A mechanistic and kinetic analysis of the interactions of the diastereoisomers of adenosine 3',5'-(cyclic)phosphorothioate with purified cyclic AMP-dependent protein kinase

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The binding affinities of the diastereoisomers of adenosine 3',5'-(cyclic)phosphorothioate, Sp-cAMP[S] and Rp-cAMP[S], for the cyclic AMP- (cAMP-)binding sites on purified and reconstituted pig heart type II cAMP-dependent protein kinase holoenzyme were determined by measuring the ability of these compounds to displace [³H]cAMP from this enzyme. Sp-cAMP[S], a cAMP agonist, displaced 50 % of the [³H]cAMP bound to the holoenzyme at a concentration 10-fold higher than that of cAMP; Rp-cAMP[S], a cAMP antagonist, required a 100-fold higher concentration relative to cAMP. Activation of the isolated holoenzyme, determined as phosphotransferase activity, was measured in the presence of the agonist and in the absence and in the presence of increasing concentrations of the antagonist. The results of fitting the activation data to sigmoid curves with a non-linear-regression program and to Hill plots by using a linear-regression program showed that Rp-cAMP[S] had no effect on $V_{\max}$, increased the EC_{50} values for agonist activation and had no effect on the co-operativity of activation (h). A K_i value of 11 μ M was determined for Rp-cAMP[S] inhibition of cAMP-induced activation of purified type II cAMP-dependent protein kinase. Electrophoresis of the holoenzyme on polyacrylamide gels under non-denaturing conditions in the presence of saturating concentrations of the diastereoisomers resulted in 100 % dissociation of the subunits with Sp-cAMP[S] and 0 % dissociation with Rp-cAMP[S]. Sp-cAMP[S], the isomer with an axial exocyclic sulphur atom, binds to the holoenzyme, releases the catalytic subunit and activates the phosphotransferase activity. Rp-cAMP[S], the isomer with an equatorial exocyclic sulphur atom, binds to the holoenzyme but does not result in dissociation, and thus acts as a competitive inhibitor of phosphotransferase activity.

INTRODUCTION

The diastereoisomers of adenosine 3',5'-(cyclic)-phosphorothioate, Sp-cAMP[S] and Rp-cAMP[S], have a sulphur substitution in one of the two exocyclic oxygen positions of cAMP. Although they differ structurally from one another only in the orientation of the sulphur atom with respect to the chiral phosphorus atom, cAMP-dependent protein kinase, the target enzyme, recognizes them as different molecules. Thus the analogues have a 10-fold difference in binding affinity for the holoenzyme as measured by [³H]cAMP displacement and opposite effects on enzyme activity measured *in vitro* as phosphotransferase activity (De Wit *et al.*, 1982, 1984) and in isolated hepatocytes as glucose production (Rothermel *et al.*, 1983, 1984a,b; Dragland-Meserve *et al.*, 1986; Marks & Parker Botelho, 1986). Sp-cAMP[S], which has an axial sulphur atom, binds to the holoenzyme with a 10-fold lower affinity compared with cAMP and promotes dissociation of the holoenzyme. Rp-cAMP[S], which has an equatorial sulphur atom, binds to the holoenzyme with a 100-fold lower affinity compared with cAMP and does not promote dissociation. Thus the orientation of the exocyclic sulphur atom determines both the affinity of the analogues for the cAMP-binding sites as well as their effect on the tetrameric structure of the holoenzyme.

Previously, studies to elucidate the molecular mechanisms responsible for the differences in the kinetic properties of these diastereoisomers with regard to their

interaction with cAMP-dependent protein kinase have been delayed by the lack of sufficient quantities of pure compounds. Recently, an improved procedure for synthesis of diastereometrically pure Sp-cAMP[S] and Rp-cAMP[S] has been developed (Eberle & Brzechffa, 1988), allowing adequate amounts to be prepared for further investigation of these properties. The purpose of the present study was to determine accurate activation and inhibition constants for the analogue-enzyme interactions and to explore the mechanism by which Rp-cAMP[S] acts as a cAMP antagonist. The following experiments are described: (1) measurement of the relative binding affinities of the diastereoisomers for purified type II cAMP-dependent protein kinase; (2) determination of various kinetic parameters for Rp-cAMP[S] inhibition of purified and partially purified type I and type II isoenzymes from different tissues and different species; (3) determination of the degree of dissociation of cyclic nucleotide-saturated holoenzyme by non-denaturing polyacrylamide-gel electrophoresis.

