Two Soluble Forms of Guanosine 5'-(β,γ-imino)triphosphate and Fluoride-activated Adenylate Cyclase*

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SUMMARY

Soluble and membrane-bound adenylate cyclase from the canine renal medulla are very slowly activated by guanosine 5'- (β,γ-imino)triphosphate (Gpp(NH)p) or fluoride. At 25°, 8 to 10 h of incubation of the soluble enzyme, and 6 to 8 h of incubation of the membrane-bound enzyme are required to reach the maximal activity. The dependence of activation on concentration of Gpp(NH)p changes with time. Half-maximal activation of soluble adenylate cyclase occurs at 3 ± 1 x 10^-6 M Gpp(NH)p if the activity is measured without preincubation with Gpp(NH)p and at 4 ± 2 x 10^-5 M if it is measured after 6 to 22 h of incubation with Gpp(NH)p at 25°. The activation occurs over a rather broad range of nucleotide concentration and cannot, therefore, be simply interpreted to reflect a function of nucleotide binding.

Two forms of soluble adenylate cyclase are resolved by Sepharose gel filtration. One form, with a Stokes radius of 71 Å, is rapidly activated by Gpp(NH)p; the other, with a Stokes radius of 55 Å, requires long incubation with Gpp(NH)p to be activated. The sedimentation coefficient of the two forms is 7.3 S. The apparent range of nucleotide concentration and cannot, therefore, be simply interpreted to reflect a function of nucleotide binding.

Since the initial observation by Rodbell et al. (1) that CTP enhances the activation of hepatic adenylate cyclase by glucagon, many laboratories have demonstrated the activation of adenylate cyclase by guanine nucleotides. The nucleotides seem to have two kinds of action. One is to modulate the response to hormone. For instance, in a number of systems (although not in all (2, 3)) guanine nucleotides have been shown to increase the apparent affinity of the enzyme for hormone (4, 5). Secondly, guanine nucleotides affect basal adenylate cyclase activity even in the absence of any hormone. The effects of the naturally occurring nucleotides are somewhat variable, but the synthetic analogue, Gpp(NH)p, seems to be a universal activator of adenylate cyclase (6). Integrity of the plasma membrane is not essential for activation by guanine nucleotides since they have been shown to activate solubilized adenylate cyclase (7-9). These findings suggest that the guanine nucleotides act on the enzyme itself.

The present studies describe the activation of soluble adenylate cyclase from the canine renal medulla by Gpp(NH)p and fluoride. Analysis of the solubilized enzyme by Sepharose gel filtration shows that there are two physically distinguishable forms of the enzyme which differ in the rate at which they are activated by Gpp(NH)p and fluoride. The finding that there exist different forms of the enzyme with different functional properties must be taken into account in the interpretation of kinetic data obtained with the enzyme in intact membranes.

EXPERIMENTAL PROCEDURES

Preparations of Adenylate Cyclase—Mongrel dogs were killed by exsanguination. The kidneys were immediately removed and chilled in isotonic saline (0.9% NaCl solution). The medulla was homogenized in a Waring Blender for 15 s in 5 volumes of 0.2 M Tris-HCl, pH 7.6, 0.075 M sucrose, 10 mM MgCl₂, 1 mM EDTA, 1 mM dithiothreitol, 1% Lubrol PX (a kind gift of ICI, America, Inc.). After straining through two layers of cheesecloth, the homogenate was centrifuged at 100,000 x g for 60 min. The supernatant contained the soluble enzyme.

The procedure for preparing plasma membranes from canine renal medullas was identical to that already described for the preparation of partially purified plasma membranes from the rat renal medulla except that a Waring Blender was used for the initial homogenization (10).

Adenylate Cyclase Assay—The assay used was a modification of the method of Krishna et al. (11) and has been described previously (10). In addition to the enzyme, the 70-μl assay mixture contained 9 mM MgCl₂, 30 mM KCl, 8 mM creatine phosphate, 4 mg/ml of creatine kinase, 0.08 to 0.15 μM Tris- HCl, pH 7.6, 0.1% bovine serum albumin, 1.6 mM cyclic AMP, and [3H]ATP (70 to 80 cpm/pmol). All samples were assayed in duplicate.

The product of the reaction in the absence and in the presence of Gpp(NH)p was shown to be cyclic AMP by two methods. It was destroyed by digestion with cyclic nucleotide phosphodiesterase (Sigma). It co-chromatographed with authentic cyclic AMP on EC-TROLA (epichlorohydrin triethanolamine cellulose) paper (Whatman) using the system described by Rao et al. (12). Protein was determined by the method of Lowry et al. (13).

