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# ALTERED REGULATION OF CYCLIC AMP-DEPENDENT PROTEIN KINASE IN A MOUSE LYMPHOMA CELL LINE

MEIRA LASSER and VIOLET DANIEL

*The Department of Biochemistry, Weizmann Institute of Science, Rehovot (Israel)*

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## Summary

The ability of cyclic AMP to inhibit growth, cause cytolysis and induce synthesis of cyclic AMP-phosphodiesterase in S49.1 mouse lymphoma cells is deficient in cells selected on the basis of their resistance to killing by 2 mM dibutyryl cyclic AMP. The properties of the cyclic AMP-dependent protein kinase (ATP:protein phosphotransferase, EC 2.7.1.37) in the cyclic AMP-sensitive (S) and cyclic AMP-resistant (R) lymphoma cells were comparatively studied. The cyclic AMP-dependent protein kinase activity of R cells cytosol exhibits an apparent $K_a$ for activation by cyclic AMP 100-fold greater than that of the enzyme from the parental S cells. The free regulatory and catalytic subunits from both S and R kinase are thermolabile, when associated in the holoenzyme the two subunits are more stable to heat inactivation in R kinase than in S kinase. The increased heat stability of R kinase is observed however only for the enzyme in which the catalytic and cyclic AMP-binding activities are expressed at high cyclic AMP concentrations ($10^{-5}$—$10^{-4}$ M), the activities expressed at low cyclic AMP concentrations ($10^{-9}$—$10^{-6}$ M) being thermolabile. The regulatory subunit of S kinase can be stabilized against heat inactivation by cyclic AMP binding both at $2 \cdot 10^{-7}$ and $10^{-5}$ M cyclic AMP concentrations. In contrast, the regulatory subunit-cyclic AMP complex from R kinase is stable to heat inactivation only when formed in the presence of high cyclic AMP concentrations ($10^{-5}$ M).

The findings indicate that the transition from a cyclic AMP-sensitive to a cyclic AMP-resistant lymphoma cell phenotype is related to a structural alteration in the regulatory subunit of the cyclic AMP-dependent protein kinase which has affected the protein's affinity for cyclic AMP and its interaction with the catalytic subunit.

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Abbreviation: C8-$NC_6$-$NH_2$-cAMP, 8-(6-aminohexyl)-aminoadenosine 3′,5′-monophosphate.

## Introduction

It was recently shown that $N^6$, $O^2$,-dibutyryl cyclic AMP induced growth inhibition and cytolysis of cultured S49.1 mouse lymphoma cells and that growth of these cells in the presence of increasing concentrations of $N^6$,$O^2$-dibutyryl cyclic AMP, resulted in the selection of cyclic AMP-resistant variants [1,2]. Both endogenous cyclic AMP and exogenous $N^6$,$O^2$-dibutyryl cyclic AMP inhibited the uptake of precursors of macromolecular synthesis and induced the synthesis of cyclic AMP-phosphodiesterase in the sensitive parental cell line, but not in the cyclic AMP-resistant mutant [3—5]. In addition, cell-free extracts from cyclic AMP-sensitive lymphoma cells previously exposed to $N^6$,$O^2$-dibutyryl cyclic AMP showed an increased capacity to translate exogenous mRNA [6]. Compared to the parental lymphoma, the resistant cells had reduced activity of cyclic AMP-binding protein and cyclic AMP-dependent protein kinase (ATP:protein phosphotransferase, EC 2.7.1.37) [1]. These observations led to the conclusion that the biological effects of cyclic AMP in lymphoma cells were mediated by a cyclic AMP-dependent protein kinase and that the altered regulation of this enzyme was responsible for the lack of cyclic AMP activity in the resistant cells [1,3]. Other investigators have also described an altered regulation of protein kinase in HTC (hepatoma) and neuroblastoma cells [7—9].

In the presence report we have studied the properties of the cyclic AMP-dependent protein kinase in the cytosols of cyclic AMP sensitive (S) and cyclic AMP-resistant (R) S49.1 lymphoma cells in order to determine the changes which have occurred in this enzyme in the transition from S to R cell phenotype.