EXPERIMENTAL

Materials

The phosphorothioates were prepared as previously described (Baraniak *et al.*, 1979a) with modifications to improve the yield (Eberle & Brzechffa, 1988). In order to reach diastereoisomeric purity and to eliminate traces of cAMP, the analogues were repurified by h.p.l.c. on a

reversed-phase C_{18} column with the use of separation techniques optimized in accordance with Van Haastert (1981). Unlabelled cAMP, bovine serum albumin, 2-mercaptoethanol, EDTA and CM-Sepharose CL-6B resin were obtained from Sigma Chemical Co. The [3H]-cAMP (30 Ci/mmol) was obtained from New England Nuclear. Coomassie Blue and acrylamide were obtained from Bio-Rad Laboratories, and NNN' -tetramethylethylenediamine and ammonium persulphate were obtained from Kodak. All other chemicals were obtained from Fisher Chemical Co., and were reagent grade or better. The isolated subunits of pig heart type II and bovine muscle type I cAMP-dependent protein kinase were obtained from Dr. Susan Taylor, University of California, San Diego, CA, U.S.A. Partially purified rabbit muscle type I and bovine heart type II cAMP-dependent protein kinase were obtained from Sigma Chemical Co.

Isolation and purification of pig heart and bovine muscle cAMP-dependent protein kinase holoenzymes

The isolated subunits of pig heart and bovine muscle cAMP-dependent protein kinase were purified as previously described (Beavo *et al.*, 1975; Kerlavage & Taylor, 1982) and were frozen immediately in a buffer containing 25 mM- KH_2PO_4 , 25 mM- K_2HPO_4 , 1 mM-EDTA and 5 mM-2-mercaptoethanol. The holoenzyme was reconstituted by mixing regulatory subunit with a slight excess (5–10%) of catalytic subunit and dialysing exhaustively against a buffer containing 12.5 mM- K_2HPO_4 , 12.5 mM- KH_2PO_4 (pH 6.1), 5 mM-2-mercaptoethanol, 2 mM-EDTA and 5% (v/v) glycerol. After dialysis, a slurry of CM-Sepharose CL-6B resin equilibrated with the dialysis buffer was added to the holoenzyme mixture at a concentration of 2 ml of resin/mg of excess catalytic subunit, and the mixture was dialysed exhaustively as before. After the second dialysis, the resin slurry was spun at 100 g for 10–15 s and the supernatant containing the purified holoenzyme was removed.

Equilibrium binding studies

The equilibrium binding studies were done as previously described (Døskeland & Haga, 1978; Døskeland *et al.*, 1977). A 2 pmol portion of pig heart type II holoenzyme (0.4 μ g) and increasing concentrations of [3H]-cAMP (33.5 Ci/mmol) were combined in 50 μ l of a buffer containing 25 mM- K_2HPO_4 , 25 mM- KH_2PO_4 , 2 mM-EDTA, 5 mM-2-mercaptoethanol and 1% (w/v) bovine serum albumin. After a 16 h incubation at 4 °C, 450 μ l of ice-cold 80%-saturated $(NH_4)_2SO_4$ was added to each sample, and the incubation was continued at 4 °C for an additional 1.5 h. Two 200 μ l portions from each sample were transferred to reservoirs containing 5 ml of ice-cold 80%-saturated $(NH_4)_2SO_4$ and filtered on Millipore membranes (HAWP, 0.45 μ m) under vacuum. Each sample was washed with an additional 15 ml of cold 80%-saturated $(NH_4)_2SO_4$, dried thoroughly and incubated at room temperature for 16 h in 5 ml of scintillation fluid (ACS). The radioactivities of the samples were counted in a Beckman LS6800 scintillation counter. The concentration of [3H]-cAMP required to obtain maximum binding was determined to be 320 nM (16 pmol) or a 4-fold molar excess of cAMP over binding sites.

