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Cyclic Nucleotides Modulate the Release of [^{3}H]Adenosine Cyclic 3′,5′-Phosphate Bound to the Regulatory Moiety of Protein Kinase I by the Catalytic Subunit of the Kinase†

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ABSTRACT: The rate of release of bound c[^{3}H]AMP from the two types (A and B) of cAMP binding sites on the regulatory subunit dimer (R_2^I) of rabbit muscle protein kinase I was studied in the presence of the catalytic (C) subunit of protein kinase. Rebinding of released c[^{3}H]AMP was avoided by using highly diluted reactants or adding unlabeled cAMP or its analogues. No significant C-induced dissociation of R_2^I-(c[^{3}H]AMP)$_4$ occurred in the absence of Mg^{2+}-ATP. Of the two options that one or two molecules of C are required to induce the release of c[^{3}H]AMP bound to R_2^I, only the first one was compatible with the first-order dependence on [C] of the rate of release of c[^{3}H]AMP observed over a wide range of C concentrations. In the absence of added unlabeled cyclic nucleotide, the rate of the C-induced release of c[^{3}H]AMP was the same from site A and site B. The apparent second-order rate constant for the association of C to R_2^I(c[^{3}H]AMP)$_4$ was 6×10^6 M^{-1} s^{-1} (37 °C, 0.15 M KCl). Raising the concentration of unlabeled cAMP in the medium up to 1 μM decreased by up to 50% the rate of the C-induced release of bound c[^{3}H]AMP from both sites. This is explained by assuming that the association of one molecule of C to R_2^I(c-[^{3}H]AMP)$_4$ leads to the release of c[^{3}H]AMP first from one R subunit and subsequently, by a process that can be blocked by about 1 μM cAMP, from the other R subunit. A further rise of the cAMP concentration decreased the rate of release from site B only, so that the C-induced release of c[^{3}H]AMP occurred almost exclusively from site A at very high concentrations of cAMP. This suggests that c[^{3}H]AMP is released first from site A and that this vacant site by interacting with cAMP inhibits the release of c[^{3}H]AMP from site B of the same R subunit. The role of site A in controlling the C-induced release was further supported by the finding that several cAMP analogues inhibited the release with potencies correlating with their affinities for site A. The C-induced release of c[^{3}H]AMP from aged R_2^I was about 10 times slower than that from fresh R_2^I. No significant C-induced release of c[^{3}H]AMP was observed from the monomeric fragment obtained by limited trypsin treatment of R_2^I.

The cAMP-dependent protein kinase (cAK)[1] exists in two isozyme forms (cAKI and cAKII), both of which are composed of a regulatory subunit dimer and two catalytic subunits. Activation of the enzyme by cAMP is accompanied by dissociation (Krebs, 1972) according to the overall equation:

$$R_2C_2 + 4cAMP \rightleftharpoons R_2(cAMP)_4 + 2C \quad (1)$$

where R_2 is the regulatory subunit dimer and C the catalytic subunit of cAK (Corbin et al., 1978; Weber & Hilz, 1979).

It is known that R_2^I (Døskeland, 1978) and R_2^{II} (Rannels & Corbin, 1980a; Øgreid & Døskeland, 1980) have two types of cAMP binding sites, termed A and B (Døskeland & Øgreid, 1981) according to the rate with which they exchange bound labeled cAMP.

The present study is concerned with the reverse reaction of eq 1. The primary aims were to find the order in which cAMP dissociated from site A and B in the process of the C-induced release of cAMP from its complex with R_2^I and the possible significance of interactions between the binding sites in this process. Another concern was whether one or, as commonly assumed (Smith et al., 1981; Builder et al., 1981; Flockhart & Corbin, 1982), two C subunits must bind to the R_2^I(cAMP)$_4$ complex to effect the release of any bound cAMP.

Experimental Procedures

Materials. c[8-^{3}H]- or c[5′,8-$^3H_2$]AMP (of specific activities varying from 26 to 60 Ci/mmol) and [γ-^{32}P]ATP were from the Radiochemical Centre, Amersham, U.K. Adenosine, AMP, ADP, ATP, cAMP, cIMP, cGMP, and N^6-monobutyryl-cAMP were from Sigma Chemical Co., St. Louis, MO. 8-Amino-cAMP and 2-*n*-butyl-cAMP were kindly supplied by Dr. Jon P. Miller and Dr. R. H. Suva, SRI-International, Menlo Park, CA.

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[1] Abbreviations: cAKI, cAMP-dependent protein kinase, isozyme form I; cAKII, cAMP-dependent protein kinase, isozyme form II; R_2^I, the regulatory subunit dimer of cAKI; R_{t1}^I, partially proteolyzed monomeric fragment of R_2^I; R_2^{II}, regulatory subunit dimer of cAKII; C, catalytic subunit of cAKI or cAKII; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; k_a, apparent second-order rate constant for the association between two molecules; k_d, apparent first-order dissociation rate constant for the dissociation of a complex; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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Active charcoal was pretreated as described (Øgreid & Døskeland, 1981b) except that the heat-stable protein fraction was absent from the solutions.

Buffer A is 15 mM Hepes–NaOH, pH 7.0, 0.3 mM EGTA, 0.15 M KCl, 6 mM magnesium acetate, 20 mM 2-mercaptoethanol, 0.5 mM dithiothreitol, and 0.5 mg/mL bovine serum albumin.

Preparation of R_2^I. cAKI holoenzyme was prepared from fresh rabbit muscle by conventional purification methods, essentially as described (Døskeland, 1978). In some cases, the homogenization buffer contained 10 mM benzamidine, and chromatography on DEAE-agarose replaced hydroxylapatite chromatography.

R_2^I complexed with c[^{3}H]AMP was obtained in about 80% yield as follows: 2 mL of cAKI (0.1 μM with respect to the R subunit) was incubated with 0.4 μM c[^{3}H]AMP for 1 h at 0 °C in buffer A without KCl and serum albumin and run through a column (0.9 × 3 cm) of carboxymethylcellulose equilibrated in the same buffer containing 5 nM c[^{3}H]AMP. The flow-through fractions were mixed with 3 volumes of 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 20 mM 2-mercaptoethanol and applied to a column (0.9 × 7 cm) of DEAE-agarose equilibrated with the Tris buffer containing 5 nM c[^{3}H]AMP, and the column was washed with 25 mL of the same buffer. R_2^I complexed with c[^{3}H]AMP was eluted with a 100-mL linear NaCl gradient (0–0.25 M) in the Tris buffer. Free (uncomplexed) R_2^I was obtained by a modification (Døskeland & Kvinnsland, 1980) of the urea dissociation method of Schwechheimer & Hofmann (1977) in about 20% yield. The solutions of urea contained 25 mM methylamine. Relative to the cAMP binding capacity, both free and complexed R_2^I had less than 1% as much protein kinase activity as cAKI. The preparations could be stored up to 7 days at 4 °C. Upon prolonged storage, progressively more of site B was converted to a form releasing bound c[^{3}H]AMP more rapidly.

Preparation of R_2^I with c[^{3}H]AMP Preferentially Bound to Site A or B. cAMP associates more rapidly to site A than to site B of R_2^I or R_2^{II} (Døskeland & Øgreid, 1981; Øgreid & Døskeland, 1981b). Reaction of free R_2^I (5 nM with respect to R subunits) with 30 nM c[^{3}H]AMP for 20 s at 0 °C in buffer A containing 3 M KCl, followed by a brief (5 s) treatment with 10 mg/mL charcoal (which was removed by sedimentation: 13000*g* for 3 min at 0 °C), resulted in a preparation of R_2^I with about 40% saturation of site A and 3–5% saturation of site B.

For the production of R_2^I with c[^{3}H]AMP selectively bound to site B, advantage was taken of the considerably faster rate of release of cAMP from site A than site B. R_2^I complexed with c[^{3}H]AMP was incubated for 3 h at 4 °C in buffer A with 3 M KCl and 10 mg/mL charcoal with constant shaking, and the charcoal was removed by centrifugation. The resulting preparation had 30–40% saturated site B and less than 4% saturated site A.

The above preparations were used for experiments less than 15 min after they had been prepared.

Preparation of R_{t1}^I by Limited Proteolysis. R_2^I(c[^{3}H]-AMP)$_4$ (50 nM with respect to the R subunit) was treated for 20 min at 0 °C with 0.4 mg/mL trypsin in buffer A with 0.5 μM c[^{3}H]AMP. The reaction was stopped by the addition of soybean trypsin inhibitor (final concentration 1 mg/mL). The incubate was applied to a column (0.6 × 30 cm) of Ultrogel AcA-34 (LKB, Bromma, Sweden) equilibrated with buffer A containing 20 nM c[^{3}H]AMP. The c[^{3}H]AMP binding activity eluted corresponding to a Stokes radius of 2.7 nm,[2] confirming that a monomeric fragment (R_{t1}^I) of R_2^I had been formed (Potter & Taylor, 1979; Rannels & Corbin, 1980b).

Preparation of the Catalytic Subunit of cAK. The C subunit of bovine cardiac muscle cAKII or rabbit muscle cAKI was prepared as described (Øgreid & Døskeland, 1981b). The preparations of C had less than 0.5% of the c[^{3}H]AMP binding activity of cAK with comparable phosphotransferase activity. The catalytic activity and the ability to enhance the rate of dissociation of the R_2^I(c[^{3}H]AMP)$_4$ complex were unaltered during storage for up to 10 weeks at 4 °C.

Preparation of Heat-Stable Protein Kinase Inhibitor. Partially purified protein kinase inhibitor was prepared by a modification (Ueland & Døskeland, 1976) of the procedure of Walsh et al. (1971).