## Materials and Methods

Mouse lymphoma line S49.1 obtained from the Salk Institute, San Diego, was propagated in Dulbecco's modified Eagle's medium containing 5% heat-inactivated fetal calf serum (Grand Island Biological Co.). Cells resistant to 2 mM $N^6$,$O^2$-dibutyryl cyclic AMP were selected from the S49.1 sensitive lymphoma line as previously described [1,10]. The resistant cells do not differ from the sensitive parental cells in their morphology, chromosome content and growth rate (20—22 h doubling time in 5% fetal calf serum).

*Chemicals.* [$\gamma$-$^{32}$P]ATP from Nuclear Research Center, Negev, Israel and Radiochemical Centre, Amersham; cyclic [$^3$H]AMP from New England Nuclear Corp.; cyclic AMP, calf thymus histone and salmon sperm protamine were from Sigma Chemical Co. and Sepharose 4B from Pharmacia.

*Assay for protein kinase.* The standard incubation mixture contained in a final volume of 0.2 ml: 50 mM potassium phosphate buffer, pH 6.5, 7 mM $MgCl_2$, 2 mM theophylline, 5 mM dithiothreitol, 0.03 mM [$\gamma$-$^{32}$P]ATP ($2.5 \cdot 10^4$ cpm/nmol), 100 $\mu$g of protamine and enzyme fraction. Incubations were at 30°C for 10 min with or without 2—5 $\mu$M cyclic AMP. Protein-bound [$^{32}$P]-phosphate was determined by the method of Kabat [11]. Cyclic AMP binding was measured by the assay of Gilmon [12].

*Separation of subunits of cyclic AMP-dependent protein kinase by affinity*

*chromatography*. 8-(6-aminohexyl)-amino-adenosine 3′,5′-monophosphate (C8-$NC_6NH_2$-cAMP) coupled to Sepharose was utilized as an affinity chromatography medium for separation of cyclic AMP-dependent protein kinase subunits and for purification of the cyclic AMP receptor protein following the procedure of Ramseyer et al. [13]. For the separation of subunits, lymphoma cyclic AMP-dependent protein kinase was partially purified on a DEAE-cellulose column. Protein concentrations were determined according to the method of Lowry et al. [14].

## Results

*Partial purification of protein kinases on DEAE-cellulose columns*

The cyclic AMP-dependent protein kinase activities from S and R lymphoma cell cytosol were partially purified on a DEAE-cellulose column as described in Fig. 1. When assayed with histone or protamine as phosphate acceptors the protein kinase activity appeared as a peak of cyclic AMP-dependent protein kinase containing all the cyclic AMP-binding activity present in the S cell cytosol followed by a broad distribution of a cyclic AMP-independent protein kinase (Fig. 1A). Chromatography of the R cell cytosol showed it to contain reduced