Displacement of [3H]-cAMP by cAMP, Sp-cAMP[S] or Rp-cAMP[S]

A 2 pmol portion of pig heart type II holoenzyme (0.4 μ g), 16 pmol of [3H]-cAMP (33.5 Ci/mmol) and increasing concentrations of cAMP, Sp-cAMP[S] or Rp-cAMP[S] were incubated, filtered and the radioactivities counted as described above.

cAMP-dependent protein kinase activation curves

The holoenzyme was activated by the addition of concentrations of 0.006 μ M- to 10 μ M-cAMP in the presence of 0 μ M-, 10 μ M-, 30 μ M- or 100 μ M-Rp-cAMP[S]. Percentage activation was measured in accordance with Cherrington *et al.* (1976) with the following minor modifications. In addition to the concentrations of cyclic nucleotides stated above, assay tubes contained 10 mM-potassium phosphate, pH 6.5, 25 mM-magnesium acetate, 5 mM-NaF, 50 μ M-Kemptide, 0.5–1.0 μ Ci of [^{32}P]-ATP plus sufficient unlabelled ATP for a final concentration of 100 μ M. The assay was initiated by addition of 20 μ l of enzyme. Preparations of type I (bovine muscle) and type II (pig heart) enzyme contained 20 μ g/ml, and preparations of type I (rabbit muscle) and type II (bovine heart) enzyme contained 200 μ g/ml. After incubation for 8 min at 30 °C, the reaction was terminated in accordance with the procedure of Witt & Roskoski (1980) by pipetting a 60 μ l portion from the 75 μ l incubation volume on to squares (2.5 cm $\times$ 2.5 cm) of Whatman P-81 chromatography paper. The paper squares were rinsed in deionized water (10 ml/square) twice for 20 min each time and their radioactivities counted in a scintillation counter.

Measurement of K_i values for Rp-cAMP[S] inhibition of isolated cAMP-dependent protein kinase

A K_i value for inhibition of isolated cAMP-dependent protein kinase by a cAMP antagonist can be obtained by measuring cAMP activation in the presence of several concentrations of the cAMP antagonist. If it is assumed that cAMP-dependent protein kinase activation and cAMP binding are linearly coupled processes showing positive co-operativity (Ogreid & Døskeland, 1982), then the concentration-dependence of the activation process can be described by the following parameters (Swillens, 1983): the maximal activity, the concentration of cAMP that gives half-maximal activity, and the degree of sensitivity of the activation process to changes in cAMP concentration. The sensitivity parameter, or the measure of co-operativity, can be adequately characterized by the Hill coefficient, which can be obtained either from a non-linear-regression analysis of each set of activation data by using the function describing a sigmoid curve:

$$f(x) = \text{fractional activation} (f) = Kx^h / (1 + Kx^h) \quad (1)$$

where x is equal to concentration of the activator, K is a constant and h is the Hill coefficient, or from the slopes of a Hill plot of these data, $\log[f/(1-f)]$ versus $\log[cAMP]$. The value for h in eqn. (1) describes the co-operativity of the activation process using all the data points defining the sigmoid curve; the value for h (called h') calculated from the slopes of the Hill plots uses only the data points that define the linear portion of the sigmoid curve. The concentrations of cAMP required for half-maximal activation (EC_{50}) in the presence of inhibitor are obtained from the abscissa-axis intercept of the Hill plot. The K_i value, the concentration of inhibitor

required for half-maximal inhibition of cAMP-induced activation, can then be calculated from a plot of the EC_{50} values as a function of the inhibitor concentration. The K_i value is the product of the ordinate-axis intercept and the reciprocal of the slope of the regression line.

Non-linear-regression and linear-regression curve-fitting was done with the RS/1 statistics and graphics software package from Bolt, Beranek and Newman, Cambridge, MA, U.S.A.

