CYCLIC 3',5'-NUCLEOTIDE PHOSPHODIESTERASE
Effect of Binding Protein on the Hydrolysis of Cyclic AMP

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SUMMARY

The rate of cyclic AMP hydrolysis by a cyclic 3',5'-nucleotide phosphodiesterase was diminished by the presence of a cyclic AMP binding protein in the reaction mixture. The reduction was proportional to the concentration of the binding protein; and was more pronounced at 0° than at 30°, presumably because the affinity of cyclic AMP to the binding protein was greater at 0° ("apparent dissociation constant" = 3×10^{-8} M) than at 30° ("apparent dissociation constant" = 4×10^{-7} M). These experiments indicate that cyclic AMP bound to the binding protein is not susceptible to the action of phosphodiesterase. It is hydrolyzed only when dissociated from the protein, and the rate of dissociation appears to be the limiting factor. The possible physiological significance of these results is discussed.

INTRODUCTION

Cyclic AMP mediates the effects of a wide variety of hormones, and thus affects many enzymic reactions and cellular processes (1,2). There is considerable evidence to indicate that the common denominator among the various reactions and processes is that the nucleotide functions as a versatile allosteric effector. This hypothesis was based on a critical review of the studies on protein kinase, "cyclic AMP-receptor protein," and cyclic AMP binding proteins (3).

Protein kinase is thus far the most studied system relevant to the mechanism of action of cyclic AMP. The enzyme is composed of a regulatory subunit and a catalytic subunit. The regulatory subunit binds cyclic AMP, and the nucleotide protein complex is dissociated from the catalytic subunit, which becomes active. In the absence of cyclic AMP, the association of the regulatory subunit to the catalytic subunit renders the enzyme inactive (4-7).

Pastan and Perlman found that the induction of β-galactosidase by cyclic AMP in *Escherichia coli* required cyclic AMP and a "cyclic AMP receptor protein" isolated from the bacteria. The receptor protein binds cyclic AMP, and presumably the nucleotide-protein complex interacts with the lac promoter region of the operon and facilitates the transcription of β-galactosidase. The receptor protein possesses no detectable protein kinase activity (8).

The only physiological mechanism known to terminate the action of cyclic AMP is through its hydrolysis to 5'-AMP catalyzed by cyclic 3',5'-nucleotide phosphodiesterase. In the protein kinase system, for example, termination of the action of cyclic AMP *in vivo* involves the hydrolysis of not

only the cyclic AMP free in the cytosol, but also that bound to the regulatory subunit. The question arises as to whether the bound form is accessible to phosphodiesterase. Experiments with cyclic AMP binding protein to be described in this paper indicate that bound cyclic AMP is not hydrolyzed and that it is susceptible to the action of phosphodiesterase only when dissociated from the binding protein. These experiments further indicate that the rate limiting factor of the hydrolysis of the bound form is the dissociation of cyclic AMP from the binding protein.

MATERIALS AND METHODS

Chemicals

Isotope-labeled and non-labeled cyclic AMP was purchased from Schwarz Bio-Research and were used without further purification. Bovine serum albumin, Fraction V, was obtained from Sigma Chemical Company.

Preparation of Binding Protein

A survey of bovine tissues indicated that kidney was rich in the binding protein (9). Kidney was obtained from a local slaughterhouse, rinsed with water and used fresh or stored at -20° until use. Subsequent steps were performed at 0-4°. The tissue was homogenized in three volumes of 20 mM Tris-HCl (pH 7.5), and the homogenate was centrifuged at 40,000 X *g* for 30 min. Solid $(NH_4)_2SO_4$ was added with stirring to the supernatant fluid to 30% saturation. After 30 min, the solution was centrifuged at 20,000 X *g* for 30 min and more $(NH_4)_2SO_4$ was added to the supernatant fluid to 45% saturation. The 30-45% $(NH_4)_2SO_4$ fraction was dialyzed extensively against Tris-HCl and the dialyzed sample was stored at -20° until use. The binding protein at this stage represents a several-fold purification over the crude homogenate. The preparation was stable and could be stored at -20° for weeks without a significant loss of activity.