RESULTS AND DISCUSSION

Gpp(NH)p and fluoride activate and stabilize both solubilized and membrane-bound adenylate cyclase. At 25°, the activation by both of these agents is a slow process requiring 8 to 10 h of incubation for maximal activation of the soluble enzyme (see Fig. 1) and 6 to 8 h for the membrane-bound one. The control, incubated at 25° without any activator, demonstrates the characteristic lability of adenylate cyclase.

At 37°, the activation of membrane-bound adenylate cyclase by Gpp(NH)p occurs much more rapidly than at 25°. The maximal rate is achieved in 30 to 40 min of incubation (see Fig. 2). In the absence of the activating nucleotide, enzymatic activity decays rapidly. In contrast, solubilized adenylate cyclase is much less stable at 37° than it is at 25°. The enzymatic activity is stimulated about 10-fold by Gpp(NH)p in the first 5 min of incubation but declines steadily thereafter. Nevertheless, as can be seen in Fig. 2, Gpp(NH)p stabilizes the enzyme so that it loses activity more slowly than the control incubated without nucleotide.

The activation of adenylate cyclase by Gpp(NH)p or fluoride which was found in these studies is slower than that which has
Further addition of Gpp(NH)p. In this respect, the behavior of longest time tested) during which it was kept at 25° without treatment and remained unchanged for the next 5 h (the irreversible. Solubilized enzyme which had been activated by such a cumulative curve.

The rate of adenylate cyclase activity is to measure the accumulation of cyclic AMP as a function of time for 5 to 15 min. However, an increase in enzymatic activity which occurs very slowly might be difficult to detect as an upward concavity in such a cumulative curve.

Activation by Gpp(NH)p does not appear to be readily reversible. Solubilized enzyme which had been activated by incubation for 16 h at 25° with 10^{-5} M Gpp(NH)p was freed of unbound nucleotide by passage over a Sephadex G-25 column. The specific activity of the enzyme was unchanged by this treatment and remained unchanged for the next 5 h (the longest time tested) during which it was kept at 25° without further addition of Gpp(NH)p. In this respect, the behavior of the solubilized enzyme is similar to that which has been reported for the membrane-bound enzyme (16). The lack of reversibility is also similar to that which has been reported for activation by fluoride (17).

The dependence of activation of solubilized adenylate cyclase upon the concentration of Gpp(NH)p was measured without preincubation and after 6 to 22 h at 25°. The results of a typical experiment are shown in Fig. 3. Two things are evident from the data. First, there is a time-dependent change in the apparent affinity of the enzyme for Gpp(NH)p. In four such experiments with three different preparations of solubilized adenylate cyclase, the concentration of Gpp(NH)p which gave half-maximal stimulation without preincubation was 3 ± 1 x 10^{-5} M, while after preincubation at 25° the value was changed to 4 ± 2 x 10^{-4} M. The second thing to notice is that activation occurs over a rather broad range of concentration. A Line-weaver-Burk plot of the data is not linear. It cannot, therefore, be simply interpreted as a function of nucleotide binding.

One possible explanation of these data is that the solution contains different forms of adenylate cyclase which differ in their kinetic properties. This hypothesis was tested with the following experiments. Solubilized adenylate cyclase which had never been exposed to Gpp(NH)p was passed over a column of Sepharose 4B or 6B. The column was equilibrated with buffer without the nucleotide. Gpp(NH)p (5 x 10^{-6} M) was added to the effluent fractions which were then divided into portions and assayed for adenylate cyclase activity immediately, after a 1-h incubation and after a 17-h incubation at 25°. Fig. 4 shows that two activities are clearly distinguishable by their elution position. One form with a Stokes radius of 71 ± 2 A (n = 5) is activated rapidly while the form with a Stokes radius of 56 ± 2 A (n = 5) requires long exposure to Gpp(NH)p to show activity. These results were obtained in five gel filtration experiments with three separate enzyme preparations. The only variation was in the relative amounts of the two forms. The same pattern of activity was seen when 10^{-2} M NaF was the activating agent (data not shown). This result indicates that prior to exposure to any activator there are two forms of adenylate cyclase which differ in their Stokes radius and in the rate at which they are activated by Gpp(NH)p or fluoride.

In order to confirm the presence of two physically and kinetically different forms of adenylate cyclase, the time course and the Gpp(NH)p concentration dependence of activation were determined separately for enzyme in each of the peaks. Fractions from the areas of such chromatograms indicated by the shaded bars in Fig. 4 were used in these studies. The comparison of the rate of activation by 10^{-5} M Gpp(NH)p at 25° is shown in Fig. 5. Peak I enzyme is 75% activated in 10 min while Peak II adenylate cyclase activity does not begin to rise before 1 h and the maximum is not reached for 17 h. 1

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1 Cuatrecasas et al. (18) mentioned a time-dependent change in the apparent affinity for Gpp(NH)p of adenylate cyclase in fat cell membranes but gave no data.