Determination of the Concentration of cAK Subunits. Since each mole of R subunit binds 2 mol of cAMP (Corbin et al., 1978; Weber & Hilz, 1979), the concentration of R subunit in a preparation was taken to be half that of the cAMP binding capacity of that preparation.

Since the composition of the cAK holoenzyme is R_2C_2, the concentration of C in a preparation of holoenzyme was taken to be equal to that of R. The concentration of free C was determined by comparing its phosphotransferase activity with that of the holoenzyme (in the presence of a maximally activating concentration of cAMP) from which the C was derived. Except when otherwise indicated, the concentration of R_2^I refers to the concentration of R subunit.

Determination of Bound c[^{3}H]AMP. The amount of c-[^{3}H]AMP bound to both sites (A + B) of R was determined by mixing an aliquot of the sample to be measured with 9 volumes of ice-cold 3.2 M ammonium sulfate solution and collecting the precipitated bound nucleotide on a membrane filter. This method has previously been shown (Døskeland et al., 1977; Øgreid & Døskeland, 1980) to give complete recovery of c[^{3}H]AMP bound to R_2^I. In one particular experiment, bound and free c[^{3}H]AMP's were separated by chromatography on Sephadex G-25 (see the legend to Figure 3 for details).

Measurement of the C-Induced Release of c[^{3}H]AMP from Sites A and B. The incubations (5 mL) were routinely started by the addition of ice-cold R–c[^{3}H]AMP complex (100 μL) to a final concentration of 0.02–0.2 nM (with respect to R).

Samples (450 μL) were removed at 10 different times of incubation, and the reaction was stopped by mixing them with 4.5 mL of quench solution (15 mM Hepes–NaOH, pH 7.0, 3.5 M NaCl, 10 mM EDTA, 0.1 mM cAMP, 20 mM 2-mercaptoethanol, 0.5 mg/mL albumin, and 0.2 mg/mL heat-stable protein kinase inhibitor). Aliquots (1 mL) were removed immediately and after 7, 18, and 24 h for the determination of bound c[^{3}H]AMP. c[^{3}H]AMP bound to site A exchanges more rapidly ($k_d \sim 2 \times 10^{-4}$ s^{-1}) than that bound to site B ($k_d \sim 10^{-6}$ s^{-1}) in the quench solution at 0 °C (Døskeland, 1978). Thus, less than 2% of the c[^{3}H]AMP initially bound to site A remains bound after 7 h in the quench solution. The amount of c[^{3}H]AMP bound to site B at the moment of quenching could be determined by plotting the logarithm of the values found for bound c[^{3}H]AMP after 7, 18, and 24 h of exchange vs. time and extrapolating to zero

[2] The column had been calibrated with the following standard proteins whose assumed Stokes' radius is indicated in parentheses: cytochrome *c* (1.7 nm), myoglobin (1.9 nm), chymotrypsinogen (2.1 nm), soybean trypsin inhibitor (2.3 nm), ovalbumin (2.8 nm), bovine serum albumin (3.55 nm), transferrin (3.6 nm), catalase (5.2 nm), and ferritin (7 nm). R_2^I eluted at a position corresponding to a Stokes radius of 4.6 nm.

time. The amount of c[^{3}H]AMP bound to site A was calculated as total nucleotide bound (site A + site B) minus nucleotide bound to site B. The C subunit did not affect the release of c[^{3}H]AMP when added after the quench solution.

When the release of bound c[^{3}H]AMP was expected to be very rapid, the reaction was started in a different manner: R_2^I(c[^{3}H]AMP)$_4$ was preincubated for 2 min with 30 nM c[^{3}H]AMP in buffer A at the temperature to be used in the experiment. R_2^I was more than 95% saturated with c[^{3}H]-AMP under such preincubation conditions. The reaction was started by mixing equal volumes (50 μL) of R_2^I(c[^{3}H]AMP)$_4$ and C subunit (preincubated at the same temperature) and stopped by the addition of 0.9 mL of quench solution. Since these incubations contained a very high concentration (15 nM) of c[^{3}H]AMP, rebinding of dissociated c[^{3}H]AMP could only be prevented by the presence of high concentrations of unlabeled cyclic nucleotides.

The C-induced release of c[^{3}H]AMP is defined as the release observed in the presence of C minus the release observed in the absence of C[3] under otherwise identical conditions.

For most of the experiments to be reported, the rate of this release in each particular incubate would be expected to be pseudo first order with respect to the concentration of the R–c[^{3}H]AMP complex, since other reactants (C, Mg^{2+}-ATP) were present in excess and thus at virtually constant concentrations during the incubation. The rate of the C-induced release of c[^{3}H]AMP bound to R is expressed as k_d (apparent dissociation rate constant) throughout this study. This parameter can be estimated from plots of ln (fractional saturation of R in the absence of C minus fractional saturation in the presence of C) vs. incubation time. For a pseudo-first-order reaction, such a plot will be linear with a slope of $-k_d$ (Figures 1, 2, 4, and 8 show examples of such plots). As will be shown in the first paragraph under Results, most of the experiments were conducted under conditions where the reverse reaction (rebinding of c[^{3}H]AMP to the newly formed R–C complex) was so slow relative to the C-induced release of c[^{3}H]AMP from R that it could be neglected.

Measurement of Protein Kinase Activity. This was by an earlier described version (Fossberg et al., 1978) of the filter disk method (Corbin & Reimann, 1974).

Affinity of cAMP Analogues for Sites A and B. The ability of a number of cAMP analogues to compete with c[^{3}H]AMP for binding to sites A and B of R_2^I at 37 °C, pH 7.0, 0.15 M KCl, has recently been determined (Døskeland et al., 1983). The relative affinity of an analogue for site A or B is expressed by the ratio $K_{I,cAMP}/K_{I,analogue}$, where $K_{I,cAMP}$ and $K_{I,analogue}$ are the apparent inhibition constants for the inhibition of c[^{3}H]-AMP binding by unlabeled cAMP and analogue, respectively.

For the cAMP analogues used in the present study, the values of $K_{I,cAMP}/K_{I,analogue}$ for site A and site B were the following: N^6-monobutyryl-cAMP, 3.6/0.093; cAMP, 1.0/1.0; 2-*n*-butyl-cAMP, 0.26/0.72; cIMP, 0.11/0.027; cGMP, 0.0046/0.014 (Døskeland et al., 1983). The value of $K_{I,cAMP}/K_{I,analogue}$ determined for 8-amino-cAMP was 0.17/3.9, which is similar to that previously reported by using a slightly different method (Corbin et al., 1982).

Results

Examination of the Assay for the C-Induced Release of c[^{3}H]AMP from R. The reaction of interest in the present study is the release of c[^{3}H]AMP from R brought about by the C subunit of cAK. The rate of this reaction is expressed by the value of k_d, which is calculated from the slopes of semilogarithmic plots like those shown in Figures 1, 2, 4, and 8 (see Experimental Procedures for details). Such plots will deviate from linearity and show a slope that is too shallow if (1) c[^{3}H]AMP rebinds to the complex between R and C formed during the incubation or (2) the concentration of free C subunit decreases during the incubation due to trapping in a complex with R or instability. If C promotes any irreversible denaturation of R, the k_d will be overestimated.

Both R and C were stable during the incubation. Thus, more than 90% of the phosphotransferase activity of C and of the c[^{3}H]AMP binding capacity of R were intact after coincubation in buffer A with 0.3 mM ATP for 10 min at 37 °C or for 60 min at 0 °C.

High concentrations of unlabeled cyclic nucleotide will prevent rebinding of c[^{3}H]AMP by isotopic dilution. They also prevent trapping of C by dissociating any complex between R and C (see eq 1 and the last paragraph under Results). Figure 2B shows that the C-induced release of c[^{3}H]AMP from both sites A and B of R_2^I(c[^{3}H]AMP)$_4$ obeys apparent first-order kinetics in the presence of 10 μM unlabeled cAMP and various concentrations of C subunit.

When unlabeled cyclic nucleotide is absent, depletion of free C can be avoided by using a much ($\geq$10 times) higher concentration of C than of R. The degree of dilution of R_2^I(c-[^{3}H]AMP)$_4$ required to avoid rebinding of nucleotide was inferred from a series of pilot experiments conducted at various concentrations (0.01–2 nM) of R_2^I(c[^{3}H]AMP)$_4$. When the concentration of the latter complex was 0.2 nM or less, the plots of ln (bound c[^{3}H]AMP) vs. time were always linear, suggesting that this dilution was sufficient to avoid significant rebinding of c[^{3}H]AMP. This was further supported by the demonstration that only when more than 0.6 nM c[^{3}H]AMP or cAKI holoenzyme was added was the determination of k_d affected under standard experimental conditions (Figure 1).

A comparison of the data of panels A and B of Figure 2 shows that unlabeled cAMP slowed down the rate of release of c[^{3}H]AMP, especially from site B. As shown in Figure 3, an inhibition of the Mg^{2+}-ATP–C-induced release of c[^{3}H]-AMP by unlabeled cAMP was observed also when gel filtration (rather than ammonium sulfate precipitation) was used to separate bound and free c[^{3}H]AMP. This finding was thus not dependent on the method used to determine bound c-[^{3}H]AMP.

