Partial Purification and Properties of Guanosine 3'-5'-Monophosphate-dependent Protein Kinase from Pig Lung

Kinya Nakazawa and Mamoru Sano

From the Section of Cytocytology, Department of Morphology, Institute for Developmental Research, Aichi Prefectural Colony, Kasugai, Aichi 480-03, Japan

Guanosine 3'-5'-monophosphate (cyclic GMP)-dependent protein kinase which catalyzes the phosphorylation of histone was purified about 200-fold from the soluble fraction of pig lung by pH 5.5 precipitation, DEAE-cellulose column chromatography, and Sephadex G-200 gel filtration. The apparent $K_0$ values for guanosine 3'-5'-monophosphate and adenosine 3'-5'-monophosphate were determined to be about 17 and 360 nm, respectively. Mg$^{2+}$ was essential for the activity exhibiting biphasic stimulation behavior and neither Mn$^{2+}$ nor Ca$^{2+}$ could substitute for Mg$^{2+}$. However, these divalent ions markedly inhibited the protein kinase activity stimulated by cyclic GMP in the presence of Mg$^{2+}$.

The role of cyclic AMP* as an intracellular mediator of hormone actions has been well documented (1), and various effects elicited by this cyclic nucleotide are proposed to be mediated through activation of cyclic AMP-dependent protein kinase (2, 3). Recently, insulin (4), cholinergic agents (5–10), and mitogenic agents, such as plant lectin (11), were shown to cause an increase in cyclic GMP levels in some mammalian tissues. Cyclic GMP-dependent protein kinase has been found in several species of arthropods (12) and purified partially from lobster tail muscle (13) and from silkworm (14, 15). Several mammalian tissues also were found to contain cyclic GMP-dependent protein kinases, although their levels were considerably lower than those of the cyclic AMP-dependent enzymes (16–19).

Through the survey of cyclic GMP distribution in mammals, it has been observed that lung tissues contain amounts of cyclic GMP comparable to those of cyclic AMP; however, most other tissues contain a much less amount of cyclic GMP than cyclic AMP (5, 20). It also was demonstrated that cholinergic agonists increase cyclic GMP levels in lung slices (21). The activity of guanylate cyclase was also much higher in lung than other tissues (22–24). In our preliminary study (25), the occurrence of a cyclic GMP-dependent protein kinase in rat lung was confirmed. Based on various lines of evidence cited above, in the present study, purification of a cyclic GMP-dependent protein kinase from pig lung was attempted. This paper reports the partial purification and some properties of the enzyme.

**EXPERIMENTAL PROCEDURE**

**Materials and Chemicals**—Fresh pig lung was obtained from the slaughterhouse. [γ-32P]ATP was prepared by the method of Glynn and Chappel (20). Cyclic [γ-32P]AMP was purchased from New England Nuclear; cyclic [8-14C]GMP, from the Radiochemical Centre. Calf thymus histone (type II), cyclic GMP (free acid), cyclic AMP (sodium salt), and ATP (magnesium salt) were obtained from Sigma; DEAE-cellulose (DE52) was from Whatman. Other chemicals were obtained from commercial sources.

**Protein Kinase Assay**—The standard reaction mixture (0.20 ml) contained 1 μmol of potassium phosphate at pH 7.0, 0.5 μmol of theophylline, 2 μmol of sodium fluoride, 10 μmol of magnesium acetate, 5 nmol of [γ-32P]ATP (20 to 60 × 10⁴ cpm/nmol), 150 μg of calf thymus histone, and an enzyme preparation. Cyclic nucleotides (50 nM) were added where indicated. Incubation was carried out for 5 min at 30° in a shaking water bath. The reaction was terminated and the phosphorylated histone was precipitated by the addition of 4 ml of 5% trichloroacetic acid containing 0.25% sodium tungstate, pH 2.0; 0.2 ml of 0.63% bovine serum albumin was added as a carrier protein. The precipitated protein was collected by centrifugation and was dissolved in 0.1 ml of 1 N NaOH, then 2 ml of the trichloroacetic acid·sodium tungstate solution were added, and protein was repurified by precipitation with 0.1 M NaOH of 1.2 N H₂SO₄. This procedure (12) was repeated twice more.² The protein was finally collected by centrifugation, then dissolved in 0.1 ml of 1 N NaOH, and its radioactivity was determined in a Beckman liquid scintillation spectrometer, LS-233, with a scintillator solution consisting of 4 g of 2,5-diphenyloxazole, 100 mg of 1,4-bis[2-(5-phenyloxazolyl)] benzene, and 0.5 liter of Triton X-100 in a liter of toluene (27).

