

Initial velocities for the phosphorylation of peptide **1** were measured in the presence of 0.5, 1.0, 5, 10, and 100 μM cyclic nucleotide at two different concentrations of reconstituted holoenzyme, 13 and 6.5 nM. The capacities of the cAMPS diastereomers to activate the reconstituted holoenzyme were calculated as the percentage of the initial rate achieved by equivalent concentrations of cAMP (Figures 1 and 2). Similar results were achieved by both concentrations of the enzyme; in both cases, regulatory protein was in 2-fold excess over the catalytic subunit. The activation by (S_P)-cAMPS relative to that by equimolar cAMP increases with concentration and appears to plateau at approximately 85% at a concentration of 10 μM. The (R_P)-cAMPS diastereomer is a poor activator

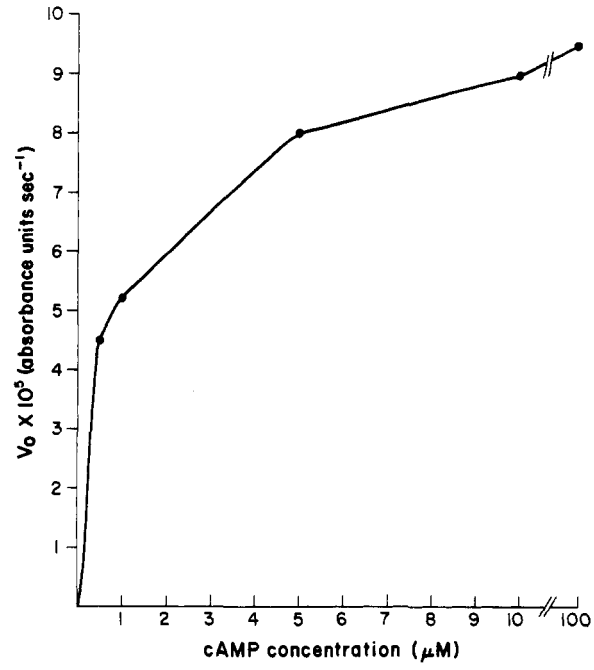

FIGURE 1: Activation of protein kinase by cAMP. The initial rates of the transfer of the γ P of ATP to the serine peptide **1** were monitored by the decrease in absorbance at 430 nm. Catalytic subunit was 13 nM; other incubation conditions are described in the text. V_0 represents the magnitude of the initial change in absorbance at 430 nm that results from the phosphorylation of peptide **1**.

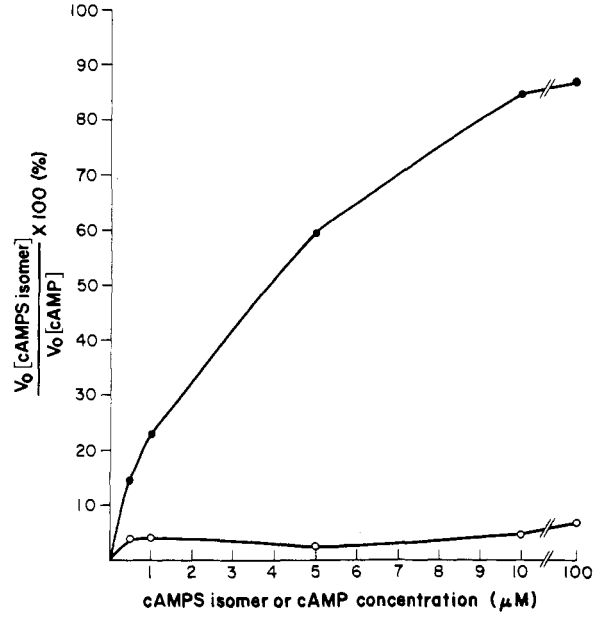

FIGURE 2: Activation of protein kinase holoenzyme by (R_P)- and (S_P)-cAMPS. The activation of protein kinase by cyclic nucleotides was monitored by the phosphorylation of peptide **1**, as described in the legend of Figure 1. Incubation conditions are given in the text; each point represents an average of two experiments, with catalytic subunit concentrations of 13 and 26 nM. Percent V_0 represents [the initial rate of phosphotransferase activity in the presence of (R_P)- or (S_P)-cAMPS divided by the initial rate observed in the presence of an equivalent concentration of cAMP] × 100. (○) Percent V_0 achieved by (R_P)-cAMPS; (●) percent V_0 achieved by (S_P)-cAMPS.