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**FIG. 1 (left).** Activation of solubilized adenylate cyclase by Gpp(NH)p or NaF at 25°. Solubilized adenylate cyclase was incubated with 10^{-6} M Gpp(NH)p (O—O) or 10^{-5} M NaF (C—C). At the times indicated, 50-μl samples were removed from the incubation mixture and added to 20 μl of reagents for adenylate cyclase assay. After incubating at 37° for 10 min, the enzyme assays were stopped. The amount of cyclic AMP formed was analyzed for all the samples at once as previously described (10). The concentration of detergent during the incubation was 0.2% Lubrol PX. The control (O——O) was incubated without activator but assayed with 10^{-4} M Gpp(NH)p. Basal activity is that measured without activator in the assay.

**FIG. 2 (center and right).** Activation of membrane-bound and soluble adenylate cyclase by Gpp(NH)p at 37°. Partially purified plasma membranes (center) or soluble adenylate cyclase (right) were incubated with 10^{-5} M Gpp(NH)p (open bars) for the times indicated (note the difference in time scale from Fig. 1). Adenylate cyclase activity was determined in 5-min assay periods at the times indicated. The control (closed bars) was incubated without Gpp(NH)p but the nucleotide was added at the time of enzyme assay.
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FIG. 3. Concentration dependence of activation of soluble adenylate cyclase by Gpp(NH)p. Gpp(NH)p at the indicated concentrations was added to the solubilized enzyme. The concentration of Gpp(NH)p in the stock solution from which further dilutions were made was determined by its absorption at 252 nm using the molar extinction coefficient reported for GTP of 13.7 \times 10^2 M^{-1} cm^{-1} (18). One sample was assayed immediately in duplicate for adenylate cyclase activity (GO). Other samples from the same mixture were assayed after 6 (O-O) and 22 (A-A) h of incubation at 25°. In the adenylate cyclase assay, the concentration of Gpp(NH)p was 71% of that indicated in the figure. Basal activity is that measured without any activator. The concentration of detergent during the incubation was 0.2% Lubrol PX. The arrows indicate the points of half-maximal stimulation.

the Gpp(NH)p concentration dependence of activation is shown. At high Gpp(NH)p concentration, 10^{-3} M, Peak I enzyme can be maximally activated without any incubation at 25°. The concentration for half-maximal activation without incubation at 25° is 4 \pm 2 \times 10^{-6} M Gpp(NH)p (n = 2). After 17 h at 25° the concentration for half-maximal activation is 5 \pm 3 \times 10^{-5} M (n = 2). In contrast, Peak II enzyme cannot be maximally activated without long exposure to the guanine nucleotide even at 10^{-3} M Gpp(NH)p. After 17 h of incubation, the concentration needed for half-maximal activation is 8 \pm 1 \times 10^{-5} M (n = 2).

Material from the leading edge of the rapidly activated enzyme peak and from the back edge of the slowly activated enzyme peak was separately rechromatographed on Sepharose 4B. The enzyme activity appeared at the position predicted from the results shown in Fig. 4 in each case. There was no evidence of interconversion of the two forms.

Although the two forms can be distinguished by gel filtration, their sedimentation coefficients are too similar to permit resolution by sucrose density centrifugation under the conditions used in these studies. Analysis of enzyme centrifuged in a sucrose density gradient with the rate of sedimentation of calibrating enzymes of known s_{20,w}. The sedimentation coefficient of adenylate cyclase from the canine renal medulla is 7.3 \pm 0.3 S (n = 7).

With this sedimentation coefficient, the value for the Stokes radius determined by gel filtration and a value of 0.71 ml/g (20), the molecular weight of the rapidly and slowly activated forms of adenylate cyclase can be calculated. The rapidly activated form (Peak I) has a molecular weight of 200,000 and a frictional ratio, f/f_{0}, of 1.6 assuming a solvation of 0.2 g of
The finding that there exist at least two species of adenylate cyclase which can be physically distinguished and which differ in the rate at which they are activated by Gpp(NH)p or fluoride complicates the interpretation of kinetic studies using intact plasma membranes or unpurified soluble adenylate cyclase. It also makes more difficult the maintenance of an intact plasma membranes or unpurified soluble adenylate cyclase. It makes more difficult the maintenance of an intact plasma membranes or unpurified soluble adenylate cyclase. It makes more difficult the maintenance of an intact plasma membranes or unpurified soluble adenylate cyclase.

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