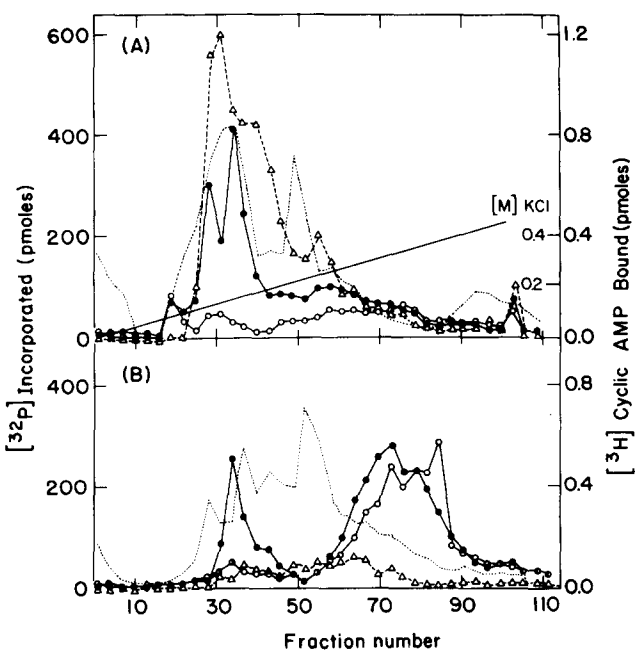


Fig. 1. DEAE-cellulose chromatography of protein kinase activity from cyclic AMP sensitive (A) or resistant cells (B). Cytosols from S and R cells prepared as previously described [1] were adjusted to pH 5.0 by dropwise addition of 1 M acetic acid. After 10 min the precipitate was removed by centrifugation and the supernatant neutralized to pH 6.5 by 0.5 M NaOH. 100 mg of pH 5 supernatant fraction was applied to a DEAE-cellulose column (1.5 × 20 cm) equilibrated with pH 7.4 buffer (TMG) containing 0.02 M Tris · HCl, 6 mM mercaptoethanol and 10% glycerol and was then eluted with a 200 ml linear (0.02—0.5 M) KCl gradient in the same buffer. 2-ml fractions were collected and 50-µl samples were assayed for 0.2 µM cyclic [$^3$H]AMP binding (△———△), protein kinase activity measured in the absence (○———○) or presence (●———●) of $2 \cdot 10^{-6}$ M cyclic AMP (. . . . . .) protein distribution.

amounts of the cyclic AMP-dependent protein kinase and cyclic AMP-binding protein (Fig. 1B). In addition, the R cell contained an increased activity of a cyclic AMP-independent protein kinase which appeared as a large broad peak (Fig. 1B).

*Properties of cyclic AMP-dependent protein kinase*

The properties of the partially purified cyclic AMP-dependent protein kinase from S and R lymphoma cells (DEAE-cellulose column) were comparatively studied. The time course of protein phosphorylation by the two kinase preparations was linear up to 30 min at 30°C, in the presence or absence of 2 $\mu$M cyclic AMP. The optimal pH for activity was 6.5—7.0; protamine and histone but not casein served as phosphate acceptors. The kinase activity was studied as a function of cyclic AMP concentration. Fig. 2 shows that although the kinase from both S and R lymphoma cells is stimulated by increasing cyclic AMP concentrations, the enzyme from R cells requires higher amounts of cyclic AMP to reach the same level of activity. The apparent $K_a$ values for cyclic AMP activation of the R and S protein kinase (measured at the same protein concentration) are approx. 0.01 and 1—2 $\mu$M, respectively. These results suggest a lower affinity of the regulatory subunit of R protein kinase for cyclic AMP. Direct experiments on cyclic [$^3$]AMP binding presented in Fig. 3 show indeed that the cyclic AMP-dependent kinase from R cells has a lower affinity for cyclic AMP than that from S cells. Although the passage of S cell cytosol through a DEAE-cellulose column resulted in an increased cyclic AMP binding, probably due to

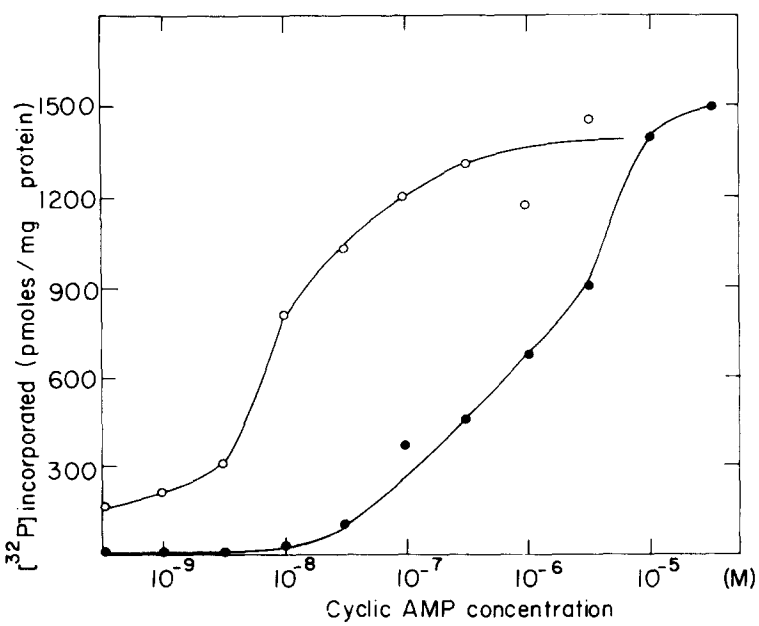


Fig. 2. Effect of cyclic AMP concentration on the cyclic AMP-stimulated protein kinase from S and R cells. Cyclic AMP-stimulated protein kinase preparations from S (○———○) and R (●———●) cells (Fig. 1) were assayed for protamin phosphorylation (Materials and Methods) in the presence of increasing concentrations of cyclic AMP.