Standardization of K_i values

The binding of cAMP to the holoenzyme is highly dependent on the concentration of the enzyme used in the assay (Baraniak *et al.*, 1979b; Schwechheimer & Hofmann, 1977; Beavo *et al.*, 1974). Thus K_i values obtained with the different isoenzymes of different degrees of purity, obtained from different tissues of different species, were standardized in accordance with a method suggested by de Wit *et al.* (1982) by using the following equation:

$$\delta\Delta G = -RT \cdot \ln \left(\frac{K(\text{cAMP})}{K(\text{Rp-cAMP[S]})} \right) \quad (2)$$

$K(\text{cAMP})$ is the concentration of cAMP experimentally determined to activate the enzyme half-maximally and $K(\text{Rp-cAMP[S]})$ is the concentration of Rp-cAMP[S] experimentally determined to inhibit the cAMP-induced activation half-maximally. By conversion into thermodynamic units all the activation constants for the different isoenzymes can be related to those of cAMP measured under the same experimental conditions.

Non-denaturing polyacrylamide-gel electrophoresis

Polyacrylamide-gel electrophoresis was run in accordance with Laemmli (1970) in the absence of a denaturing agent. A 100 pmol (19 μg) portion of purified type II pig heart holoenzyme was dissolved in 80 μl of a buffer containing 20 mM- K_2HPO_4 , 20 mM- KH_2PO_4 , 50% (w/v) sucrose, 6% (v/v) 2-mercaptoethanol and 0.1% Bromophenol Blue. Each sample contained 3 μg of holoenzyme and either no additions or a 20-fold molar excess of cAMP, a 200-fold molar excess of Sp-cAMP[S] or a 2000-fold molar excess of Rp-cAMP[S]. Three additional samples were prepared as above with 50 pmol (5.5 μg) each of regulatory subunit instead of holoenzyme. After a 1 h incubation at room temperature, the samples were applied to a 150 mm $\times$ 150 mm $\times$ 1.5 mm polyacrylamide gel. The upper gel contained 5% acrylamide, 10 mM- K_2HPO_4 , 10 mM- KH_2PO_4 , 0.8% ammonium persulphate and 0.15% NNN' -tetramethylethylenediamine. The lower gel contained 7.5% acrylamide, 10 mM- K_2HPO_4 , 10 mM- KH_2PO_4 , 0.4% ammonium persulphate and 0.083% NNN' -tetramethylethylenediamine. The gel was run for 20 h at 20–35 mA at 4 $^\circ\text{C}$, and the results represent the dissociation state of the holoenzyme upon entering the gel and do not require the constant association of the respective cAMP analogues throughout the run. The gels were stained in a solution containing 0.25% Coomassie Blue R250, 25% (v/v) propan-2-ol and 10% (v/v) acetic acid. Destaining was done in a solution containing 25% (v/v) propan-2-ol and 10% (v/v) acetic acid.

Table 1. Apparent K_d values of cAMP, Sp-cAMP[S] and Rp-cAMP[S] for type II cAMP-dependent protein kinase

Apparent K_d values were obtained by equilibrium-binding displacement of [^3H]cAMP from purified reconstituted pig heart type II holoenzyme as described in the Experimental section. Experimental data are shown in Fig. 1 and experimental details are given in the legend to Fig. 1.

Cyclic nucleotide	Apparent K_d (nM)
cAMP	83
Sp-cAMP[S]	785
Rp-cAMP[S]	7400

RESULTS

Comparison of the binding affinities of cAMP, Sp-cAMP[S] and Rp-cAMP[S]

The apparent binding affinities of the diastereoisomers, expressed as K_d values, were determined by measuring the displacement of [^3H]cAMP from purified pig heart cAMP-dependent protein kinase type II holoenzyme. The concentration of cAMP or analogue isomer required to displace 50% of the [^3H]cAMP was measured, and the apparent K_d value was calculated.

As shown in Fig. 1, incubation with increasing concentrations of cAMP, Sp-cAMP[S] or Rp-cAMP[S] resulted in a concentration-dependent displacement of [^3H]cAMP. The apparent K_d values estimated from the Hill plots (Fig. 1 inset) are listed in Table 1. The apparent K_d for cAMP under these experimental conditions was 83 nM and the apparent K_d values for Sp-cAMP[S] and Rp-cAMP[S] were approx. 9.5-fold higher and 90-fold higher respectively.