**Cyclic Nucleotide-binding Protein Assay**—Cyclic nucleotide binding protein was assayed by measuring the binding of radioactive cyclic nucleotide with a Millipore filter (pore size, 0.45 μ) by a slight modification of the method described by Gilman (28). The standard assay mixture (80 μl) contained 4 μmol of sodium acetate at pH 4.0, 3 pmol of cyclic [γ-32P]AMP or cyclic [8-3H]GMP (2.5 × 10⁴ cpm/pmol), and a protein preparation to be assayed. Reactions were initiated by addition of protein preparations and were allowed to proceed for 60 min at 0°. Subsequently the mixtures were diluted approximately 1 ml with cold 20 mM potassium phosphate at pH 6.0 and were passed through a Millipore filter previously rinsed with the same buffer. The filter was immediately washed with 10 μl of this buffer, placed in the counting vial, and dried. The radioactivity was determined as described above.

²Control experiments showed that a third precipitation with trichloroacetic acid caused no further reduction in the amount of radioactivity per mg of protein.
RESULTS

Partial Purification of Cyclic GMP-dependent Protein Kinase—Pig lungs were placed on ice immediately upon their removal from slaughtered animals. All subsequent manipulation were carried out at 0-4°C. After removing connective and fat tissues as well as resectable trachea, 200 g of pig lung were diced and homogenized in portions with 5 volumes of 0.25 M sucrose in Buffer A (10 mM potassium phosphate at pH 7.0 containing 50 mM 2-mercaptoethanol, 2 mM EDTA, and 10% glycerin) using a Polytron PT 20 for 2 min at maximum speed. The homogenate was centrifuged for 30 min at 20,000 x g. The resultant supernatant solution (930 ml) was adjusted to pH 5.5 by the dropwise addition of 1 N acetic acid with stirring. After stirring for 15 min, the acidified solution was centrifuged at 20,000 x g for 15 min and the supernatant containing the major portion of the cyclic AMP-dependent protein kinase was discarded. The precipitate was dissolved in 700 ml of Buffer A using a Potter-Elvehjem Teflon-glass homogenizer. The enzyme solution was readjusted to pH 7.0 with 1 M dibasic potassium phosphate with stirring. After stirring for 20 min, the precipitate was removed by centrifugation at 30,000 x g for 25 min. The supernatant solution (2.9 g of protein) was applied to a DEAE-cellulose (DE52) column (20 x 3.5 cm) equilibrated with Buffer A. After washing the column with 1 liter of the same buffer, protein was eluted from the column by the stepwise application of 600 ml each of 30 and 60 mM ammonium sulfate in Buffer A, and 10-ml fractions were collected. By subjecting each fraction to the protein kinase assay in the presence of either cyclic GMP or cyclic AMP, three activity peaks were detected corresponding to the peaks of cyclic nucleotide-binding activity and optical density at 280 nm (Fig. 1). The protein kinases in the first two peaks were slightly stimulated by the addition of either cyclic AMP or cyclic GMP. The protein kinase in the third peak, eluted by the 60 mM ammonium sulfate in Buffer A, was stimulated greatly by the addition of cyclic GMP, whereas only slight stimulation was observed by cyclic AMP under the standard assay conditions. The major portion of cyclic GMP-binding protein was located in the third peak, in conformity with the protein kinase activity stimulated by cyclic GMP. The protein kinase in the third peak was subjected to further purification as follows. The enzyme in the third peak (Fractions 77 through 86) was precipitated by the addition of solid ammonium sulfate (0.34 g/ml). The precipitate was collected by centrifugation for 20 min at 20,000 x g and dissolved in 5 ml of Buffer A. This enzyme preparation was applied to a Sephadex G-200 column (90 x 2.7 cm) equilibrated previously with Buffer A. Elution was performed with the same buffer at a flow rate of 10 ml/hour and 3-ml fractions were collected. As shown in Fig. 2, the minor peak and subsequently the major peak of protein kinase activity were detected in the presence of either cyclic GMP or cyclic AMP. The major peak which had clearly separated from the main protein peak, was stimulated remarkably by the addition of cyclic GMP with a coincidence of cyclic GMP-binding activity. Cyclic GMP stimulated the protein kinase activity of the major peak more than 10 times greater than cyclic AMP. The tracer amount of cyclic AMP-dependent protein kinase was removed with the main protein peak.