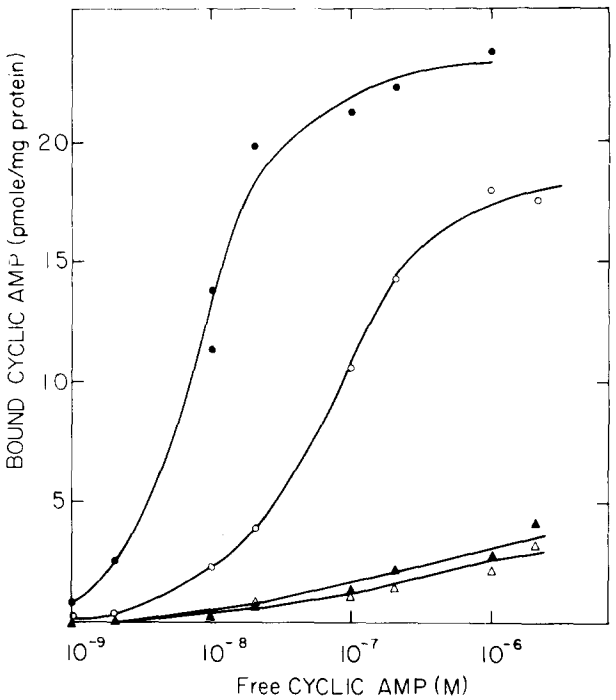


Fig. 3. Cyclic AMP binding by crude cytoplasmic extracts and DEAE-cellulose purified cyclic AMP-stimulated protein kinase from S and R cells. Reaction mixture of 0.1 ml contained 50 mM potassium phosphate (pH 6.5), 2 mM theophylline, 20—60 μg protein of sensitive cell or 150 μg protein of resistant cell preparations and increasing concentrations of cyclic [$^3$H]AMP (24 Ci/mmol) with or without 0.1 mM non-radioactive cyclic AMP. The difference between the amount of cyclic [$^3$H]AMP bound in the presence or absence of excess non-radioactive cyclic AMP gives the bound cyclic AMP. The concentration of free cyclic [$^3$H]AMP is calculated by subtracting the bound cyclic [$^3$H]AMP from total cyclic [$^3$H]AMP present in the incubation mixture. S cell cytosol (○———○) and DEAE-cellulose-purified protein kinase (●———●). R cell cytosol (△———△) and DEAE-cellulose purified-protein kinase (▲———▲).

the removal of an inhibitor, the cyclic AMP-binding activation of R cell cytosol was not affected by this purification (Fig. 3). The cyclic AMP-binding capacity of the R kinase regulatory subunit was studied also after its purification and isolation by affinity chromatography. Fig. 4 shows that the concentration of cyclic AMP producing half saturation of R kinase regulatory subunit is similar to that required for half maximum activation of R kinase described in Fig. 2.

*Heat stability of protein kinase subunits*

One may ask whether the alteration in the regulatory subunit of R kinase, which finds expression in a lowered affinity for cyclic AMP, has also affected other structural and functional properties of this protein. To this end we carried out comparative studies on the heat stability of the free and interacting subunits of S and R kinases. The results presented in Fig. 5 show that the free catalytic subunits from both kinases, prepared either by affinity chromatography on a C8-$NC_6$-$NH_2$-cAMP-Sepharose column or by the dissociation of the holoenzyme in the presence of cyclic AMP, are rapidly inactivated at 46° C. The thermolability of the catalytic subunit in the holoenzyme was studied by incubating the kinases at 46° C for different periods of time and assaying for phos-