of reconstituted holoenzyme; it activates reconstituted holoenzyme with less than 10% the potency of cAMP, even at a concentration of 640 μM. Similar results were achieved in parallel experiments with crude holoenzyme. In these studies, (R_P)-cAMPS activation never exceeded 5% of that seen for cAMP, and (S_P)-cAMPS activation was approximately the

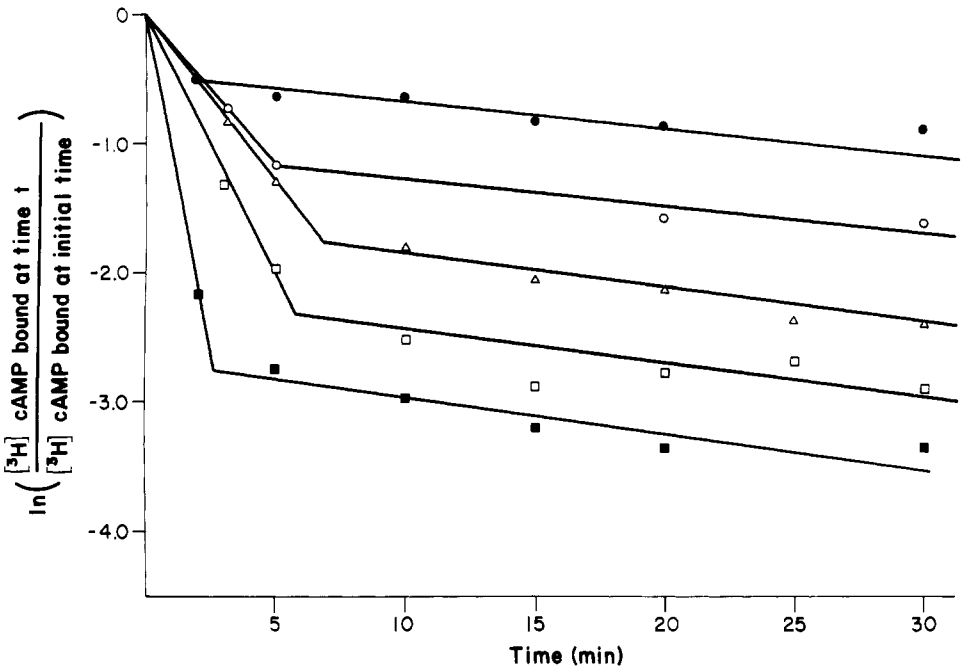

FIGURE 3: Displacement of bound [^{3}H]cAMP by cAMP in the presence and absence of cAMPS isomers. Regulatory protein was incubated with 0.8 μM [^{3}H]cAMP and specified concentrations of cAMPS isomers, as described in the text. Cold cAMP was added, and the time course of the displacement of bound [^{3}H]cAMP was monitored by Millipore filtration assays. The cAMPS isomers were present in the incubation mixtures at the following concentrations: (●) 4 mM (S_P)-cAMPS; (○) 160 μM (S_P)-cAMPS; (△) no cAMPS; (□) 90 μM (R_P)-cAMPS; (■) 1.2 mM (R_P)-cAMPS. Points represent average of duplicate experiments.

same as that observed with reconstituted holoenzyme. Gel-filtered reconstituted holoenzyme was activated by (R_P)-cAMPS to approximately 25% of the extent found for cAMP across a range of (R_P)-cAMPS concentrations from 50 to 400 μM. At higher concentrations, the extent of activation of gel-filtered holoenzyme by (S_P)-cAMPS appeared to be equivalent to that of cAMP.

Millipore filtration was employed in the investigation of the exchange between cAMP and [^{3}H]cAMP at the binding sites on the regulatory dimer. The binding of [^{3}H]cAMP to R_2·(cAMP)$_4$ was examined in two different buffers. In Mes buffer, 100 μM [^{3}H]cAMP was required to achieve binding of 1.5 pmol of [^{3}H]cAMP/pmol of R (with concentrations of R subunits ranging from 10 to 30 nM in typical experiments). With a concentration of 10 μM [^{3}H]cAMP in Mes buffer and 1 μM [^{3}H]cAMP in Mes buffer, binding reached a value of 0.5 pmol of [^{3}H]cAMP/pmol of R and 0.2 pmol of [^{3}H]-cAMP/pmol of R at equilibrium, respectively. Both (R_P)- and (S_P)-cAMPS inhibited [^{3}H]cAMP binding in Mes buffer. In phosphate buffer binding reached 1.4–1.5 pmol of [^{3}H]-cAMP/pmol of R in the presence of 1 μM [^{3}H]cAMP with the concentration of R subunit typically being 10–30 nM. At equilibrium, the inhibitions of [^{3}H]cAMP binding to the regulatory subunit by 90 μM (R_P)-cAMPS and 80 μM (S_P)-cAMPS obtained were 62 ± 6% and 85 ± 3%, respectively.