"A considerable amount of cyclic AMP-dependent protein kinase was further eluted with higher salt concentration from the column."
TABLE I
Summary of purification of cyclic GMP-dependent protein kinase from pig lung

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume</th>
<th>Protein</th>
<th>Specific activity in the presence of:</th>
<th>Total activity with cyclic GMP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ml</td>
<td>mg</td>
<td>No addition</td>
<td>Cyclic AMP</td>
</tr>
<tr>
<td>Homogenate</td>
<td>1,240</td>
<td>46,500</td>
<td>25</td>
<td>30</td>
</tr>
<tr>
<td>20,000 x g supernatant</td>
<td>950</td>
<td>14,000</td>
<td>46</td>
<td>57</td>
</tr>
<tr>
<td>pH 5.5 precipitate</td>
<td>640</td>
<td>2,880</td>
<td>121</td>
<td>150</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>94</td>
<td>137</td>
<td>191</td>
<td>282</td>
</tr>
<tr>
<td>Sephadex G-200</td>
<td>18</td>
<td>15.3</td>
<td>411</td>
<td>835</td>
</tr>
</tbody>
</table>

FIG. 3 (left). Protein kinase activity as a function of incubation time. The purified enzyme (45 µg) was employed for this experiment. Protein kinase activity in the presence of cyclic AMP (○—○) and cyclic GMP (●—●), and in the absence of cyclic nucleotide (▲—▲).

FIG. 4 (right). Protein kinase activity as a function of enzyme concentration. The standard assay conditions were employed except for the variation in enzyme concentration. Protein kinase activity in the presence of cyclic GMP (○—○) and cyclic AMP (▲—▲), and in the absence of cyclic nucleotide (▲—▲).

as shown in Fig. 4. The enzyme activity showed a broad pH optimum over the range of pH 6.5 to 7.8, using potassium phosphate buffers with different pH values under the standard assay conditions. The apparent Kₐ value for ATP, determined at various concentrations (1.25 to 12.5 µM), was calculated from the Lineweaver-Burk plot to be 1 x 10⁻⁸ M in the presence of 50 nM cyclic GMP.

Effects of Cyclic Nucleotides and Divalent Metal Ions—The relative ability of various concentrations of cyclic GMP and cyclic AMP to stimulate the protein kinase activity was illustrated in Fig. 5. The enzyme was specifically activated by cyclic AMP at lower concentrations; however, at higher concentrations it also was activated by cyclic AMP to a certain extent. The apparent Kₐ value for cyclic AMP was 17 nM, whereas Kₐ for cyclic GMP was 360 nM, which is about 20 times greater than that for cyclic GMP. With the lower concentration (10 mM) of Mg²⁺, the apparent Kₐ for cyclic GMP was 22 nM, and Kₐ for cyclic AMP was 280 nM. These results are similar to those reported by Kuo (30) with the enzyme obtained from guinea pig lung. When maximal concentrations of cyclic GMP and cyclic AMP were added together, no additive effect was obtained.