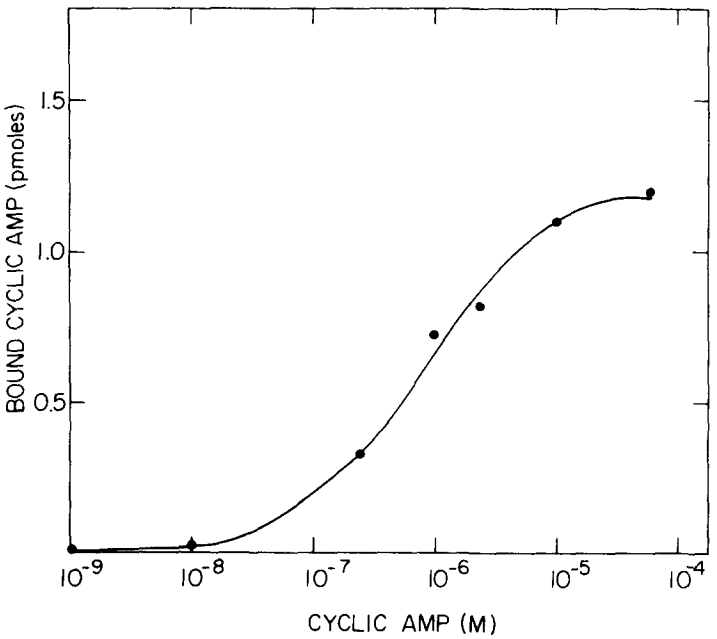


Fig. 4. Cyclic [$^3$H]AMP binding to the purified protein kinase regulatory subunit from R cells. The regulatory subunit was separated from the catalytic subunit by affinity chromatography on cyclic AMP-Sepharose column as described in Material and Methods and was then assayed for cyclic [$^3$H]AMP binding at different cyclic [$^3$H]AMP concentrations as described in Fig. 3.

photransferase activity in the absence or presence of cyclic AMP. Fig. 6 shows that the enzymatic activity of the protein kinases stimulated by $10^{-5}$ M cyclic AMP is thermolabile in S kinase but heat stable in R kinase. Our results indicate therefore a difference in the thermolability of the catalytic subunit of the two

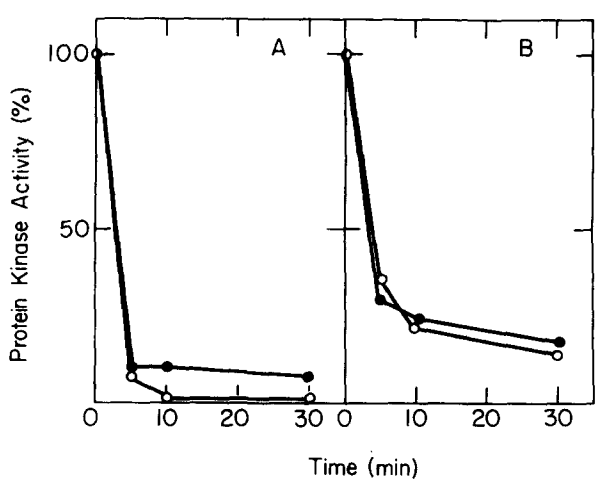


Fig. 5. Heat inactivation of free catalytic subunits. Catalytic subunit preparations from S kinase (A) and from R kinase (B), prepared either by affinity chromatography on C8-$NC_6$-$NH_2$-cAMP-Sepharose (○) or by dissociation of holoenzymes in the presence of $10^{-5}$ M cyclic AMP (●), were incubated at 46°C in TMG (Fig. 1) buffer. Samples were taken after indicated periods and assayed for phosphotransferase activity as described in Materials and Methods.

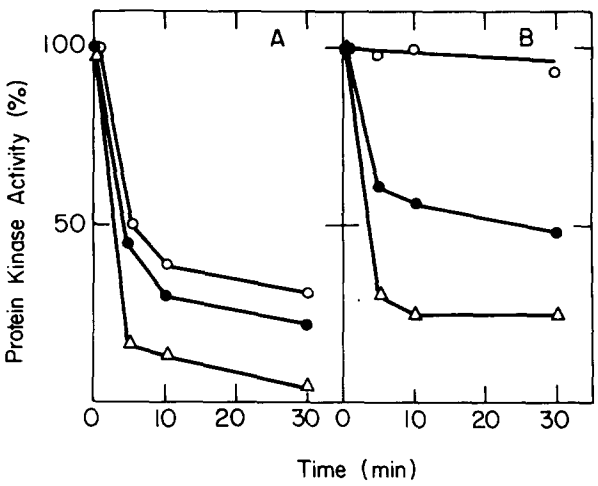


Fig. 6. Heat inactivation of the catalytic subunit in the protein kinase holoenzyme S kinase (A) and R kinase (B) were incubated at 46°C in the TMG buffer. Samples were taken after indicated periods and assayed for catalytic activity in the absence (△) or presence of $2 \cdot 10^{-6}$ (●) and $10^{-5}$ (○) M cyclic AMP.