In equilibrium dialysis experiments the amount of binding of [^{3}H]cAMP that was observed was 1.6–1.9 pmol/pmol of regulatory monomer with [^{3}H]cAMP and regulatory subunit concentrations of 3 μM and 31 nM, respectively. After 18 ± 2 h the extents of inhibition of the binding of [^{3}H]cAMP observed with concentrations of (R_P)-cAMPS that were 10-, 50-, and 100-fold that of the tritiated cyclic nucleotide (3.0 μM [^{3}H]cAMP) were 0%, 36%, and 53%, respectively. The degrees of inhibition exerted by (S_P)-cAMPS observed at 0.1-, 1-, 10-, 25-, 50-, and 100-fold the concentration of [^{3}H]cAMP were 14%, 20%, 61%, 68%, 82%, and 88%, respectively (with regulatory subunit concentrations that were 36 nM). The degrees of the inhibition of [^{3}H]cAMP binding by the R_P and S_P isomers measured after 18 ± 2 h by equilibrium dialysis were similar to those observed after 30 min with the filter assay.

The preferences of (R_P)-cAMPS, (S_P)-cAMPS, and [^{3}H]cAMP for binding to either of the two cAMP binding sites in the regulatory subunits were determined by the method of Rannels & Corbin (1980); these authors propose that there are two classes of sites on the regulatory dimer that can be distinguished on the basis of the kinetics of the displacement of [^{3}H]cAMP by unlabeled cAMP. Site 2 is characterized by a rate constant for the dissociation of [^{3}H]cAMP, k_{diss}, which is approximately 1 order of magnitude greater than that seen for site 1. The loss of bound [^{3}H]cAMP was monitored subsequent to the addition of 5 μL of 10 mM cAMP to a mixture (500 μL) in which R_2·(cAMP)$_4$ (regulatory subunit concentration 0.18–0.21 μM) was incubated with 0.8 μM [^{3}H]cAMP and a specified concentration of inhibitor for 30 min (Figure 3). In the absence of inhibitor, 33% of the displaced label was chased from site 1 with the remaining 67% being displaced from site 2. In the presence of 160 μM and 4 mM (S_P)-cAMPS, the percentage of the label chased from site 1 increased to 37% and 53%, respectively, indicating that (S_P)-cAMPS binds preferentially to site 2. In the presence of 90 μM and 1.2 mM (R_P)-cAMPS, 24% and 21% of the displaced labels were respectively attributable to site 1, indicating that (R_P)-cAMPS binds preferentially to site 1. The slope of the first-order plot of the slow phase of the displacement of [^{3}H]cAMP from the regulatory subunit represents the rate constant k_{diss} for dissociation of cAMP from site 1; k_{diss} is 0.024 ± 0.002 min^{-1}. The rate constant k_{diss} for the dissociation of cAMP from site 2 corresponds to the slope observed in a logarithmic plot of the total concentration of displaced [^{3}H]cAMP corrected for the [^{3}H]cAMP displaced from site 1 vs. time; the calculated value of the site 2 k_{diss} is 0.37 ± 0.03 min^{-1}.

For direct observation of the ability of each stereoisomer of cAMPS to displace [^{3}H]cAMP from each site, [^{3}H]cAMP was incubated with the regulatory dimer for 30 min, an aliquot was withdrawn for measurement of total cAMP bound, and a large quantity of (R_P)- or (S_P)-cAMPS was added to give final concentrations of regulatory subunit and [^{3}H]cAMP of 0.18 and 0.8 μM, respectively. Both the R_P and S_P isomers were found to displace [^{3}H]cAMP at diastereomer concentrations of 100 and 200 μM. Of the initial [^{3}H]cAMP bound, 100 and 200 μM (S_P)-cAMPS displaced 86% and 92%, respectively. The (R_P)-cAMPS diastereomer displaced only 41% and 73% of the initial [^{3}H]cAMP bound at concentrations of 100 and 200 μM (R_P)-cAMPS, respectively.