Assay of Binding Protein

Binding of cyclic AMP was assayed either by equilibrium dialysis as previously described (10), or according to a procedure slightly modified after Gilman (11). The reaction was carried out at 30° or 0° in a final volume of 0.1 ml which contained 50 mM sodium acetate (pH 4.0), 2 mM EDTA, 0.1 μM (H^3)-cAMP (sp. act. 5 μCi/nmole) and various concentrations of binding protein. Cyclic AMP was usually added last to start the reaction. EDTA was added to suppress the low level of phosphodiesterase activity present in the preparation. At the end of 60 min, the reaction mixture was diluted with 2 ml of ice-chilled 20 mM phosphate buffer (pH 6.0), containing 10 mM $MgCl_2$. The diluted solution was quickly filtered through a millipore cellulose ester membrane (diameter 2.4 cm, pore size 0.45 μ). Approximately 3 ml of the phosphate-$MgCl_2$ buffer was passed through to wash the filter. The washing was repeated once. The filter was dried at 90° for 5 min and then counted in 5 ml of a scintillation fluid containing 3 g PPO and 100 mg POPOP per 1 liter of toluene. When a higher concentration of binding protein was called for in some experiments, the reaction mixture became turbid in the acetate buffer, and a 50 mM citrate buffer (pH 7.0) was used instead. Both buffers gave essentially similar results. In other words, the turbidity of the protein solution did

not significantly affect the binding of cyclic AMP nor its subsequent susceptibility to phosphodiesterase.

Preparation and Assay of Cyclic 3',5'-Nucleotide Phosphodiesterase

Phosphodiesterase was prepared from *Serratia marcescens* according to Okabayashi and Ide (12) and was assayed by measuring the inorganic phosphate released as described previously (13). Alternatively, isotope-labeled cyclic AMP was used and at the end of the reaction 5'-AMP was separated from the cyclic AMP nucleotide on descending paper chromatography (14). Spots corresponding to 5'-AMP were cut out and the radioactivity was counted in 5 ml of the toluene scintillation fluid.

Determination of Protein

Protein was determined with the biuret reagent using bovine serum albumin as a standard, or estimated according to the spectrophotometric technique of Warburg and Christian (15).

RESULTS AND DISCUSSION

The binding protein exhibited a site specific for cyclic AMP (10). It has been found in all bovine and rabbit tissues examined. Cyclic AMP bound to the protein was recovered indistinguishable from an authenic sample, indicating that the nucleotide did not undergo any

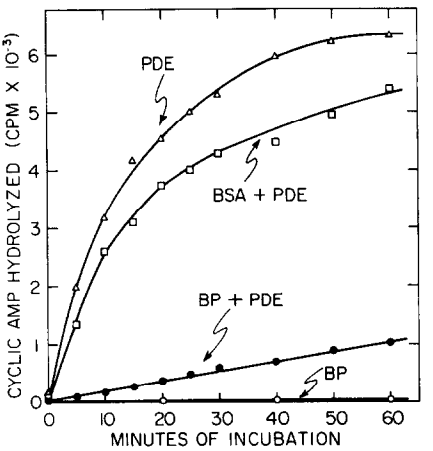

Figure I. Effect of binding protein on the hydrolysis of cyclic AMP. One ml of the incubation mixture contained 50 μmoles Na-acetate (pH 4.0), 70 pmoles (H^3)-cyclic AMP (sp. act. 14.3 μCi/nmole), 2 μmoles EDTA, and when present, 12.8 mg binding protein, 18 mg bovine serum albumin, or 0.18 μg phosphodiesterase. The incubation was carried out in two stages. The first stage at 0° allowed binding to achieve equilibrium, and was initiated by the addition of cyclic AMP. At the end of 60 min, the reaction system was transferred to a 30° water bath for the second stage of incubation. Phosphodiesterase was added immediately (0 time for the second incubation), and 100 μl aliquots of the reaction mixture were transferred at different times into clean tubes containing 10 μl of ice-chilled 2 M HCl. These samples were kept at 0° until the experiment was over. Carrier cyclic AMP and 5'-AMP were added. An aliquot of this reaction mixture was put on Whatman No. 1 paper for descending chromatography. Spots corresponding to 5'-AMP were cut and the radioactivity counted as described previously (14).

change in molecular structure. The protein bound 20-30 pmoles cyclic AMP per mg protein. Full details about the binding protein will be presented elsewhere (9).

Phosphodiesterase purified from *Serratia marcescens* according to Okabayashi and Ide (12) was estimated to hydrolyze cyclic AMP at a rate of about 70 μmoles/mg protein/min at 30°, pH 8.0, under our assayed conditions. The specific activity could only be estimated because of a very low protein concentration in the purified preparation. The apparent Km for cyclic AMP was found to be 7×10^{-4} M, in good agreement with the value of 5×10^{-4} M obtained by Okabayashi and Ide (12). Although EDTA suppresses the activity of mammalian phosphodiesterase (16), it does not affect the activity of the bacterial enzyme.