Kinetic analysis of Rp-cAMP[S] inhibition of cAMP-induced phosphotransferase activity

The cAMP-dependent activation curves for the type II isoenzyme from pig heart in the absence and in the presence of Rp-cAMP[S] are shown in Fig. 2. Curve-fitting to eqn. (1) was done with a non-linear-regression program, and the calculated h values for each activation curve are listed in Table 2. The concentrations required for half-maximal activation, EC_{50} values, were calculated from the Hill plots, $\log[f/(1-f)]$ versus $\log[\text{cAMP}]$, for the activation curves. Curve-fitting to the Hill plots was done with a linear-regression program, and values for the degree of co-operativity of the activation process were obtained from the slopes of the regression lines and are referred to as h' values. The calculated EC_{50} values and the h' values are also listed in Table 2.

Fig. 2 inset shows the plot of the EC_{50} values for each curve versus Rp-cAMP[S] concentration. Curve-fitting was done with a linear-regression program. The ordinate-axis intercept of the regression line represents a calculated EC_{50} of 84 nM for cAMP in the absence of Rp-cAMP[S], which agrees well with the directly measured experimental value of 79 nM. The calculated K_i for Rp-cAMP[S] inhibition of cAMP-induced activation of pig heart type II cAMP-dependent protein kinase is 11.2 μM .

Identical kinetic experiments were carried out with purified type I (bovine muscle) cAMP-dependent protein kinase and with partially purified type I (rabbit muscle)

Fig. 1. Displacement of $[^3\text{H}]\text{cAMP}$ by cAMP, Sp-cAMP[S] and Rp-cAMP[S]

Portions (2 pmol) of purified protein kinase were incubated with 16 pmol of $[^3\text{H}]\text{cAMP}$ (33.5 Ci/mmol) and increasing concentrations of cAMP (Δ), Sp-cAMP[S] ($\times$) or Rp-cAMP[S] ($\square$) for 16 h at 4°C . After $(\text{NH}_4)_2\text{SO}_4$ precipitation, the numbers of pmol of bound $[^3\text{H}]\text{cAMP}$ were calculated. The inset shows a linear-regression analysis of the data from which the apparent K_d values were calculated from the abscissa-axis intercept.

Fig. 2. cAMP-dependent protein kinase activation curves

Purified type II holoenzyme from pig heart was activated by the addition of $0.006\ \mu\text{M}$ - to $10\ \mu\text{M}$ -cAMP in the presence of the following concentrations of Rp-cAMP[S]: $0\ \mu\text{M}$ ($\bullet$), $10\ \mu\text{M}$ ($\blacklozenge$), $30\ \mu\text{M}$ ($\blacktriangle$) and $100\ \mu\text{M}$ ($\blacktriangledown$). Phosphotransferase activity was measured as described in the Experimental section and plotted as fractional activation. The activation data were replotted in accordance with the Hill equation as described in the Experimental section and the data points for the inset were obtained from the abscissa-axis intercepts (EC_{50} values). The ordinate-axis intercept of the inset plot divided by its slope is the K_i value for Rp-cAMP[S].

and type II (bovine heart) cAMP-dependent protein kinase. The calculated K_i values for Rp-cAMP[S] inhibition of cAMP-induced activity and the K_a values for cAMP-induced activation are listed in Table 3 under the heading 'K'. The calculated $\delta\Delta G$ values are also listed in this Table.

Correlation between binding and dissociation of the holoenzyme

Concentrations of cAMP ($0.024\ \mu\text{M}$), Sp-cAMP[S] ($0.24\ \mu\text{M}$) or Rp-cAMP[S] ($2.4\ \mu\text{M}$), calculated from Fig. 1 to displace approx. 75% of bound $[^3\text{H}]\text{cAMP}$ from purified holoenzyme, were allowed to come to equili-

Table 2. Effects of Rp-cAMP[S] on cAMP-induced activation of type II cAMP-dependent protein kinase

cAMP-dependent protein kinase activation was measured as phosphotransferase activity as described in the Experimental section. Experimental data are shown in Fig. 2 and the experimental details are given in the legend to Fig. 2 and in the Experimental section. EC_{50} is the concentration of cAMP required for half-maximal activation of phosphotransferase activity. Values were obtained from the abscissa axis intercepts of Hill plots. h is the Hill coefficient derived from a non-linear-regression analysis of each set of activation data using the function describing a sigmoid curve (eqn. 1). See the Experimental section for details. Data are shown in Fig. 2. h' is the Hill coefficient derived from the slopes of the Hill plots of the activation data.