C-Induced Release of c[^{3}H]AMP from Aged or Trypsin-Treated R. The release of c[^{3}H]AMP from preparations of R_2^I kept for more than 2 weeks at 4 °C showed two phases, each of which obeyed apparent first-order kinetics and showed similar rate dependences on the concentrations of C, ATP, and unlabeled cAMP. The C-induced release of c[^{3}H]AMP was about 10 times slower in the second phase than in the first phase. The fact that the C subunit enhanced the release of c[^{3}H]AMP from aged R_2^I served to differentiate the latter from trypsin-treated R_2^I, which showed no enhanced dissociation in the presence of C–Mg^{2+}-ATP (Figure 4A).

Effect of ATP and Other Nucleoside Derivatives on the Rate of the C-Induced Release of c[^{3}H]AMP from R_2^I. Under conditions when rebinding of c[^{3}H]AMP was prevented, the C subunit was completely dependent upon ATP to enhance

[3] In buffer A at 37 °C, the apparent k_d values for the spontaneous dissociation of c[^{3}H]AMP from site A and B, respectively, of the complex R_2^I(c[^{3}H]AMP)$_4$ were 7×10^{-3} and 7×10^{-4} s^{-1} in the presence and 12×10^{-3} and 7×10^{-3} s^{-1} in the absence of excess unlabeled cAMP. At 0 °C, the corresponding values for k_d were 7×10^{-5} and 4×10^{-6} s^{-1} in the presence and 9×10^{-5} and 5×10^{-5} s^{-1} in the absence of unlabeled cAMP. For R_{t1}^I, the k_d for the dissociation from site B was as for R_2^I, whereas the k_d for the dissociation from site A at 0 °C was 2×10^{-4} s^{-1} whether unlabeled cAMP was present or not.

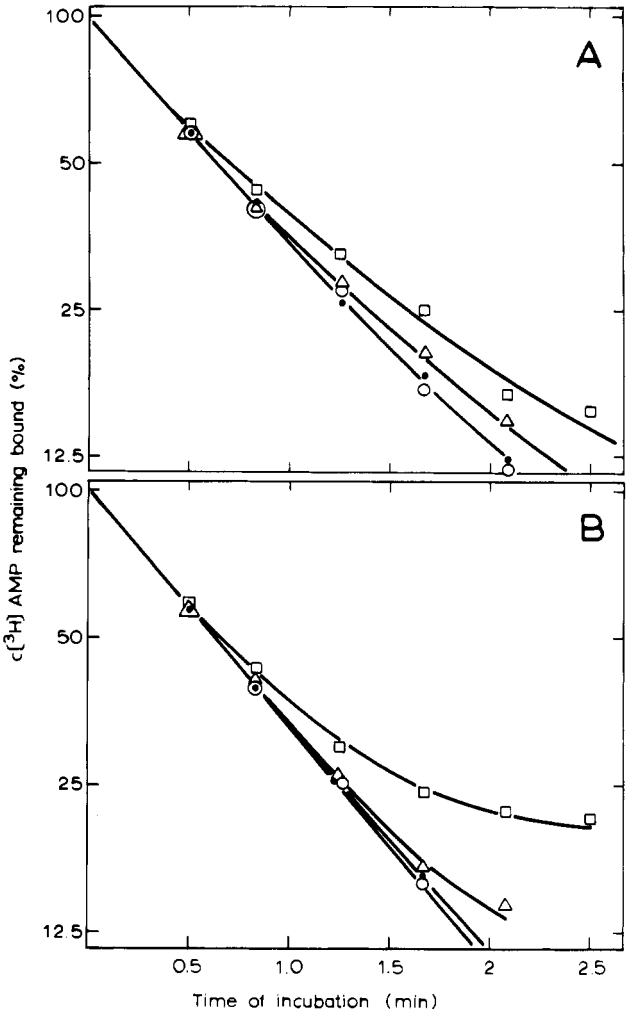

FIGURE 1: Decrease of c[^{3}H]AMP bound to R_2^I as a function of incubation time and the concentrations of added c[^{3}H]AMP or cAKI holoenzyme. R_2^I(c[^{3}H]AMP)$_4$ (0.3 nM) was incubated at 37 °C in buffer A with 3 nM C subunit and 3 mM ATP. Aliquots were removed for the determination of bound c[^{3}H]AMP (site A + B) after various periods of time. (A) Incubations contained 0 (○), 0.6 (●), 1.8 (△), or 6 nM (□) added c[^{3}H]AMP of the same specific activity as that bound to R_2^I. (B) Incubations contained 0 (○), 0.6 (●), 1.2 (△), or 6 nM (□) cAKI holoenzyme. The concentrations given for R_2^I and cAKI refer to the concentrations of R^I subunit. For both panels A and B of this figure as well as for panels A and B of Figures 2, 4, and 8, the natural logarithm of the fractional saturation of R_2^I with c[^{3}H]AMP is plotted against the incubation time (see the Experimental Procedures for details). So that interpretation could be facilitated, numbers on the ordinate refer to the percent of (initially bound) c[^{3}H]AMP remaining bound. 100% corresponds to a value of ln fractional saturation of 1, 50% corresponds to −0.69, 25% corresponds to −1.39, and 12.5% corresponds to −2.08.

the rate of release of c[^{3}H]AMP from either site A or site B of R_2^I(c[^{3}H]AMP)$_4$ (Figure 4A).

The effect of ATP was concentration dependent, being optimal at about 0.1 mM ATP and considerably less at 3 mM than at 0.3 mM ATP (Table I). The reassociation between R_2^{II} and C has also been reported to be inhibited by millimolar concentrations of ATP (Rangel-Aldao & Rosen, 1977). The inhibition of the C–Mg^{2+}-ATP-induced release of c[^{3}H]AMP by adenosine, AMP, and ADP was presumably due to competition with ATP since the inhibition was overcome by increasing the concentration of ATP (Table I). Furthermore, the potencies of adenosine, AMP, and ADP as inhibitors of the release of c[^{3}H]AMP corresponded with their known potencies as inhibitors of Mg^{2+}-ATP binding to the cAKI holoenzyme (Hoppe et al., 1977).

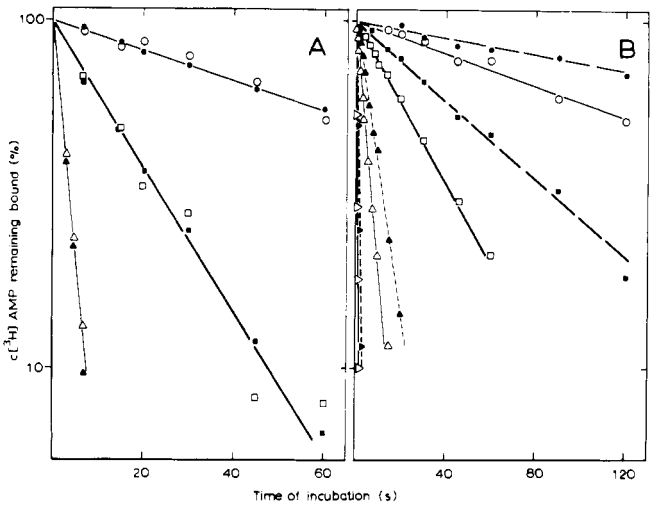

FIGURE 2: Time course of the C-induced release of c[^{3}H]AMP from R_2^I(c[^{3}H]AMP)$_4$ from sites A and B at various concentrations of C. R_2^I(c[^{3}H]AMP)$_4$ (0.2 nM) was incubated at 37 °C in buffer A with 3 mM ATP and 1.6 (○, ●), 8 (□, ■), 50 (△, ▲), or 400 nM (▷, ▶) C subunit. The amount of c[^{3}H]AMP bound to site A (open symbols) and site B (closed symbols) was determined after various periods of incubation. The incubations contained 0 (A) or 10^{-5} M (B) unlabeled cAMP.

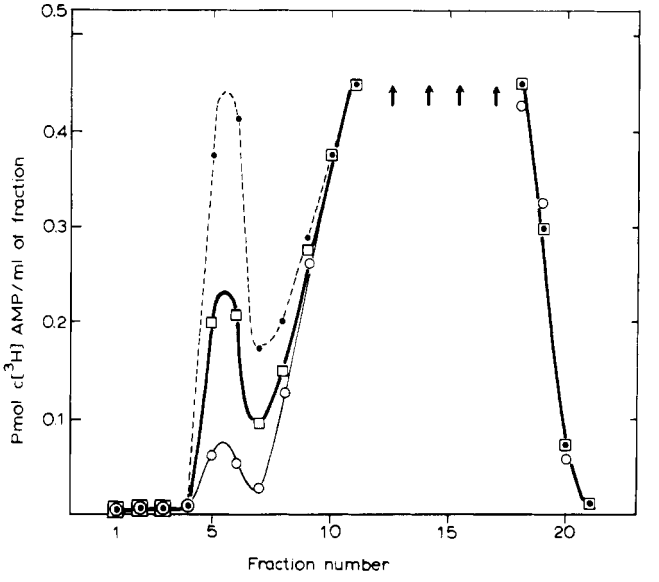

FIGURE 3: R_2^I(c[^{3}H]AMP)$_4$ (1 nM) was incubated at 37 °C in buffer A (0.4 mL) with 8 nM C subunit in the absence of ATP (●), in the presence of 3 mM ATP (○), or in the presence of 3 mM ATP and 0.1 mM unlabeled cAMP (□). After 1 min of incubation, 0.1 mL of ice-cold 300 mM aqueous Na_2EDTA–NaOH (pH 7.0) was added, and the mixture was rapidly layered on a column (0.7 × 30 cm) of Sephadex G-25 (medium) equilibrated with buffer A containing 10 mM EDTA instead of 6 mM magnesium acetate. The chromatography was performed at 4 °C, and fractions of about 0.5 mL were collected. The fractions were mixed with 0.3 mL of 2% aqueous sodium dodecyl sulfate, quantitatively transferred to scintillation vials, mixed with 10 mL of Instagel (Packard), and counted.

