Functional Specificity of Guanosine 3':5'-Monophosphate-dependent and Adenosine 3':5'-Monophosphate-dependent Protein Kinases from Silkworm*

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KAORU NISHIYAMA,† HIDEKI KATAKAMI, HIROHEI YAMAMURA, YOSHIKI TAKAI, REIKO SHIMOMURA,§ AND YASUTOMI NISHIZUKA

From the Department of Biochemistry, Kobe University School of Medicine, Kobe, Japan

SUMMARY

Adenosine 3':5'-monophosphate-dependent protein kinase partially purified from silkworm pupae shows identical functional activities with those of mammalian protein kinases; the insect and mammalian kinases are completely exchangeable in the phosphorylation of muscle glycogen phosphorylase kinase and glycogen synthetase resulting in the activation and inactivation of the respective enzymes. In contrast, guanosine 3':5'-monophosphate-dependent protein kinase obtained from the same organism is totally inactive in this role and phosphorylates different, mainly seryl and some threonyl, residues of acceptor proteins. Substrates of the latter kinase intimately involved in the regulation of biological processes have remained unknown.

The role of cyclic AMP as an intracellular mediator of hormone action has been well documented (1), and various effects elicited by the cyclic nucleotide are proposed to be mediated through activation of this class of enzymes (2, 3). Recently, insulin (4), acetylcholine (5), and mitogenic agents such as plant lectins (6) 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 and purified partially from lobster tail muscle (7, 8). More recently, this type of protein kinase was found also in mammalian tissues, such as brain (9, 10), pancreas (11), bladder, and uterine tissue (9). However, no entirely distinct pattern of differences of cyclic GMP-dependent and cyclic AMP-dependent protein kinases has been described, although the existence of the two classes of enzyme does serve to strengthen the possibility of their different roles in biological systems. In the present studies functional specificity of these enzymes obtained from silkworm pupae was investigated, and the results indicate that the cyclic AMP-dependent enzyme apparently lacks species specificity in its catalytic activity and shows closely similar properties to those of mammalian enzymes, whereas the cyclic GMP-dependent enzyme belongs to another entity with different substrate specificity. Partial purification together with some kinetic and physical properties of these two enzymes is also briefly described.

EXPERIMENTAL PROCEDURE

Materials and Chemicals—The cocoons of silkworm (Bombyx mori) were obtained from a cocoonery and stored at -20°C until use. Rat liver cyclic AMP-dependent protein kinase (protein kinase B) was prepared as described previously (12). Crystalline rabbit skeletal muscle glycogen phosphorylase b was purchased from Boehringer Mannheim. Rabbit skeletal muscle glycogen phosphorylase kinase and glycogen synthetase were prepared by the method of Cohen (13) and Villar-Palasi et al. (14), respectively. Calf thymus histone was prepared by the method of Johns (15). Salmon sperm protamine (lot 268 8000) was obtained from Sigma. Calcium phosphate gel-cellulose was prepared by the method of Gley and Chappel (17). Cyclic [8-3H]AMP (7.7 Ci per mmol) was purchased from Daiichi Pure Chemicals; cyclic [8-3H]GMP (13 Ci per mmol) from Radiochemical Centre. The radiochemical purity of these materials was examined by thin layer chromatography before use. Other chemicals were obtained from commercial sources.

Enzyme Assay—Protein kinase activity was routinely assayed with calf thymus histone as substrate under the conditions specified previously (12, 18). Where indicated, cyclic nucleotide was added in a final concentration of 4 X 10^{-8} M. Cyclic nucleotide-binding protein was assayed by measuring the binding of radioactive cyclic nucleotides with a Millipore filter ( pore size, 0.45μm) under the conditions described previously (12). The activation of glycogen phosphorylase kinase and inactivation of glycogen synthetase by protein kinase was examined as described in a preceding paper (18).

Determinations—The radioactivity of 3P and 3H samples was determined with a Nuclear-Chicago Geiger Muller gas flow counter, model 4388, and a Packard Tri-Carb liquid scintillation spectrometer, model 3320, with Bray's solution (19), respectively. Protein was determined by the method of Lowry et al. (20) with bovine serum albumin as a standard.

Other Procedures—Acid hydrolysis and trypic digestion of enzymatically fully phosphorylated histone preparations were car-
ried out as described previously (12). The relative ratio of phosphoserine and phosphothreonine was determined by measuring the radioactivity after separating them with a Dowex 50 (H+ form) column by the method of Schaffer et al. (21). Autoradiography of radioactive trypsic peptides was made as described previously (22). Isoelectrofocusing electrophoresis and estimation of molecular weight of proteins by gel filtration were performed under the conditions specified earlier (18).