kinases only when this subunit is associated with the regulatory one in the holoenzyme. It should be noted that the protein kinases from both S and R cells present a catalytic activity in the absence of cyclic AMP addition which is rapidly inactivated by heating (Figs. 6A and 6B). In addition one can see in R kinase an activity stimulated by lower cyclic AMP concentrations ($10^{-6}$ M) which is thermolabile like S kinase. These findings were extended by studying the thermolability of the protein kinases as a function of cyclic AMP concentrations required for activation. Holoenzymes from S and R cells were incubated at 46°C for 30 min and were subsequently assayed for catalytic activity at different cyclic AMP concentrations. Fig. 7 demonstrates that the enzyme from S cells, activated by cyclic AMP concentrations from $10^{-9}$ to $10^{-4}$ M, is thermolabile. In the protein kinase from R cells the activity stimulated by $10^{-9}$—$10^{-6}$ M cyclic AMP is thermolabile while the enzyme requiring $10^{-5}$—$10^{-4}$ M cyclic AMP for stimulation is resistant to heat inactivation.

Fig. 8 shows that the free regulatory subunits of both S and R kinase are inactivated by 30 min incubation at 46°C and cyclic AMP binding provides protection from heat inactivation. The regulatory subunit from S kinase is protected by cyclic AMP binding both at low ($2 \cdot 10^{-7}$ M) and high ($10^{-5}$ M) cyclic AMP concentrations. In contrast the regulatory subunit from R kinase is stabilized by cyclic AMP binding only at high cyclic AMP concentrations ($10^{-5}$ M), low concentrations ($2 \cdot 10^{-7}$ M) giving only a partial protection. To study the thermal stability of the regulatory subunit in the holoenzyme we exposed the protein kinases to 46°C for 30 min and then followed the cyclic AMP-binding activity at different cyclic AMP concentrations. The data presented in Fig. 9 show that as a result of the heat treatment, the regulatory subunit of S kinase loses its cyclic AMP binding activity. In the R holoenzyme, however, only the cyclic AMP-binding activity expressed at low cyclic AMP concentrations was

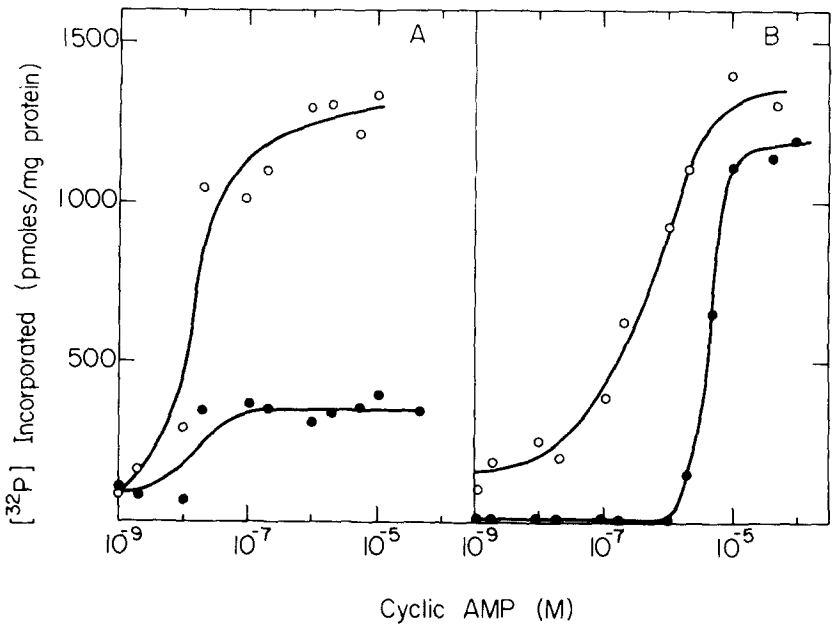


Fig. 7 Cyclic AMP stimulation of the catalytic activity of heat-treated protein kinase. S kinase (A) and R kinase (B) were incubated in the TMG buffer for 30 min at 46°C and assayed for cyclic AMP- stimulated catalytic activity as a function of cyclic AMP concentration (●). Control experiment without heating (○). Kinase activities in the absence of cyclic AMP were subtracted (S kinase : 675 and 75 pmol/mg for control and 30 min heated enzyme, respectively; R kinase: 400 and 130 pmol/mg for control and 30 min heated enzyme, respectively).