It cannot be excluded that inhibition of the C-induced release of cAMP through competition with Mg^{2+}-ATP is of significance. The data of Table I suggest that if the level of ADP increases relative to that of ATP, the C-induced release of R_2^I-bound cAMP will be decreased, leading to a higher sensitivity of cAKI for activation by cAMP according to eq 1. Since Mg^{2+}-ATP is not required for recombination of the subunits of cAKII (Rangel-Aldao & Rosen, 1977), such a mechanism would only operate for cAKI.

In contrast to the strict requirement for Mg^{2+}-ATP for the C-induced release of c[^{3}H]AMP from R_2^I(c[^{3}H]AMP)$_4$

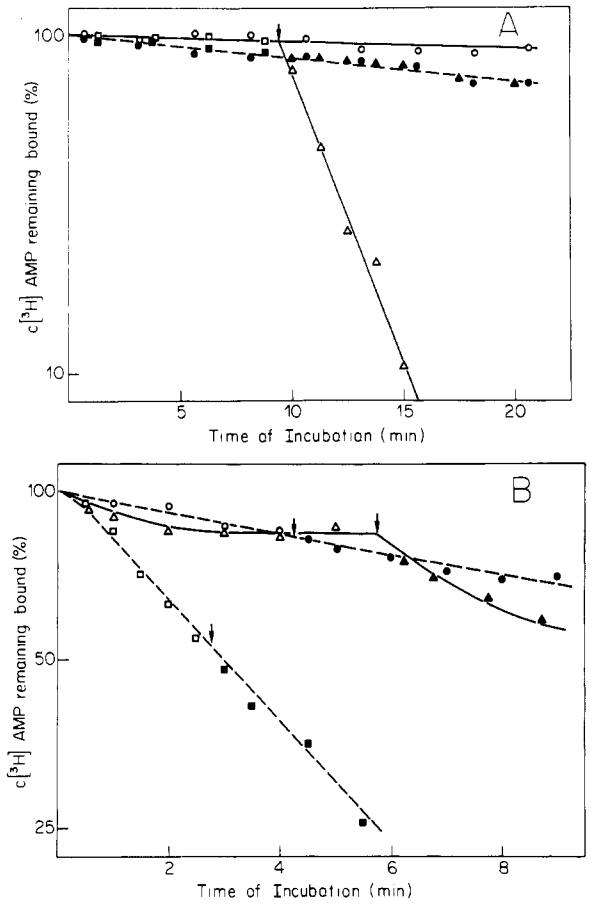

FIGURE 4: Effect of the C subunit and ATP on the rate of release of c[^{3}H]AMP bound to R_2^I or R_{t1}^I. (A) The rate of exchange of labeled cAMP bound to site A of R_2^I(c[^{3}H]AMP)$_4$ (○) or R_{t1}^I(c-[^{3}H]AMP)$_2$ (●) in the absence of C was compared to that in the presence of 160 nM C subunit before (□, ■) or after (△, ▲) the addition of ATP (final concentration 0.3 mM). The arrow indicates the time point when ATP (from a 24 mM stock solution) was added. The reactions were carried out at 0 °C in buffer A containing 50 nM unlabeled cAMP. The concentrations of R_2^I and R_{t1}^I were 0.2 nM. The data given are corrected for the dilution occurring on addition of ATP. Results similar to those shown were obtained for the rate of exchange of c[^{3}H]AMP bound to site B. (B) The rate of release of c[^{3}H]AMP from site B of R_2^I was monitored before (open symbols) or after (closed symbols) the addition of C subunit (to a final concentration of 80 nM). No ATP was present. The dashed lines show the rate of dissociation of c[^{3}H]AMP from site B of R_2^I (0.05 nM) in the absence (□, ■) or presence (○, ●) of 50 nM unlabeled cAMP. The solid line shows the amount of c[^{3}H]AMP bound to site B of R_2^I (0.3 nM) in the presence of 7.5 nM c[^{3}H]AMP (of the same specific activity as the bound cyclic nucleotide). The C subunit was added from a 2 μM stock solution at the time points indicated by the arrows. The reactions were carried out at 37 °C in buffer A. Note the difference in temperature between the experiments shown in panels A and B.

(Figure 4A), Brostrom et al. (1971) as well as Builder et al. (1981) have noted an apparent C-induced release in the absence of ATP. In the present study, the C subunit was able to decrease the amount of c[^{3}H]AMP bound to R only when the experimental conditions did not prevent rebinding of c-[^{3}H]AMP (solid line of Figure 4B). This suggests that in the absence of ATP, the C subunit lowers bound c[^{3}H]AMP by inhibiting rebinding of c[^{3}H]AMP rather than by releasing the bound nucleotide. The mechanism for the ATP-independent inhibition of rebinding shown by the C subunit may be indirect: In the absence of C, the spontaneous dissociation of R_2^I(c[^{3}H]AMP)$_4$ will lead to the formation of R_2^I and free c[^{3}H]AMP. In the presence of C, some of the R_2^I will associate with C to form the cAK holoenzyme ($R_2^IC_2$). Since

Table I: C-Induced Release of c[^{3}H]AMP from R_2^I(c[^{3}H]AMP)$_4$. Effect of Nucleoside Derivatives at Various Concentrations of ATP[a]

nucleoside derivative present	k_d(A)/[k_d(B)] 3×10^{-5} M ATP	3×10^{-4} M ATP	3×10^{-3} M ATP
	0.63/0.63	1.0/1.0	0.61/0.62
5×10^{-5} M adenosine	0.28/0.24	0.79/0.89	0.61/0.60
2×10^{-3} M adenosine	0.06/0.03	0.35/0.40	0.50/0.46
5×10^{-5} M AMP	0.59/0.63	0.98/1.2	0.56/0.66
2×10^{-3} M AMP	0.19/0.23	0.88/0.82	0.58/0.54
10^{-5} M ADP	0.50/0.46	0.95/1.0	0.61/0.68
10^{-4} M ADP	0.20/0.25	0.59/0.58	0.58/0.51
2×10^{-3} M ADP	0.12/0.08	0.26/0.23	0.28/0.21
10^{-6} M cAMP	0.43/0.30	0.68/0.55	0.46/0.26
10^{-4} M cAMP	0.32/0.04	0.58/0.03	0.34/0.05
3×10^{-3} M cAMP	0.08/0.04	0.47/0.05	0.38/0.03
3×10^{-3} M cGMP	0.30/0.16	0.43/0.31	0.29/0.12
4×10^{-4} M cIMP	0.31/0.08	0.43/0.09	0.32/0.09

[a] The rate of dissociation of the complex of c[^{3}H]AMP and the regulatory moiety of protein kinase I was assessed from plots like those of Figure 2. The concentration of C was 8 nM. The incubations contained the (final) concentrations of unlabeled nucleoside derivatives given in the first column of the table. The data are given as the relative dissociation rate which is the ratio of the apparent k_d observed for a certain set of conditions to the k_d determined at 0.3 mM ATP in the absence of other added unlabeled nucleoside derivatives.

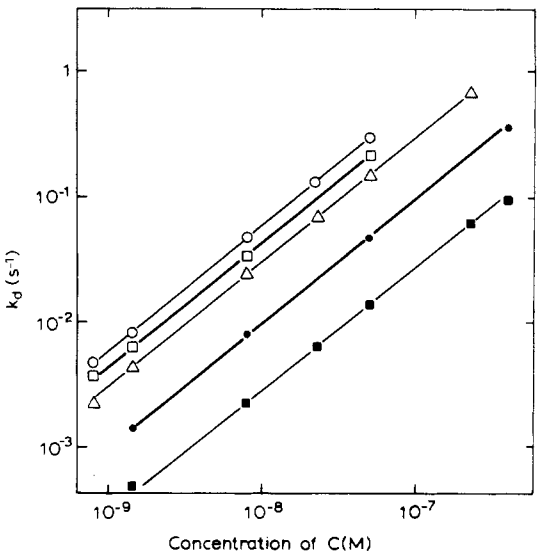

FIGURE 5: Relation between the rate of the C-induced release of c[^{3}H]AMP from R_2^I(c[^{3}H]AMP)$_4$ and the concentration of C. The apparent first-order dissociation rate constant (k_d) for the dissociation of c[^{3}H]AMP from site A or B of R_2^I(c[^{3}H]AMP)$_4$ was calculated from experiments like those of Figure 2. The figure shows the apparent k_d for the dissociation of c[^{3}H]AMP from site B as a function of the concentration of C subunit. The incubations (37 °C) contained 0 (○), 0.2 μM (□), 1.5 μM (△), 15 μM (●), or 0.1 mM (■) unlabeled cAMP. The data for site A (not shown) were similar to those for site B at 0, 0.2, and 1.5 μM added cAMP. At 15 μM and 0.1 mM cAMP, the data for site A were similar to those for site B in the presence of 1.5 μM cAMP.

cAMP associates slower with $R_2^IC_2$ than with R_2^I (Døskeland & Øgreid, 1981), the presence of C by producing the species $R_2^IC_2$ will inhibit the rebinding of c[^{3}H]AMP.

Rate of Release of c[^{3}H]AMP from R_2^I(c[^{3}H]AMP)$_4$. Dependence on the Concentrations of R_2^I(c[^{3}H]AMP)$_4$, C, and Unlabeled Cyclic Nucleotides. The rate of release of bound c[^{3}H]AMP (expressed as k_d) was independent of the concentration (0.03–5 nM tested) of R_2^I(c[^{3}H]AMP)$_4$ when measured in the presence of 10 μM cAMP.