RESULTS

Partial Purification of Protein Kinases—The naked pupae (50 g) were homogenized in a Waring Blender for 2 min at maximum speed with 3 volumes of 10 mM phenylmethylsulfonyl fluoride in Buffer A (20 mM potassium phosphate at pH 7.0 containing 6 mM 2-mercaptoethanol, 4 mM EDTA, and 5 mM magnesium acetate). All operations were performed at 0–4°. Cell debris was removed by filtration through three layers of gauze, and the filtrate was centrifuged for 20 min at 27,000 × g. The supernatant solution was filtered through a glass wool to remove fat. To the filtrate (150 ml), ammonium sulfate (56.5 g) was added to a final concentration of 60% saturation. After centrifugation for 20 min at 20,000 × g, the precipitate was dissolved in 50 ml of Buffer A, and dialyzed overnight against a large volume of the same buffer. The enzyme solution (57 ml) was applied to a calcium phosphate gel-cellulose column (13 X 3 cm) equilibrated with Buffer A. After the column was washed with 300 ml of the same buffer, the elution was carried out with a 1000-ml linear gradient from 20 mM to 400 mM potassium phosphate at pH 7.0 containing 6 mM 2-mercaptoethanol, 4 mM EDTA, and 5 mM magnesium acetate. Fractions of 15 ml each were collected. When each fraction was assayed for protein kinase activity in the presence of either cyclic AMP or cyclic GMP, two major peaks of protein kinase appeared (Fig. 1). The protein kinase in the first peak (tubes 30 through 38) was stimulated greatly by the addition of cyclic GMP, and practically no stimulation was observed by cyclic AMP under the standard assay conditions. This protein kinase is referred to hereafter as protein kinase G. In contrast, the enzyme in the second peak (tubes 54 through 70) was activated by cyclic AMP but not by cyclic GMP. The protein kinase is referred to as protein kinase A.

Each protein kinase was dialyzed overnight against a large volume of Buffer B (20 mM Tris-HCl at pH 7.5 containing 6 mM 2-mercaptoethanol, 4 mM EDTA, and 5 mM magnesium acetate), and applied separately to a DE-52 column (15 × 2 cm) equilibrated with Buffer B containing 20 mM NaCl. After washing with 100 ml of the saline-buffer, elution was performed with a 400-ml linear concentration gradient of NaCl (20 mM to 300 mM) in Buffer B, and fractions of 10 ml each were collected. Protein kinase G was eluted at about 120 NaCl as a single sharp peak which coincided exactly with cyclic GMP-binding protein. Under the same conditions protein kinase A was eluted at about 150 NaCl as a peak which coincided with cyclic AMP-binding protein.

By the procedure described above both protein kinase G and A were purified at least 70- to 100-fold with an over-all yield of, all together, approximately 30% of the original total activity. These preparations were free of endogenous phosphate acceptor under the conditions for the standard assay.

Kinetic and Physical Properties of Protein Kinase G and A—Table I summarizes the properties of protein kinase G and A in comparison with rat liver enzyme. Protein kinase G was specifically activated by cyclic GMP at lower concentrations, although the enzyme was also sensitive to cyclic AMP at higher concentrations (Fig. 2A). Similarly, protein kinase A was relatively specific for cyclic AMP at lower concentrations, but responded also to cyclic GMP at higher concentrations (Fig. 2B). These observations are similar to those described by Kuo and Greengard (7) for the enzymes obtained from other species of arthropods. Fig. 2C shows the results obtained with rat liver enzyme for comparison. The apparent Km values, the concentrations needed for half-maximum stimulation, of these protein kinases for the cyclic nucleotides are given in the table.

Table I

| Summary of properties of silkworm and rat liver protein kinases |
|------------------|------------------|
|                  | Silkworm         | Rat liver protein kinase |
| Protein kinase G | Protein kinase A |                     |
| Km value for ATP | 3.3 × 10⁻⁵ M     | 5 × 10⁻⁵ M            | 5 × 10⁻⁵ M            |
| Kα value for     |                  |                      |                     |
| Cyclic GMP       | 7.5 × 10⁻⁹ M     | 4 × 10⁻⁹ M           | 1.5 × 10⁻⁹ M         |
| Cyclic AMP       | 2 × 10⁻⁴ M       | 1.3 × 10⁻⁴ M         | 2 × 10⁻⁴ M           |
| Optimum Mg²⁺ ion | 50-100 mM        | 3.0 mM               | 3.0 mM               |
| Optimum pH       | pH 7-8           | pH 7-8               | pH 7                 |
| Isoelectric point | pH 5.4           | pH 5.4               | pH 5.2               |
| Molecular weight | 110,000          | 189,000              | 189,000              |

* The data are taken from Refs. 12 and 18.