The effect of binding protein on the hydrolysis of cyclic AMP by phosphodiesterase is depicted in Fig. I which shows that the presence of the binding protein diminishes the rate of hydrolysis markedly. The reduced rate was not due to a general protein effect because the presence of a comparable concentration of bovine serum albumin diminishes the rate to a much smaller

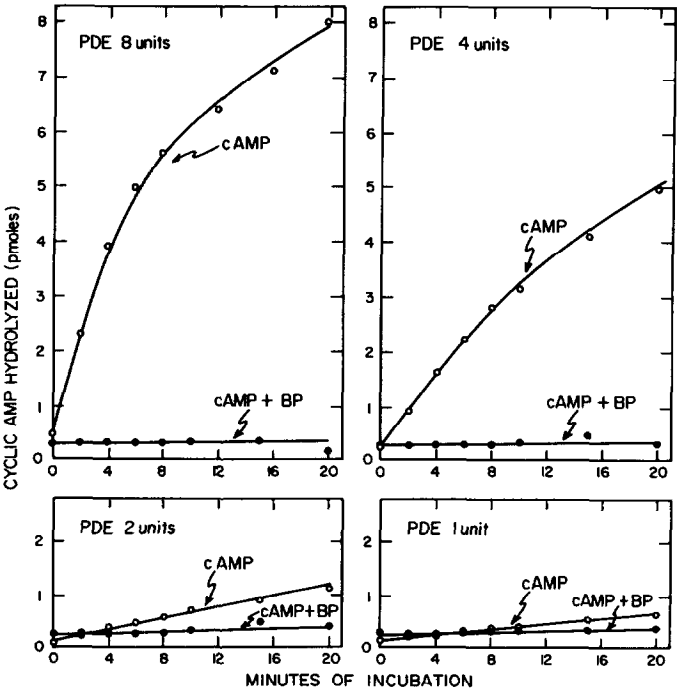

Figure II. Hydrolysis of cyclic AMP by phosphodiesterase in the presence of the binding protein. The incubation was carried out at 0° in both stages. One ml of the incubation mixture contained 50 μmoles citrate buffer (pH 7.0), 122 pmoles (H^3)-cyclic AMP (sp. act. 16.4 μCi/nmole), 2 μmole EDTA, 18.2 mg binding protein, and 8,4,2 or 1 arbitrary unit of phosphodiesterase corresponding to 1.5, 0.75, 0.38 and 0.19 μg protein, respectively. The first stage was initiated by the addition of cyclic AMP. At the end of 60 min, phosphodiesterase was added immediately (0 time for the second incubation), and aliquots of the reaction mixture were transferred at times indicated into clean tubes containing 10 μl of ice-chilled 2 M HCl. Subsequent steps were as described in the legend to Figure I.

extent. The binding protein itself under our experimental conditions exhibited virtually no phosphodiesterase activity. This experiment indicates that cyclic AMP bound to the protein is not readily susceptible to the action of phosphodiesterase.

Figure II demonstrates the effect of varying the concentration of phosphodiesterase on cyclic AMP hydrolysis in the presence of a fixed concentration of binding protein. In this experiment the binding sites were in excess of cyclic AMP, and essentially all the cyclic AMP was taken up by the binding protein. As expected, the rate of cyclic AMP hydrolyzed in the absence of binding protein was directly proportional to the concentration of phosphodiesterase. However, the rate in the presence of the binding protein was essentially zero and appeared independent of the various concentrations of phosphodiesterase. This indicates clearly that the rate limiting factor was the inaccessibility of the nucleotide to phosphodiesterase rather than the activity of the enzyme.

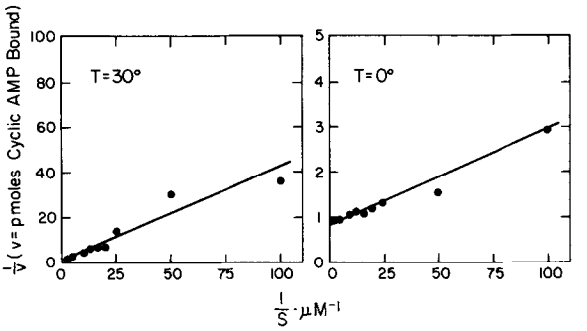

Figure III. The "apparent dissociation constant" of the binding protein for cyclic AMP at 30° or 0° was estimated by the Lineweaver-Burke plot. Binding was carried out in the Na-acetate buffer and the millipore technique was used as described under Materials and Methods. The "apparent dissociation constant" was 4 X 10^{-7} M at 30° and 3 X 10^{-8} M at 0°.

To illustrate this point further, we studied the effect of temperature on the affinity of cyclic AMP to the binding protein (Fig. III), and on its rate of hydrolysis in the presence of various concentrations of the binding protein (Fig. IV). Figure III shows the "apparent dissociation constant" of the binding of cyclic AMP at two temperatures as estimated by the double reciprocal plots. The "apparent dissociation constant" increased from 3 X 10^{-8} M at 0° to 4 X 10^{-7} M at 30°. An increase of temperature presumably accelerates random thermal motion, which facilitates the dissociation of the nucleotide from the binding site and therefore increases the "apparent dissociation constant."