The rate of the C-induced release of c[^{3}H]AMP was proportional to the concentration of C (Figure 5) within the range

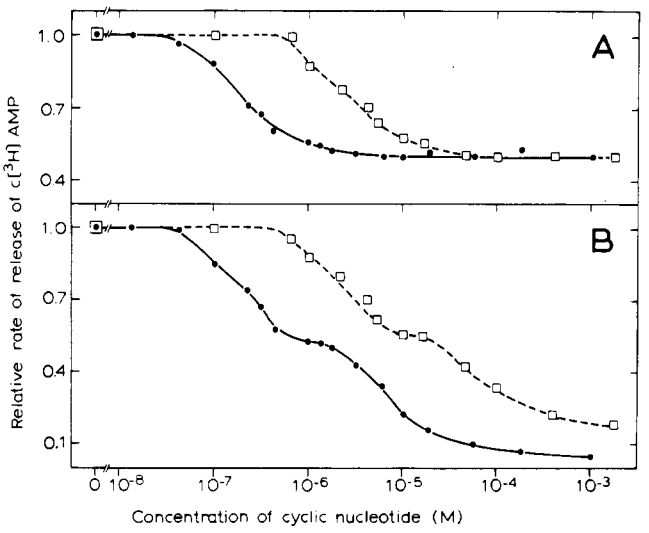

FIGURE 6: Inhibition of the C-induced release of c[^{3}H]AMP from sites A and B of R_2^I(c[^{3}H]AMP)$_4$ by various concentrations of unlabeled cAMP or cIMP. The apparent k_d for the C-induced release of c[^{3}H]AMP from site A (A) and site B (B) was determined at various concentrations of unlabeled cAMP (●) or cIMP (□). The conditions were as described in the legend to Figure 2. The data are given as the relative rate of release of c[^{3}H]AMP, which is the ratio of the k_d determined in the presence of a certain concentration of unlabeled cyclic nucleotide to the k_d determined in the absence of added cAMP or cIMP.

tested (0.8–50 nM in the absence and 1.6–400 nM in the presence of added cAMP at 37 °C). The C subunit derived from bovine cAKII or rabbit cAKI showed similar potency in releasing bound c[^{3}H]AMP.

In buffer A containing 3 mM ATP, the apparent second-order rate constant (k_a) for the interaction of C and R_2^I(c-[^{3}H]AMP)$_4$ leading to release of bound c[^{3}H]AMP was 6×10^6 M^{-1} s^{-1} in the absence of added unlabeled cyclic nucleotide (see the Appendix for the details of the determination of k_a).

In the absence of added unlabeled cyclic nucleotide, the rate of the C-induced release of c[^{3}H]AMP (Figure 2A) was the same from site A [expressed as k_d(A)] as from site B [expressed as k_d(B)]. The dissociation of ligand from the two sites was concerted also in the presence of moderately high concentrations of cyclic nucleotides. Thus, increasing concentrations of unlabeled N^6-monobutyryl-cAMP, cAMP (Figure 6), 2-*n*-butyl-cAMP, 8-amino-cAMP, cIMP (Figure 6), or cGMP decreased the values of k_d(A) and k_d(B) in parallel to about half their original values. However, the release from sites A and B was no more coupled at high concentrations of cyclic nucleotide, when only the rate of the C-induced release from site B was further decreased (Figure 6). This pattern was noted for all the unlabeled cyclic nucleotides tested.

For comparison of their potencies as inhibitors of the C-induced release, the concentrations of cAMP analogues required to decrease k_d(A) or k_d(B) to 0.75 times the original value and k_d(B) to 0.25 times the original value were determined from plots like those of Figure 6. About 30 times more cAMP analogue was required to decrease k_d(B) to 0.25 times than to 0.75 times its original value. There was a clear correlation between the potency of an analogue to decrease k_d(A) or k_d(B) and its affinity for site A (Figure 7A), but not for site B (Figure 7B). This suggested that the inhibition of c[^{3}H]AMP release by cyclic nucleotides was due to interaction with site A, rather than site B or interference with the action of ATP. This latter notion is further strengthened by the observation that the cyclic nucleotides at high concentrations

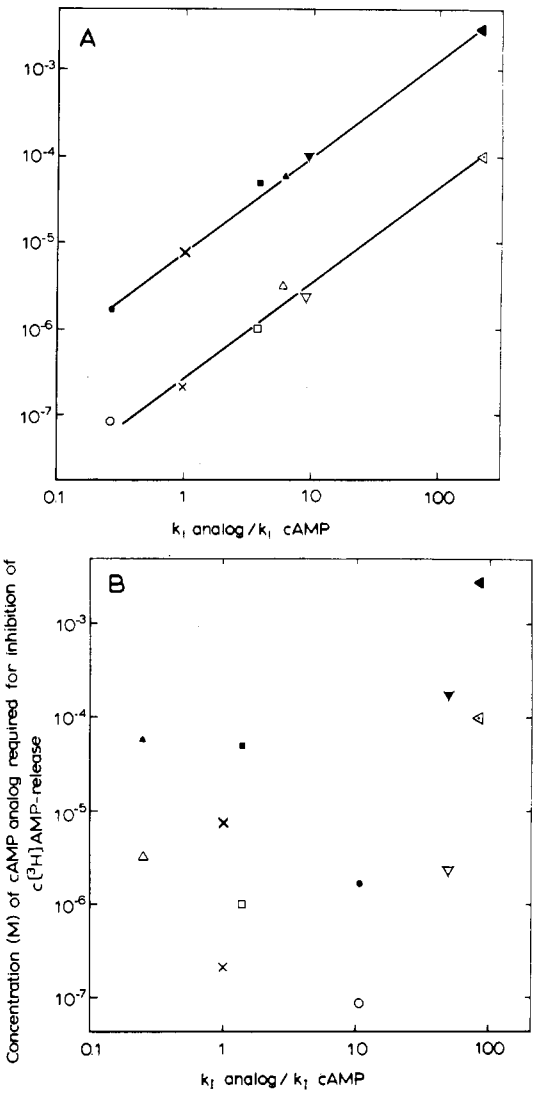

FIGURE 7: Correlation between the potency of cAMP analogues to inhibit the C-induced c[^{3}H]AMP release and their affinity for sites A and B of R_2^I. The concentration of unlabeled cAMP analogue required to decrease the release of c[^{3}H]AMP from site B to 75% (open symbols) or 25% (closed symbols) of its original rate was determined from experiments like those shown in Figure 6. Those parameters were plotted as a function of the ratio $K_{I,analogue}/K_{I,cAMP}$ for site A (A) or site B (B) of R_2^I. The ratio $K_{I,analogue}/K_{I,cAMP}$ is inversely proportional to the affinity of an analogue for its binding site (see Experimental Procedures for details). The straight lines were drawn according to a linear regression analysis by using the least-squares method. The cAMP analogues studied were N^6-monobutyryl-cAMP (○, ●), cAMP (x, X), 2-*n*-butyl-cAMP (□, ■), 8-amino-cAMP (△, ▲), cIMP (▽, ▼), and cGMP (◁, ◀).

inhibited the release from site B more than from site A (Figures 2 and 5, Table I), whereas adenosine, AMP, and ADP that presumably inhibit release by interfering with the action of ATP (see the third paragraph under Results) inhibited the release from sites A and B to the same extent (Table I). Furthermore, unlike that of adenosine, AMP, and ATP, the effect of cyclic nucleotides could not be overcome by increasing the concentration of ATP (Table I).

C-Induced Release of c[^{3}H]AMP from R_2^I Containing c[^{3}H]AMP Selectively Bound to Site A or B. The release of c[^{3}H]AMP from R_2^I with c[^{3}H]AMP chiefly bound to site A (Figure 8A) or site B (Figure 8B) was about 5 times faster than that from R_2^I(c[^{3}H]AMP)$_4$.

This may indicate that the recombination of R_2^I and C is more efficiently inhibited when both sites A and B are occupied than when only one type of binding site is occupied.