Enzyme-catalyzed interconversion of the cAMPS diastereomers might occur in the presense of the regulatory dimer if the binding interaction between the cyclic nucleotide and the regulatory subunit were to involve the reversible opening of the six-membered phosphate ring with concomitant adenylylation of the subunit (Greengard et al., 1969; Lee et al., 1971). In other words, if cAMP were covalently attached in the ring-opened form to the regulatory subunit and if pseudorotation were to occur readily, enzyme-catalyzed equilibration of the cAMPS diastereomers would be possible. However, within the limits of detection of our experiments no such interconversion was found.

Discussion

The regulatory component of the bovine heart type II protein kinase is a dimer containing four cAMP binding sites, which occur in two kinetically distinguishable classes (Rannels & Corbin, 1980). Cyclic nucleotides with substitutions at the 8 position of the adenine ring have been shown to compete preferentially with cAMP for site 1, while those with modifications at the 6 position have a preference for site 2 (Rannels & Corbin, 1980). The importance of the phosphoryl group of cAMP in its interaction with regulatory protein is suggested by the effect of modifications of the phosphate ring on the ability of the cyclic nucleotide to activate protein kinase. The requirement for a charged phosphoryl residue in the cyclic nucleotide in order to observe activation of protein kinase types I and II was inferred from the lack of activity of adenosine 3′,5′-sulfate, cAMP ethyl ester diastereomers, and cAMP dimethylphosphoramidate (Yagura & Miller, 1981). In addition, stereoselective activations of protein kinases by cAMPS diastereomers have been reported. (S_P)-cAMPS was found to be over 10-fold more potent than (R_P)-cAMPS in the activation of partially purified rabbit skeletal muscle protein kinase type I, whereas (R_P)-cAMPS was observed to be slightly (about 20%) more potent than (S_P)-cAMPS as an activator of partially purified bovine brain protein kinase type II (Yagura & Miller, 1981). The diastereomers of 5′-amido-cAMPS have been separated, and it has been determined that the S_P isomer is 4 times more potent than the R_P isomer in the activation of bovine muscle protein kinase (Jastorff & Bär, 1973). In the present paper we have reported the results of studies both on the activation of the bovine heart type II holoenzyme by the cAMPS diastereomers and on the binding of these diastereomers to the regulatory protein.

For the experiments in which we examined activation of the holoenzyme, we developed methodology for reconstitution of the holoenzyme without the use of a potential denaturant, urea (Corbin & Rannels, 1981). Specifically, we employed cGMP to elute the regulatory protein from the cAMP affinity column to which it was bound. Bound cAMP, which could be endogenous or an artifact of the preparation, and bound cGMP were quantitated by radioimmunoassays of the sample, after proteolytic destruction of the cAMP binding sites. The regulatory protein, to which was bound 1.5 ± 0.1 molecules of cGMP per monomer, was fully active in binding [^{3}H]cAMP and inhibiting the phosphotransferase activity of the equimolar catalytic subunit. The removal of cyclic nucleotide from the reconstituted holoenzyme solution was performed by gel filtration. The experiments on the holoenzyme were carried out with either reconstituted holoenzyme, gel-filtered reconstituted holoenzyme, or crude, directly isolated holoenzyme. A preparation of the gel-filtered reconstituted holoenzyme was used as a control to determine whether the cGMP present in solutions of reconstituted holoenzyme produced artifacts. The performance of a number of experiments with the crude, directly isolated holoenzyme provided a control to detect possible artifacts that might be involved in the reassociation of isolated subunits.