To examine the degradation of cyclic nucleotides during the assay, each radioactive cyclic nucleotide in final concentration of 50 nM was incubated with the purified enzyme (50 µg) for 5 min at 30°C under the standard conditions for protein kinase assay. Then, the mixture was diluted with 1 ml of cold 20 mM sodium acetate buffer, pH 4.0, together with 100 pmol of nonradioactive cyclic nucleotide as carrier, and applied on the neutral aluminum oxide-Dowex 1-X2 column as described elsewhere (24). One hundred percent of cyclic [³H]AMP and more than 90% of cyclic [³H]GMP were recovered. The results indicated that cyclic AMP was not preferentially degraded during protein kinase assay.

The effect of Mg²⁺ concentrations on the enzyme activity was shown in Fig. 6, and the maximum activity was obtained at about 50 mM Mg²⁺. Double reciprocal plots of Mg²⁺ concentration against histone phosphorylation exhibited a definite biphasic kinetic curve. Phosphate in higher concentration (50 mM) had no effect on the stimulation of the enzyme activity by high Mg²⁺ concentration.

Upon testing the effects of other divalent cations on the histone phosphorylation activity, neither Mn²⁺ nor Ca²⁺ could substitute for Mg²⁺ in the presence of cyclic GMP. However, as shown in Fig. 7, these divalent cations, especially Mn²⁺, markedly inhibited histone phosphorylation stimulated by cyclic GMP in the presence of 50 mM Mg²⁺. Table II shows the concentrations of Mn²⁺ and Ca²⁺ required for 50% inhibition on histone phosphorylation in the presence of two different concentrations of Mg²⁺ with cyclic GMP. These data suggest the inhibitory effects of Mn²⁺ or Ca²⁺ seem to be more predominant at lower Mg²⁺ concentration. The addition of either Mn²⁺ (1.5 mM) or Ca²⁺ (15 mM) did not increase the degradation of cyclic nucleotides during protein kinase assay.

DISCUSSION

The experiments presented in this paper clearly demonstrate the presence of a cyclic GMP-dependent protein kinase in the
soluble fraction of pig lung which is capable of phosphorylating histone. Recently, Kuo also described the occurrence of high levels of cyclic GMP-dependent protein kinase in mammalian tissues using the assay mixture with protein kinase modulator (30). The previous failures in isolating this type of protein kinase in mammalian tissues may be due to the relative instability together with its poor yield. The major advantage of this new purification procedure is the step of the DEAE-cellulose column chromatography following pH 5.5 precipitation. It provides the rapid and definite isolation of a cyclic GMP-dependent protein kinase from mammalian tissues (Fig. 1).

Since it has been reported that the cyclic AMP-dependent protein kinase is susceptible to proteolysis during enzyme isolation (31, 32), it cannot be ruled out that the apparent cyclic GMP-dependent protein kinase activity might arise from some modification of the cyclic AMP-dependent kinase. However, in the present study, such evidence that the present cyclic GMP-dependent protein kinase might be converted from the cyclic AMP-dependent protein kinase has not been obtained.

The change in slope in the double reciprocal plots of Mg2+ concentration against histone phosphorylation (Fig. 5) could be arbitrarily interpreted in many ways. Two enzymes having different affinities for Mg2+ may present, or negative cooperative effects may act in such a fashion that Mg2+ is less strongly bound as the enzyme becomes more saturated with this cation. Alternately, there may be the involvement of a heat-stable protein factor as reported by Donnelly et al. (33). The mechanism of inhibitory effects of Mn2+ and Ca2+ on the enzyme activity in the presence of Mg2+ (Table II) is also difficult to interpret. In order to elucidate these mechanisms, a more highly purified enzyme preparation seems to be required.

Acknowledgments—We are grateful to Drs. Y. Nishizuka, H. Yamamura and K. Nishiyama, Kobe University School of Medicine, for making their unpublished manuscript available to us and for stimulating discussions. The technical assistance of Miss Yasuko Isogai is gratefully acknowledged.

REFERENCES