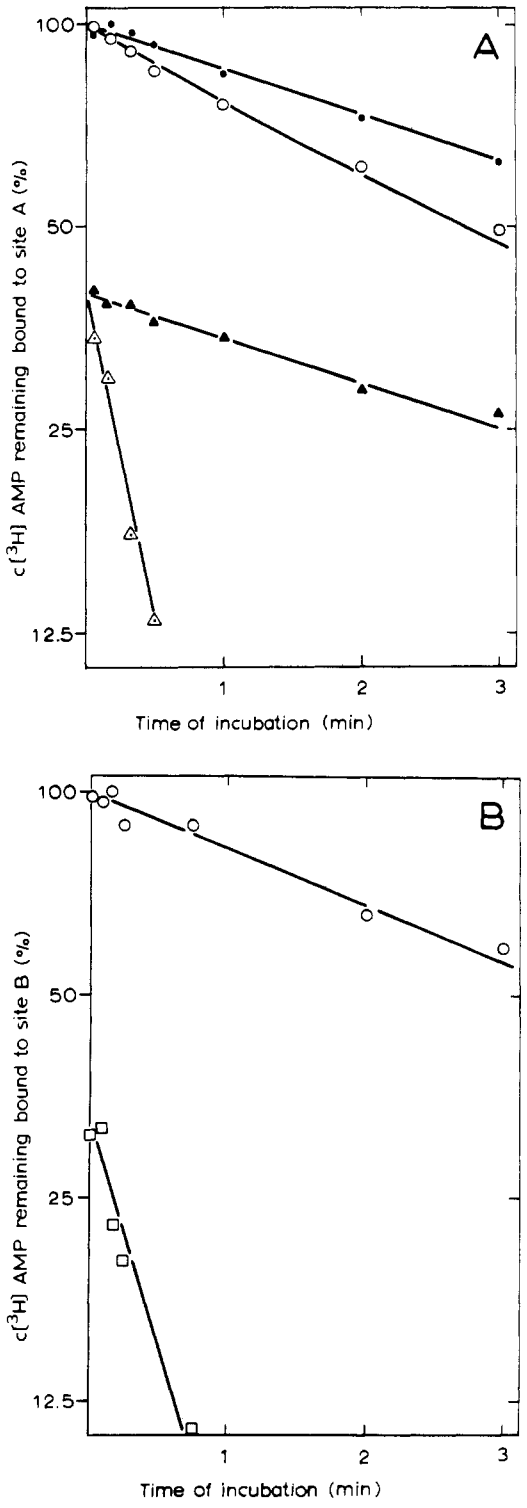

FIGURE 8: Rate of the C-induced release of c[^{3}H]AMP from preparations of R_2^I with c[^{3}H]AMP selectively bound to site A or site B. Preparations of R_2^I with 40% of site A and less than 5% of site B occupied and with 30% of site B and less than 5% of site A occupied were prepared as described under Experimental Procedures. The rate of the C-induced release of c[^{3}H]AMP from such preparations was compared with that from fully saturated R_2^I(c[^{3}H]AMP)$_4$. The incubations were performed at 0 °C in buffer A with 3 mM ATP and 80 nM C subunit. (A) The rate of C-induced release of nucleotide from R_2^I with c[^{3}H]AMP selectively bound to site A (△, ▲) and from fully saturated R_2^I(c[^{3}H]AMP)$_4$ (○, ●) was studied in the absence of added unlabeled nucleotide (open symbols) and in the presence of 0.1 mM cAMP (closed symbols). (B) The rates of C-induced release of c[^{3}H]AMP from R_2^I with the nucleotide selectively bound to site B (□) and from R_2^I(c[^{3}H]AMP)$_4$ (○) were compared in the absence of added unlabeled cyclic nucleotide.

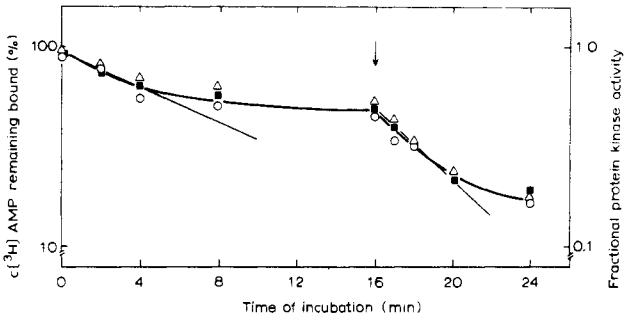

FIGURE 9: Recombination of R_2^I(c[^{3}H]AMP)$_4$ and C. Comparison of the rates of dissociation of c[^{3}H]AMP from its complex with R_2^I and the rate of inhibition of C. R_2^I(c[^{3}H]AMP)$_4$ and C (both at a concentration of 4 nM) were incubated in buffer A without KCl and with 0.1 mM ATP for 16 min at 0 °C and then (arrow) transferred to a water bath kept at 30 °C. The fractional protein kinase activity (■) and the amounts of c[^{3}H]AMP bound to site A (△) or site B (○) were measured in aliquots removed from the same incubations.

However, since less than 50% of either site was saturated in the preparations tested, several of the dimers (R_2^I) presumably carried c[^{3}H]AMP bound to only one of the R subunits. An alternative explanation of the data of Figure 8 is thus that C recombines faster with that R subunit which has no bound c[^{3}H]AMP and that this interaction may lead to release of c[^{3}H]AMP from the other R subunit.

Effect of Temperature on the C-Induced Release of c-[^{3}H]AMP from R. All types of experiments reported in the present study were performed at 0 °C as well as 37 °C. Qualitatively, similar results were obtained at the two temperatures. For each experiment, therefore, only data obtained at one of the two temperatures are shown. Except for experiments involving R_{tI}^I (Figure 4A) or partially saturated R_2^I (Figure 8), whose rapid rate of spontaneous dissociation from site A at 37 °C[3] rendered the estimation of k_d less precise at this temperature than at 0 °C, the data given are those obtained at the more physiological temperature (37 °C).

The rate of the C-induced release of c[^{3}H]AMP from R^I was about 150 times faster at 37 °C than at 0 °C, indicating a considerable activation energy for the reaction. This high temperature dependence of the release also indicates that it is not diffusion controlled, at least at 0 °C.

Correlation between the C-Induced Release of c[^{3}H]AMP and Inhibition of C. When stoichiometric proportions of R_2^I(c[^{3}H]AMP)$_4$ and C were added in the presence of Mg^{2+} and ATP, the fractional saturation of R_2^I and the fractional kinase activity decreased proportionally (Figure 9). The above data indicated a gross proportionality between the amount of R_2^I complexed with cAMP and the amount of catalytically active C during the process of recombination of the subunits.

The above type of experiment does not give information about the existence of putative complexes like R_2^I(cAMP)$_4$–C or R_2^I(cAMP)$_4$–C$_2$ in which C might be catalytically active. So that an estimate of the maximum amount of such complexes in a mixture of R_2^I(cAMP)$_4$ and C could be obtained, a gel filtration column[2] was equilibrated at 37 °C with R_2^I (120 nM with respect to the R subunit) in buffer A containing 3 mM ATP and 0.1 mM cAMP. A sample (0.2 mL) of 60 nM C and 120 nM R_2^I in the equilibration buffer was applied, and 50 fractions (of 0.2 mL) were collected and assayed for phosphotransferase activity.

The C activity eluted at the same position (corresponding to a Stokes radius of 2.6 nm) as when R_2^I was not present. On the basis of experience with marker proteins, a shift in the apparent Stokes radius from 2.6 to 2.9 nm would have been detected. If all the C were complexed with R_2^I(cAMP)$_4$, it

would be expected to elute as $R_2^IC_2$ (whose Stokes' radius was 5.3 nm in this system). We conclude, therefore, that less than 10% of C was complexed with R_2^I at any time during the chromatography, i.e., $[R_2^I(cAMP)_4–C] < 6$ nM and $[C] > 54$ nM.

The apparent K_D for the interaction between $R_2^I(cAMP)_4$ (concentration given with respect to the R_2^I dimer) and C is

$$K_D = \frac{[R_2^I(cAMP)_4][C]}{[R_2^I(cAMP)_4–C]} = \frac{\{60\text{ nM} - [R_2^I(cAMP)_4–C]\}[C]}{[R_2^I(cAMP)_4–C]} > 486\text{ nM} \quad (2)$$

The minimal value of K_D was thus estimated to be about 5×10^{-7} M. The above estimate was not based on any assumptions regarding the catalytic activity of $R_2^I(cAMP)_4–C$ or $R_2^I(cAMP)_4–C_2$. On the basis of the assumption that C is considerably inhibited when complexed with $R_2^I(cAMP)_4$, Builder et al. (1981) have recently estimated the K_D for the interaction between C and $R_2^I(cAMP)_4$ to be 15 μM, which is well above the minimal estimate of K_D given above.

Discussion

The present study is concerned with the reverse reaction of the overall equation:

$$R_2^IC_2 + 4cAMP \rightleftharpoons R_2^I(cAMP_4) + 2C \quad (1)$$

for activation of protein kinase I (cAKI) by cAMP. This reaction has previously been studied by Brostrom et al. (1971), Chau et al. (1980), and Builder et al. (1981), but the question of the roles of the two types of cAMP binding sites (A and B) of R^I in that reaction has not been addressed.

We found that in the absence of added unlabeled cyclic nucleotide the C-induced release of c[^{3}H]AMP occurred with the same rate from sites A and B (Figure 2, Table I).

When the rate of the C-induced release of c[^{3}H]AMP was measured at increasing concentrations (10^{-8}–10^{-6} M) of unlabeled cAMP, it decreased progressively to reach half its original value (Figure 6).

One explanation for this would be that cAMP perturbed the C-induced release of c[^{3}H]AMP by competing with Mg^{2+}-ATP for the high-affinity Mg^{2+}-ATP binding site on the R^I–C interface (Hoppe et al., 1977). This is unlikely, however, since the inhibition of c[^{3}H]AMP release by cAMP was not much affected by varying the concentration of Mg^{2+}-ATP (Table I). Since sites A and B are the only binding sites for cAMP known to exist on R_2, C, or R_2C_2, cAMP may only affect the rate of release by interacting with site A or B, and those sites are only vacant as the result of the release of bound c[^{3}H]AMP. A reduction of about 50% in the rate of release from both sites A and B can be explained if the association of C and $R_2^I(c[^3H]AMP)_4$ leads to a random release of c[^{3}H]AMP from sites A and B, interaction of unlabeled cAMP with the site first vacant blocking the release from the other site. However, several cAMP analogues inhibited the release of c[^{3}H]AMP from sites A and B with potencies paralleling their affinities for site A (Figure 7A) rather than site B (Figure 7B) of R_2^I. A more likely explanation is therefore that the interaction of C with $R_2^I(c[^3H]AMP)_4$ leads to dissociation of c[^{3}H]AMP from both subunits in the absence of added cyclic nucleotide, the release of c[^{3}H]AMP from one subunit preceding the release from the other one. By binding to site A of the subunit that first released its bound c[^{3}H]AMP, cyclic nuleotides at moderate concentrations (e.g., 1 μM cAMP) confine the release to this subunit and thereby decrease the apparent rates of release from both sites to half their original values.