In experiments using reconstituted holoenzyme, gel-filtered holoenzyme, or crude, directly isolated holoenzyme and employing high concentrations of the cyclic nucleotide, the degrees of activation by (R_P)-cAMPS appear to level off at values that are considerably less than those obtained in the presence of cAMP. At apparent saturation with (R_P)-cAMPS, the activation observed relative to that seen in the presence of cAMP with crude holoenzyme, reconstituted holoenzyme, and gel-filtered reconstituted holoenzyme corresponds to approximately 5%, 10%, and 25%, respectively. These qualitative differences may reflect the presence of phosphodiesterases and of other proteins that bind cyclic nucleotides in the crude holoenzyme solutions, the 2:1 ratio of regulatory subunit to catalytic subunit in the reconstituted holoenzyme, the process of the reassociation of the isolated subunits, and the presence of cGMP in the reconstituted holoenzyme solution. Because the activation observed at saturating concentrations of the (S_P)-cAMPS isomer appears to be equivalent to that attained by cAMP in experiments with gel-filtered reconstituted holoenzyme, the somewhat lower levels of activation observed for (S_P)-cAMPS in studies with reconstituted holoenzyme and crude holoenzyme may be due to artifacts such as those mentioned above in connection with the qualitative differences seen for the levels of activation by (R_P)-cAMPS of different types of protein kinase holoenzyme preparations. Several lines of evidence have been advanced that suggest that there is formation of a complex between the holoenzyme and cyclic nucleotide prior to the release of the catalytic subunit (Armstrong & Kaiser, 1978; Builder et al., 1980; Tsuzuki & Kiger, 1978). A possible explanation for the kinetic results found at high concentrations of the (R_P)-cAMPS diastereomer is that the complex formed between (R_P)-cAMPS and the holoenzyme does not release the catalytic subunit as readily as does the corresponding complex formed with cAMP. In other words, under the activation conditions it seems possible that an appreciable amount of the holoenzyme may be present even at saturating concentrations of (R_P)-cAMPS. Experiments to examine this possibility and other possible reasons for the incomplete activation of the holoenzyme by high concentrations of (R_P)-cAMPS diastereomer are now under way. In any event, it is clear that the bovine heart type II cAMP-dependent protein kinase is activated more effectively by the S_P isomer of cAMPS than by the R_P isomer.

By experiments on the regulatory subunit in which the cAMPS diastereomers were allowed to compete with [^{3}H]-cAMP for the cyclic nucleotide binding sites and bound radioactive cAMP was displaced by unlabeled cAMP, we were able to show that (S_P)-cAMPS preferentially binds to site 2 and (R_P)-cAMPS is selective for site 1. These site-selectivity

data, together with the activation data discussed above, are consistent with the finding that [^{3}H]cIMP is a potent activator of bovine heart protein kinase type II and has a strong preference for site 2 (Rannels & Corbin, 1981). When the cAMPS diastereomers were employed to displace directly [^{3}H]cAMP from the regulatory subunit, we found that (S_P)-cAMPS was able to displace 86% and 92% of the total bound [^{3}H]cAMP at concentrations of the sulfur-labeled nucleotide that were 100- and 200-fold greater, respectively, than those of [^{3}H]cAMP. Under similar conditions (R_P)-cAMPS displaced only 41% and 73% of the bound [^{3}H]cAMP.

The activation results, the results on the degrees of displacement of [^{3}H]cAMP, and the results of measurements on selectivity for sites 1 and 2 indicate that there is a very significant difference between the interactions of the S_P and R_P diastereomers of cAMPS with bovine heart type II protein kinase. The importance of the location of charge on the phosphoryl group in the cyclic phosphate ring may be considerable, in view of the absence of activation of the holoenzyme by cyclic nucleotides lacking charge in the phosphate ring (Yagura & Miller, 1981). Hydrogen bonding of a functional group on the regulatory subunit to the negatively charged phosphoryl residue may be involved in the observed selectivity of cAMPS binding and activation. In particular, the observed stereoselectivity may reflect the relative strengths of hydrogen bonding between a functional group on the regulatory subunit and exocyclic oxygen or sulfur in the negatively charged thiophosphoryl residue of cAMPS. It seems unlikely that stereoselectivity of the magnitude observed in our studies on the cAMPS diastereomers reflects steric hindrance due to the increase in the van der Waals radius when oxygen is replaced by sulfur, but this cause of the difference, although unlikely, cannot be completely ruled out.

In summary, we have found a pronounced stereoselectivity in the activation of bovine heart protein kinase type II by cAMPS diastereomers. The S_P isomer is more potent than the R_P species in both the activation of the reconstituted holoenzyme and the competitive inhibition of [^{3}H]cAMP binding to the regulatory subunit. There is a marked preference for the binding of the S_P isomer to site 2 in the regulatory subunit, and this could reflect the interaction of the thiophosphoryl group with a hydrogen-bond-donating residue in this site with the hydrogen bond being preferentially oriented toward the oxygen rather than the sulfur of the thiophosphoryl residue.

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