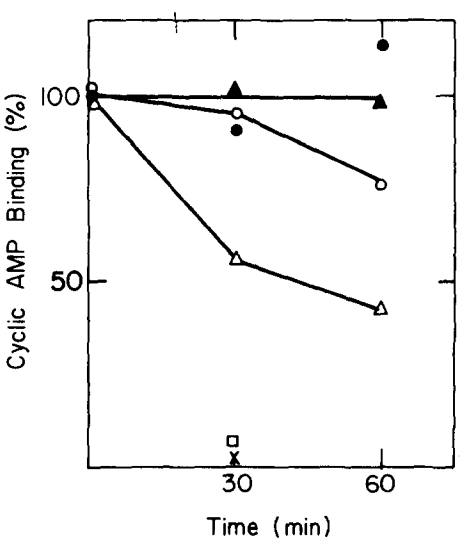


Fig. 8. Heat inactivation of free regulatory subunits. Free regulatory subunits from S kinase (x) and from R kinase (□), isolated by affinity chromatography on C8-$NC_6$-$NH_2$-cAMP-Sepharose were incubated at 46°C for 30 min and assayed for cyclic [$^3$H]AMP binding activity. S (circles) and R (triangles) kinase regulatory subunits were preincubated for 60 min with $2 \cdot 10^{-7}$ M (○,△) and $10^{-5}$ M (●, ▲) cyclic [$^3$H]-AMP, heated at 46°C for the times indicated and the bound cyclic AMP determined.

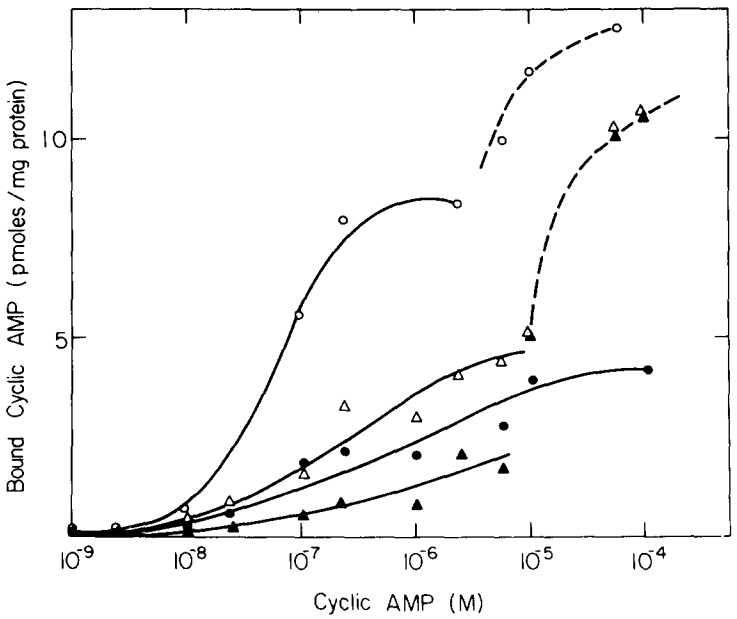


Fig. 9 Heat inactivation of regulatory subunit of protein kinase holoenzyme S (●) and R (▲) kinase were assayed for cyclic AMP binding after exposure to 46°C for 30 min as described in legend to Fig. 3. ○ and △ represent the control experiment for S and R kinase, respectively.

lost upon heating, the activity expressed at high cyclic AMP concentrations ($10^{-5}$—$10^{-4}$ M) being heat stable under the conditions of the experiment (Fig. 9).