When the concentration of unlabeled cAMP was increased beyond 1 μM, the rate of release of c[^{3}H]AMP from site B only decreased further (Figure 6). Thus, at high concentrations of cyclic nucleotide, c[^{3}H]AMP is chiefly released from site A of $R_2^I(c[^3H]AMP)_4$. This must mean that the release of ligand from site A precedes the release from site B of the same subunit. Since only site A is unoccupied by c[^{3}H]AMP to an appreciable extent in the presence of high concentrations of cyclic nucleotide, the effect of the latter on the release of ligand from site B is almost bound to be due to interaction with site A. As expected, the potency of cAMP analogues to selectively inhibit the release from site B did correlate with the affinity for site A (Figure 7).

The above result suggests that the interaction between (one or two molecules of) C and $R_2^I(c[^3H]AMP)_4$ leads to the release of ligand from site A of one subunit followed by the release from site B of the same subunit and sites A and B of the second subunit. Interaction of cyclic nucleotides with site A of the subunit that first lost its ligand may block the release from both sites of the second subunit (interchain interaction) and from site B of the first subunit (intrachain interaction).

It is of interest that whereas binding to site B precedes and controls bindig to site A when cAMP interacts with the cAK holoenzyme (Døskeland & Øgreid, 1981; Øgreid & Døskeland, 1981a), the opposite (see above) holds true for the reverse process of recombination of $R_2^I(cAMP)_4$ and C.

It is also of interest that the concentrations of cAMP required to inhibit the release of c[^{3}H]AMP are in the physiologically relevant range and that the effects were observed under presumably near-physiological conditions (37 °C, pH 7.0, 0.15 M KCl). It is possible that this inhibition of the C-induced release of bound cAMP by counteracting the "retrooperative" effect of the C subunit (Swillens & Dumont, 1976) is partly responsible for the high "control efficiency" (Swillens & Dumont, 1976) observed for the activation of cAK in vivo [see Døskeland & Øgreid (1981) and Flockhart & Corbin (1982) for recent reviews].

It has been suggested that only one (Døskeland & Øgreid, 1981) or two (Smith et al., 1981; Builder et al., 1981) molecules of C must bind to $R_2^I(c[^3H]AMP)_4$ to effect the release of the first molecule of bound c[^{3}H]AMP. The first option is described by

$$R_2^I(c[^3H]AMP)_4 + C \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} R_2^I(c[^3H]AMP)_4–C \xrightarrow{k_2} R_2^I(c[^3H]AMP)_3–C \quad (3)$$

k_1 is the second-order rate constant for the formation of the complex between $R_2^I(c[^3H]AMP)_4$ and C and k_{-1} the first-order rate constant for its dissociation. k_2 is the first-order rate constant for the release of c[^{3}H]AMP. When negligible rebinding of c[^{3}H]AMP occurs, the second step above can be considered irreversible.

The second option is described by

$$R_2^I(c[^3H]AMP)_4 + 2C \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} R_2^I(c[^3H]AMP)_4–C_2 \xrightarrow{k_2} R_2^I(c[^3H]AMP)_3–C_2 \quad (4)$$

where k_1 is a third-order rate constant, or by

$$R_2^I(c[^3H]AMP)_4 + C \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} R_2^I(c[^3H]AMP)_4–C$$

$$k_{-1}/k_1 = K_{D1}$$

$$R_2^I(c[^3H]AMP)_4–C + C \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} R_2^I(c[^3H]AMP)_4–C_2 \xrightarrow{k_3} R_2^I(c[^3H]AMP)_3–C_2 \quad (5)$$

A realistic scheme must be compatible with the observation (Figure 5) that the release of c[^{3}H]AMP is proportional to the concentration of C in the range 0.8–50 nM. This rules out the scheme of eq 4 since it predicts a second-order relationship between the release rate and [C]. The scheme of eq 3 satisfies this criterion if $k_2 >> k_{-1}$. If it is similarly assumed for eq 5 that $k_3 >> k_{-2}$, it must still be shown for this scheme to be plausible that $k_2[C] >> k_{-1}$ for concentrations of C between 0.8 and 50 nM. The estimated theoretical upper limit for the association rate constant (k_2) for the association between R_2^I(c[^{3}H]AMP)$_4$–C and C is about 10^9 M^{-1} s^{-1} (see the Appendix). In the presence of 0.8 nM C, the maximal value of $k_2[C]$ is therefore 0.8 s^{-1}. Since $k_{-1} = K_{D1}k_1$, and the minimal value of K_{D1} has been estimated to be 5×10^{-7} M (see the last paragraph under Results) and that of k_1 is the observed apparent k_a ($=6 \times 10^6$ M^{-1} s^{-1}) for the association between C and R_2^I(c[^{3}H]AMP)$_4$ leading to release of c-[^{3}H]AMP, the minimal value of $k_{-1} = 3$ s^{-1}. Obviously, the criterion $k_2[C] >> k_{-1}$ is not satisfied for the scheme of eq 5. Under the conditions of the present study, therefore, the interaction of one molecule of C with R_2^I(c[^{3}H]AMP)$_4$ suffices to release at least one molecule of bound c[^{3}H]AMP.

The concentration of unlabeled cAMP required to counteract the C-induced release of c[^{3}H]AMP was independent of the concentration of C (data for 0.3 μM, 1.5 μM, and 0.1 mM cAMP are shown in Figure 5). This is expected if one hit of C subunit (in the absence of added cyclic nucleotide) leads to the release of all four molecules of bound c[^{3}H]AMP but is not expected for most schemes requiring a second hit of C subunit to release the c[^{3}H]AMP molecules remaining bound after the first one has dissociated. Additional studies, preferably using rapid mixing devices, will be required to distinguish between these options [one or two hits of C required to release all four ligands from R_2^I(cAMP)$_4$].

If it is assumed that one hit of C is sufficient to dissociate all four molecules of c[^{3}H]AMP bound to R_2^I, the close correlation observed between the release of c[^{3}H]AMP and the inhibition of the catalytic activity of C upon recombination of R_2^I(c[^{3}H]AMP)$_4$ and C (Figure 9) suggests that the association between R_2^I and C may be more rapid than that between R_2^I(cAMP)$_4$ and C. In fact, Builder et al. (1980a) have determined the k_a for the association between R_2^I and C at 4 °C to be 3×10^6 M^{-1} s^{-1}, which is 75 times higher than the apparent k_a (4×10^4 M^{-1} s^{-1}) we have determined for the interaction between R_2^I(c[^{3}H]AMP)$_4$ and C at 0 °C.

Partially proteolyzed R^I or R^{II} complexed with cAMP does not efficiently recombine with C (Sugden & Corbin, 1976; Weber & Hilz, 1979). However, since those experiments were conducted under conditions where rebinding of cAMP could not be excluded, they do not tell whether the inefficient formation of recombined holoenzyme was due to a lack of C-induced release of bound cAMP or to a lowered affinity of C for partially proteolyzed ligand-free R. The present study showed a complete lack of C-induced release of c[^{3}H]AMP from either site A or site B of the monomeric R_{t1}^I(c[^{3}H]AMP)$_2$ (Figure 4A) but did not tell whether the binding between ligand-free R_{t1}^I and C was also impaired.

Under conditions when c[^{3}H]AMP dissociated from R_2^I-(c[^{3}H]AMP)$_4$ did not rebind, the C-induced release of c-[^{3}H]AMP was strictly dependent on Mg^{2+}-ATP (Figure 4A). A similar Mg^{2+}-ATP requirement was noted by Chau et al. (1980). Their incubations were carried out in the presence of a nitrocellulose disk and terminated by filtering the incubate through the disk. When this method is used, binding of cAMP to site A is usually not detected (Øgreid & Døskeland, 1980; de Wit & Hoppe, 1981), presumably due to interaction between R(cAMP)$_4$ and the filter matrix leading to release of bound cAMP (Builder et al., 1980b). It is therefore possible that the data of Chau et al. (1980) refer to the effect of C–Mg^{2+}-ATP on the release of c[^{3}H]AMP from site B of R_2^I complexed with nitrocellulose and having a vacant site A. The apparent k_a for the C–Mg^{2+}-ATP-induced release of c[^{3}H]-AMP from site B calculated from their data is about 10 times higher than the k_a found in the present study. It is of interest that the rate of release of c[^{3}H]AMP from R_2^I with c[^{3}H]-AMP selectively bound to site B was higher than that from R_2^I with c[^{3}H]AMP bound to both sites A and B (Figure 8).

In conclusion, the present study has presented evidence that one hit of C subunit may lead to the release of at least one molecule of c[^{3}H]AMP from R_2^I(c[^{3}H]AMP)$_4$. The release occurs orderly; i.e., c[^{3}H]AMP bound to site A of one R subunit dissociates before c[^{3}H]AMP bound to site B of the same subunit. Furthermore, the release of c[^{3}H]AMP from sites A and B of one subunit may precede release from the other subunit. The kinetic data presented suggest intrasubunit interaction between sites A and B as well as intersubunit interaction, both of which are modulated by ligand binding to site A. Cyclic nucleotides interacting with site A retard the C-induced release of c[^{3}H]AMP at near-physiological temperature, pH, ionic strength, and ATP concentration. This mechanism would be expected to decrease the reverse reaction of eq 1 (reassociation of protein kinase subunits) and may be operative in vivo.