Concn. of Rp-cAMP[S] (μ M)	EC_{50} (nM)	h	h'
0	79	1.77	1.69
10	148	1.66	1.58
30	330	1.70	1.66
100	830	1.60	1.56

brium with the holoenzyme. After incubation at room temperature for 1 h, each preparation was subjected to polyacrylamide-gel electrophoresis in the absence of SDS. The experiment was performed twice with identical results, and a photograph of one of the gels is shown in Fig. 3. The dissociated regulatory subunits (lanes 1, 4 and 7) are not observable on the portion of the gel shown, but the holoenzyme band is seen at 190 kDa (lanes, 2, 5 and 8). The addition of a 20-fold molar excess of cAMP resulted in the total loss of the holoenzyme band (lane 3), addition of a 200-fold molar excess of Sp-cAMP[S] resulted in loss of approx. 85% of the holoenzyme band (lane 6) and addition of a 2000-fold molar excess of Rp-cAMP[S] resulted in no measurable loss of the holoenzyme band (lane 9).

DISCUSSION

Since both diastereoisomers bind to the cAMP-binding sites on cAMP-dependent protein kinase, but have the opposite effects on the tetrameric structure of the holoenzyme, activation of this enzyme appears to involve

Table 3. Inhibition constants for Rp-cAMP[S] interaction with the protein kinase isoenzymes from different tissues and species

The preparation, sources and purity of the various enzymes are given in the Experimental section. K (cAMP) is the concentration of cAMP experimentally determined to activate the enzyme half-maximally, and K (Rp-cAMP[S]) is the concentration of Rp-cAMP[S] experimentally determined to inhibit the cAMP-induced activation half-maximally. The derivation of $\delta\Delta G$ values is described in the Experimental section.

Holoenzyme	Analogue	K (nM)	$\delta\Delta G$ (kJ/mol)
Type I (rabbit muscle)	cAMP	47	
	Rp-cAMP[S]	5800	13.7
Type I (bovine muscle)	cAMP	57	
	Rp-cAMP[S]	8300	14.0
Type II (bovine heart)	cAMP	52	
	Rp-cAMP[S]	6300	13.7
Type II (pig heart)	cAMP	79	
	Rp-cAMP[S]	11200	14.0

two separate and dissociable events. The initial event presumably involves the binding of two molecules of cAMP to two non-identical binding sites on the regulatory subunit (Corbin *et al.*, 1978; Weber & Hilz, 1979; OGREID & DØSKELAND, 1980); subsequently or simultaneously, an additional molecular interaction results in a conformational change in the holoenzyme leading to dissociation of the subunits.

Sp-cAMP[S], a cAMP analogue with a sulphur atom substitution in the axial exocyclic oxygen position, is capable of triggering both the binding and the dissociation events. The 10-fold decrease in the binding affinity of this analogue as compared with cAMP is presumably due to a less favourable interaction between the analogue and the enzyme resulting from the substitution of a sulphur atom for the axial exocyclic oxygen atom. Rp-cAMP[S], a cAMP analogue with a sulphur atom substitution in the equatorial exocyclic oxygen position, binds to the regulatory subunit but does not induce dissociation of the holoenzyme. There is a 90-fold decrease in the binding affinity of Rp-cAMP[S] compared with cAMP for the holoenzyme (de Wit *et al.*,

Fig. 3. Dissociation of the holoenzyme by cAMP, Sp-cAMP[S] or Rp-cAMP[S]

Holoenzyme (1.2 nM) was incubated for 1 h at room temperature with 24 nM-cAMP, 240 nM-Sp-cAMP[S] or 2400 nM-Rp-cAMP[S]. The samples were applied to a non-denaturing polyacrylamide electrophoresis gel as described in the text. The different lanes of the gel contained the following samples: lanes 1, 4 and 7, 0.6 nM regulatory subunit (5.5 μ g); lanes 2, 5 and 8, 1.2 nM holoenzyme (2.4 μ g); lane 3, 1.2 nM holoenzyme plus 24 nM-cAMP; lane 6, 1.2 nM holoenzyme plus 240 nM-Sp-cAMP[S]; lane 9, 1.2 nM holoenzyme plus 2400 nM-Rp-cAMP[S].