The effect of varying the concentrations of binding protein on cyclic AMP hydrolysis at the two temperatures in the presence of a constant concentration of phosphodiesterase is examined in Fig. IV. The rate of hydrolysis in the presence of each concentration of the binding protein was reduced more at 0° than at 30°, even though the rates without the binding protein were comparable at both temperatures. The differential rates at the two temperatures were not due to

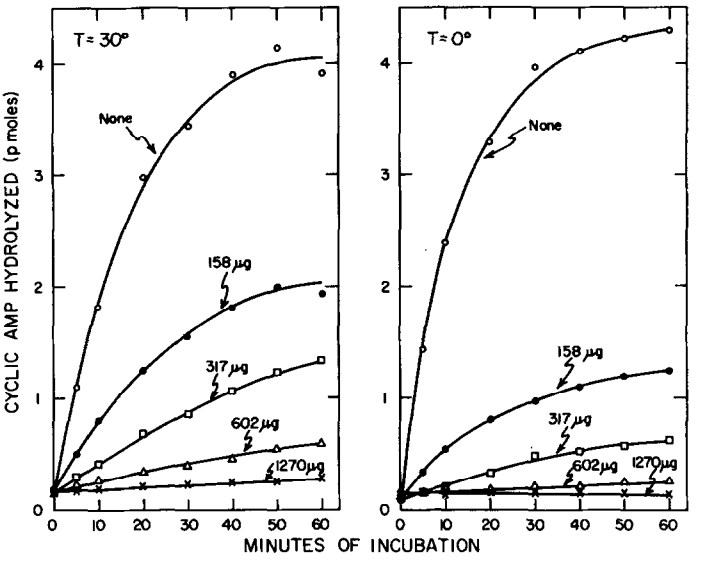

Figure IV. Effect of various concentrations of binding protein on the hydrolysis of cyclic AMP at 30° and 0°. One set of the experiment was carried out at 30° in both stages, and the other at 0° as indicated in Figure IV. One ml of the incubation mixture contained 50 µmoles citrate buffer (pH 7.0), 61 pmoles (H^3)-cyclic AMP (sp. act. 16.7 µCi/nmoles), 2 µmoles EDTA, 0.5 µg phosphodiesterase and when present, various concentrations of binding protein. At the end of 60 min, phosphodiesterase was added to initiate the second stage of incubation. Subsequent steps were as described in the legend to Figure I.

limiting activities of phosphodiesterase but rather to a differential affinity of the binding protein for the nucleotide at these temperatures. A higher affinity of the binding protein for the nucleotide at 0° makes the nucleotide less susceptible to the hydrolytic action of the enzyme and hence a reduced rate of hydrolysis; conversely, a diminished affinity at 30° makes it more susceptible to degradation.

These experiments show that cyclic AMP bound to the protein is not hydrolyzed. It is accessible to the action of phosphodiesterase only when dissociated from the protein, and the rate of hydrolysis apparently is a function of the rate of dissociation.

The "apparent dissociation constant" of the regulatory subunit of protein kinase for cyclic AMP is 1 X 10^{-8} M (11), and thus compares favorably with that of the binding protein described here. It seems likely that the effect of the binding protein on the hydrolysis of cyclic AMP may also apply to the protein kinase. Indeed, O'Dea *et al.* noted that cyclic AMP in complex with the regulatory subunit of protein kinase was inaccessible to phosphodiesterase (17). The binding protein in our preparation exhibited protein kinase activity; whether the enzyme contributed significantly to the binding capacity was not assessed.

The recent observation of Chambaut *et al.* that an extract of rat liver contains a cyclic AMP binding protein devoid of any kinase activity underscores the possibility that the binding protein

may serve a role other than its involvement with the protein kinase (see also ref 8). One such role may be that the binding protein regulates the availability of free cyclic AMP in the cell. However, the high affinity of the protein for cyclic AMP would render it difficult for the nucleotide to dissociate readily from the protein, and therefore would not constitute a flexible regulatory mechanism.

Within the context of the concept that cyclic AMP serves as a versatile allosteric effector (3), termination of the action of cyclic AMP involves the hydrolysis of not only the free form but more importantly the bound and active form. In considering the magnitude and duration of cyclic AMP action, the bound and active pool is perhaps more pertinent than the unbound pool. Further, termination of cyclic AMP action involved not only the factors affecting the activity of phosphodiesterase but also those affecting the rate of dissociation of the bound and active nucleotide.

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