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Appendix

(A) Estimation of the Apparent Association Rate Constant for the Interaction between C and R Leading to Release of cAMP Bound to R. Equation 6 describes the reaction of a macromolecule with a bound ligand (R–L_1) and a second ligand (L_2), whose interaction with R leads to the release of the originally bound ligand.

$$R\text{–}L_1 + L_2 \rightleftharpoons R\text{–}L_2 + L_1 \quad (6)$$

If the reverse of the above reaction (rebinding of L_1) is very slow compared to the forward reaction, then the apparent association rate constant (k_a) is given by

$$k_a t = \frac{-1}{[R\text{–}L_1]_0[L_2]_0} \ln \frac{[L_2]_0[R\text{–}L_1]_t}{[R\text{–}L_1]_0([L_2]_0 - [R\text{–}L_2]_t)} \quad (7)$$

where $[R\text{–}L_1]_0$ and $[L_2]_0$ are the initial concentrations of bound L_1 and free L_2, respectively, and $[R\text{–}L_1]_t$ and $[R\text{–}L_2]_t$ the concentrations of bound L_1 and L_2, respectively, after the reaction has proceeded for a period of time t.

When $[L_2]_0$ is in great excess of $[R\text{–}L_1]_0$ (pseudo-first-order conditions), the expression simplifies to

$$k_a t = \frac{1}{[L_2]_0} \ln \frac{[R\text{–}L_1]_0}{[R\text{–}L_1]_t} \quad (8)$$

In the present study, rebinding of c[^{3}H]AMP (L_1) was avoided by either working with highly diluted reactants or working in the presence of excess unlabeled cAMP. The concentration of C (L_2) was at least 10 times higher than that of site A or B (R) in the experiments used to calculate k_a. Therefore, eq 4 could be used to calculate the apparent k_a for the interaction between C and R, leading to release of c-[^{3}H]AMP from site A or B.

(B) Estimation of the Upper (Diffusion-Controlled) Limit for the Association Rate Constant for the Interaction between C and R_2^I–C. The upper limit for a second-order rate constant as calculated from diffusion theory is

$$k_a = \frac{4\pi N}{1000} r(D_1 + D_2) f k \quad (9)$$

where N is Avogadro's number, r is the interaction radius for the two reactants (in centimeters), D_1 and D_2 are the diffusion constants for the two reactants (in square centimeters per second), f is a (unitless) electrostatic factor, and k is a (unitless) steric interaction factor corresponding to the fraction of the total collisions that are successful. When it was assumed that M_r = 40 500 for C (Shoji et al., 1981) and M_r = 48 000 for R_1^I (Hofmann et al., 1975), with partial specific volumes of 0.74 cm^3/g, r was estimated to the 5.7 nm. Taylor & Stafford (1978) have calculated the values of D for C (7.9×10^{-7} cm^2 s^{-1}), R_2^I (4.9×10^{-7} cm^2 s^{-1}), and $R_2^IC_2$ (3.9×10^7 cm^2 s^{-1}). Taking r as 5.7 nm, D_1 as 7.9×10^{-7} cm^2 s^{-1}, and D_2 as about 4.3×10^{-7} cm^2 s^{-1}, and substituting into eq 9, gives

$$k_a = (5 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}) f k \quad (10)$$

as the upper limit for k_a.

The estimation of values of f and k is uncertain for macromolecular associations in solution. The value of f depends on the difference in net charge between the areas of the reactants in contact as well as the difference in the overall net charge of the reactants (Hammes & Alberty, 1959). The isoelectric pH of R_2^I is about 5.5 (Geahlen et al., 1982) and that of C about 7.5 (Peters et al., 1977), suggesting that R_2^I–C and C may be oppositely charged at pH 7.0. C has a basic region surrounding the Mg^{2+}-ATP binding site and an acidic region near its carboxy-terminal end, both of which may interact with oppositely charged regions of R (Takio et al., 1982; Geahlen et al., 1982). The above suggests that f may be above 1 for the interaction between R_2^I–C and C. As the ionic strength of the medium is increased, f approaches unity (Hammes & Alberty, 1959; Berg & Blomberg, 1978). At the relatively high ionic strength (presence of 0.15 M KCl) of the present study, therefore, f would be expected to be close to 1. Most estimates of k for the interaction of a macromolecule with a ligand of low molecular weight or with another macromolecule range from 10^{-4} to 10^{-1} (Hill, 1975; Koren & Hammes, 1976; Samson & Deutch, 1978; Northrup & Hynes, 1979; Winter et al., 1981). If f is considered to be 2 and k to be 10^{-1}, the upper limit for the k_a for the association of R_2^I–C and C would be 10^9 M^{-1} s^{-1} (see eq 10), which is about 10 times higher than any value of k_a experimentally determined for protein–protein interactions (Koren & Hammes, 1976).

Registry No. Protein kinase, 9026-43-1; cAMP, 60-92-4; Mg, 7439-95-4; ATP, 56-65-5; AMP, 61-19-8; ADP, 58-64-0; cGMP, 7665-99-8; cIMP, 3545-76-4; adenosine, 58-61-7.

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Reaction of 5-Ethynyl-2′-deoxyuridylate with Thiols and Thymidylate Synthetase†

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ABSTRACT: Thymidylate synthetase has been found to catalyze addition reactions at the normally inert acetylene moiety of 5-ethynyl-dUMP (EdUMP). A chemical counterpart for this reaction has also been found in which 5-ethynyl-dUrd (EdUrd) and EdUMP react with 2-mercaptoethanol to give 5-[1-[(2-hydroxyethyl)thio]vinyl]-dUrd (HETVdUrd) and -dUMP (HETVdUMP). If one uses 6-tritiated EdUrd, the model reaction proceeds with a large α-secondary inverse isotope effect (k_T/k_H = 1.23), indicating that nucleophilic attack of thiolate at the 6 position of the heterocycle is an early event in the reaction. Kinetic studies are in accord with the proposal that the next step of this reaction involves rate-determining general acid catalyzed protonation of the acetylene moiety and rearrangement to an allene conjugated with the 4-carbonyl group of the heterocycle. Subsequent reaction of thiol with the reactive Michael center of the allene and β-elimination across the 5,6 double bond account for the formation of the product, HETVdUrd. In the presence of CH_2-H_4folate, EdUMP causes a time-dependent inactivation of dTMP synthetase [Barr, P. J., Nolan, P. A., Santi, D. V., & Robins, M. J. (1981) *J. Med. Chem. 24*, 1385–1388]. However, in the absence of the cofactor, dTMP synthetase catalyzes the conversion of EdUMP and 2-mercaptoethanol to HETVdUMP. As in the model chemical counterpart, a large inverse α-secondary hydrogen isotope effect (k_T/k_H = 1.22–1.23) is observed with 6-tritiated EdUMP, which indicates that nucleophilic attack of the catalytic thiol group of the enzyme at the 6 carbon of EdUMP is an early step in this reaction. In analogy with model reactions, it is proposed that subsequent steps involve the formation of a reactive, enzyme-bound allene intermediate that undergoes reaction with a thiol group at the Michael center and β-elimination across the 5,6 double bond of the heterocycle to provide HETVdUMP and catalytically competent enzyme. Interestingly, certain nucleophilic buffers such as *N*-methylmorpholine and morpholine can effectively compete with 2-mercaptoethanol for reaction at the Michael center of the enzyme-bound allene intermediate. Such reactions are not observed in model chemical counterparts and may result from binding of these buffers to the site occupied by the cofactor in the normal enzymic reaction.

Thymidylate synthetase (EC 2.1.1.45) catalyzes the conversion of dUMP and CH_2-H_4folate to dTMP and H_2folate. It is known that an early event in catalysis involves covalent bond formation between a thiol group of the enzyme—Cys-198 in the *Lactobacillus casei* enzyme (Bellisario et al., 1979)—and the 6 position of dUMP to give transient 5,6-dihydropyrimidine intermediates [cf. Pogolotti & Santi (1977)]. A similar reaction occurs with a number of 5-substituted analogues of dUMP, giving rise to either mechanism-based inhibitors (Danenberg, 1977; Santi, 1980) or alternate substrates (Pogolotti et al., 1979; Garrett et al., 1979). Detailed studies of the interactions of such inhibitors and substrates have provided much information regarding the mechanism of dTMP synthetase. With mechanism-based inhibitors, the covalent bond that is formed may be stable, or it may activate a latent reactive chemical group at the 5 position that subsequently forms a covalent bond with the enzyme. Position-5 substituents of alternate substrates are activated in a similar manner but react with nucleophiles of the media, and the modified analogue is released to regenerate the catalytically active enzyme. Interestingly, the presence of the cofactor, CH_2-H_4folate, may alter the course of many such interactions, either by serving as a chemical partner in the reaction(s) or by indirectly modifying the binding or reactivity of the nucleotide analogue.

Interest in the interaction of EdUMP[1] with dTMP synthetase has arisen from observations that EdUrd potently

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[1] Abbreviations: EdUrd, 5-ethynyl-2′-deoxyuridine; EdUMP, 5-ethynyl-2′-deoxyuridylate; HETVdUrd, 5-[1-[(2-hydroxyethyl)thio]-vinyl]-2′-deoxyuridine; HETVdUMP, 5-[1-[(2-hydroxyethyl)thio]-vinyl]-2′-deoxyuridylate; NMMVdUMP, 5-[1-(4-methylmorpholinium-4-yl)vinyl]-2′-deoxyuridylate; AcdUMP, 5-acetyl-2′-deoxyuridylate; CH_2-H_4folate, (±)-L-5,10-methylenetetrahydrofolic acid; TEAB, triethylammonium bicarbonate; NMM, *N*-methylmorpholine; Tes, 2-[[tris(hydroxymethyl)methyl]amino]ethanesulfonate; EDTA, ethylenediaminetetraacetic acid; RV, retention volume.

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