1982), implying that the substitution of a sulphur atom for the equatorial exocyclic oxygen atom results in an even less favourable analogue-enzyme interaction. The fact that the same 90-fold loss in binding affinity is obtained with Rp-cAMP[S] and the isolated regulatory subunit dimer (de Wit *et al.*, 1982) suggests that the decreased affinity results from an unfavourable binding interaction and is unrelated to the inability of this compound to induce the molecular interaction that results in dissociation of the holoenzyme. These data clearly demonstrate that the cyclic phosphate portion of the molecule and, in particular, the integrity of the exocyclic oxygen atoms are intimately involved in both the binding affinity and the initiation of the dissociation event.

Thus, as has been previously proposed, and has been physically demonstrated here in the polyacrylamide-gel electrophoresis experiment, Rp-cAMP[S] binds to the cAMP-binding sites on the regulatory subunits without causing dissociation of the holoenzyme. Presumably it is this property that enables Rp-cAMP[S] to inhibit cAMP-induced activation of cAMP-dependent protein kinase, and the effects of Rp-cAMP[S] on the various kinetic parameters of the cAMP-activated enzyme imply that this analogue is a competitive inhibitor. Thus the binding of Rp-cAMP[S] to the holoenzyme does not affect the ability of cAMP to induce maximal velocity ($V_{\max}$), but does shift the EC_{50} value for cAMP-activation of the enzyme to higher concentrations of agonist. In addition, in the presence of concentrations of Rp-cAMP[S] that affect the EC_{50} for cAMP the degree of co-operativity (h) of the cAMP activation process is not significantly different, suggesting that Rp-cAMP[S] competes with cAMP for both of the cAMP-binding sites on the regulatory subunit. Further experiments designed to look specifically at the individual binding sites are required to determine whether this is true.

Rp-cAMP[S] inhibits the cAMP-induced activation of the various cAMP-dependent protein kinases with an average K_i value of $7.9 \pm 1.2 \mu\text{M}$. The experimental values obtained for K_i are slightly lower for the type I isoenzyme ($6.05 \pm 0.35 \mu\text{M}$) than for the type II isoenzyme ($9.75 \pm 2.05 \mu\text{M}$), but the differences are not statistically significant. Thus the data imply that there is no significant difference in the experimental K_i values obtained for inhibition of the type I isoenzyme compared with the type II isoenzyme or for inhibition of the purified reconstituted isoenzymes compared with the partially purified isoenzymes from different tissues of different species. Calculations of the $\delta\Delta G$ values confirm the lack of a significant difference between the two isoenzymes obtained from different tissues and species. Also, the average $\delta\Delta G$ value of $13.9 \pm 0.09 \text{ kJ/mol}$ determined from the different enzymes listed in Table 3 agrees very well with the value of 14.3 kJ/mol obtained by de Wit *et al.* (1982) for type I holoenzyme from rabbit skeletal muscle.

In summary, Sp-cAMP[S] and Rp-cAMP[S] are diastereoisomeric compounds that have a 10-fold difference in binding affinity to cAMP-dependent protein kinase and that have opposite effects on the tetrameric structure and thus the activity of this enzyme. Since these analogues differ from cAMP only in the substitution of a sulphur atom for an exocyclic oxygen atom, this part of the cAMP molecule must be very important in both the

molecular events that determine the binding affinity as well as those involved in the dissociation of the holoenzyme. Rp-cAMP[S], which has an equatorial exocyclic sulphur atom, is a competitive inhibitor of cAMP-induced dissociation of the holoenzyme with a K_i of $7.9 \pm 1.2 \mu\text{M}$ measured for both isoenzymes obtained from different tissues and species.

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