## Discussion

S49.1 mouse lymphoma cells contain a cyclic AMP-dependent protein kinase which appears to be present only in the cytosol and purifies as a single peak on a DEAE-cellulose column. The cytoplasmic extracts of cells which have undergone the transition from a cyclic AMP sensitive (S) to a cyclic AMP-resistant (R) phenotype show a very low activity of cyclic AMP-binding and cyclic AMP-stimulated protein kinase. The apparent lack of cyclic AMP-dependent protein kinase in R cell cytosol may be explained by the presence of an inhibitor, a deficiency of the regulatory subunit of the protein kinase, or a low affinity of this subunit for cyclic AMP. Our finding that the cyclic AMP-dependent kinase of R cells can be brought up to a level of activity similar to that of the enzyme from the parental cell by a 100-fold higher cyclic AMP concentration (Fig. 2) indicates that the R kinase is not deficient in its regulatory subunit but that the latter has a reduced affinity for cyclic AMP. The fact that the regulatory subunit of R kinase even after extensive purification by affinity chromatography maintains its low affinity for cyclic AMP rules out the presence of an inhibitor in this enzyme. The cyclic AMP-resistant character of R cells seems therefore to be due to an alteration in the regulatory subunit of the cyclic AMP-dependent protein kinase which is reflected in a reduced affinity for cyclic AMP.

The free regulatory and catalytic subunits from both S and R kinases appear

to be thermolabile (Figs. 5 and 8). In contrast the two subunits in the holoenzyme form have become more stable to heat inactivation in R kinase than in S kinase (Figs. 7 and 9). The differences observed in the stability to heat inactivation of S and R kinases may be taken as an indication for changes which have occurred in the interaction between the subunits.

The molecular alteration of the regulatory subunit in R kinase seems therefore to be structural and to affect not only the protein's binding site for cyclic AMP but also its interaction with the catalytic subunit. Cyclic AMP-dependent protein kinases with different affinities between subunits or with different temperature stabilities have been recently described for several mammalian tissues [15,16]. These studies have not determined however a correlation between these two properties and the affinities of the regulatory subunits for cyclic AMP.

The lymphoma R kinase appears heterogeneous in its behaviour towards heat inactivation and interaction with cyclic AMP (Figs. 7 and 9). The enzyme which can bind cyclic AMP at low concentrations ($2 \cdot 10^{-7}$ M) is thermolabile while the enzyme which binds cyclic AMP at high concentrations ($10^{-5}$ M) is much more stable to heat inactivation. In addition, the regulatory subunit-cyclic AMP complex from R kinase is stable to heat inactivation only when formed in the presence of high cyclic AMP concentrations (Fig. 8). These findings may be explained by the presence of two or more molecular populations of R kinase or by assuming a heterogeneity in the cyclic AMP binding sites on the regulatory subunit of the enzyme. The presence of cyclic AMP-sensitive revertants in the resistant cell population is unlikely since the R cells were often checked and reselected for resistance to 2 mM $N^6,O^2$-dibutyryl cyclic AMP.

The alteration of R kinase which seems to be responsible for the lack of biological effects of cyclic AMP in S49.1 lymphoma cells is an inherited phenotype which persists in the absence of selective pressure [1]. The simplest explanation for the alteration in kinase structure would be a mutation in the structural gene of the regulatory subunit [17]. We have observed however that, during the selection of cells resistant to 2 mM $N^6,O^2$-dibutyryl cyclic AMP by stepwise increase of $N^6,O^2$-dibutyryl cyclic AMP concentration, variants with intermediate resistance to cyclic AMP were isolated (unpublished results). The existence of such lymphoma variants with an intermediate cyclic AMP-resistant phenotype and a protein kinase partially altered in its affinity for cyclic AMP [17,18] is difficult to accommodate with a single mutation hypothesis. The chromatography of R cell cytosol on a DEAE-cellulose column (Fig. 1) had revealed also a peak of an increased cyclic AMP-independent protein kinase which was not observed in the parental S cell. Although this enzyme does not seem to be derived from a cyclic AMP-stimulated kinase by loss of receptor, since it did not interact with purified regulatory subunit from S kinase (data not shown), we cannot exclude also an alteration in the catalytic R kinase.

Considering the role attributed to the cyclic AMP-dependent protein kinase as a mediator of cyclic AMP effects in the eukaryotic cell [19] the fact that the R lymphoma cell resistant to 2 mM $N^6,O^2$-dibutyryl cyclic AMP still grow in culture normally, with the same division times as that of the wild-type S cells, is puzzling. If cyclic AMP is totally inactive in R cells it may be assumed that

no vial function requires it and as in bacteria [20] the cyclic nucleotide plays a role only in specific inducible functions. More studies are necessary to determine the role of the cyclic AMP-dependent protein kinase and the cellular components involved in the regulation of cell growth.

## Acknowledgements

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