* The data represent values for holoenzymes.
FIG. 2. Effects of varying concentrations of cyclic GMP and cyclic AMP on protein kinases from silkworm and rat liver. ●●●● and ○○○○, protein kinase activity with cyclic GMP and cyclic AMP, respectively. A, protein kinase G; B, protein kinase A; C, rat liver protein kinase.

Both protein kinase G and A showed an absolute requirement for a divalent metal ion; either Mg\(^{2+}\), Mn\(^{2+}\), or Co\(^{2+}\) supported enzymatic activity. It may be pointed out that protein kinase G was most active in the presence of 50 to 100 mM Mg\(^{2+}\). Protein kinase G and A showed slightly different Ka values for ATP, but revealed an identical isoelectric point of pH 5.4. The molecular weight of protein kinase G was smaller than that of protein kinase A. It may be noted that protein kinase A exhibited almost identical properties to those of the mammalian enzyme.

Catalytic Properties of Protein Kinase G and A—Both protein kinase G and A phosphorylated histone and protamine; protamine was 30% more active than histone for protein kinase G, whereas, similar to mammalian enzymes, it was 20% less active than histone for protein kinase A in the reaction rate. Acid hydrolysis of each histone preparation fully phosphorylated by protein kinase G or A resulted in the formation of mainly phosphoserine. Quantitative analysis revealed that the amount of phosphothreonine produced by protein kinase G and A was 1.3% and 8%, respectively, of that of phosphoserine. Under the same conditions rat liver enzyme gave the value of 6.5%.

In another set of experiments the radioactive histone preparations were subjected to tryptic digestion and, subsequently, to paper chromatography followed by high voltage paper electrophoresis. As illustrated in Fig. 3, A and B, different patterns of radioactivity peaks were obtained for protein kinase G and A. The pattern obtained for protein kinase A was closely similar but not exactly identical with that obtained for rat liver enzyme (Fig. 3C). Nevertheless, the insect and mammalian cyclic AMP-dependent enzymes were functionally identical as described below. Under these conditions the histone preparations were equally digested to produce identical sets of more than 30 peptide spots visualized by the ninhydrin reaction.

Functional Dissimilarity of Protein Kinase G and A—The experiments shown in Fig. 4 were designed to show that protein kinase G and A were functionally distinct enzymes. When protein kinase A was incubated with muscle glycogen phosphorylase kinase and glycogen phosphorylase b in the presence of ATP, the phosphorylase activity was enhanced significantly as judged by the formation of glucose 1-phosphate during the subsequent incubation with glycogen and radioactive inorganic orthophosphate. The results clearly indicate that the efficiency of protein kinase A in the activation of phosphorylase system was...
essential identical with that of rat liver cyclic AMP-dependent protein kinase, whereas protein kinase G was totally inactivated in this capacity. When ATP was omitted from the reaction mixture, practically no radioactive phosphate was incorporated into glucose 1-phosphate. Some activity observed in the absence of protein kinase was probably due to autocatalytic phosphorylation of glycogen phosphorylase kinase as described by Krebs (24), or due to the activated form of phosphorylase kinase which slightly contaminated the preparation.

In a similar set of experiments muscle glycogen synthetase was shown to be inactivated progressively by the preincubation with ATP and increasing amounts of protein kinase A as well as rat liver enzyme in a parallel fashion. Neither protein kinase nor ATP alone was active in the conversion of I-form to D-form of glycogen synthetase. Under the same conditions protein kinase G was again totally inactive.

**DISCUSSION**

Although cyclic GMP-dependent protein kinase has been found together with cyclic AMP-dependent enzyme in various species of arthropods and mammals (7-11), there appears to be a wide variation in the relative distribution of these two enzymes among different tissues of a species and among similar tissues of different species (25). In addition, a heat-stable protein factor has been reported by Donnelly et al. (26) which stimulates cyclic GMP-dependent enzyme, but inhibits cyclic AMP-dependent enzyme. This factor is also shown to alter the effectiveness of several histone species as substrates for both cyclic GMP-dependent enzyme from this organism. Of these two classes of protein kinases, increasing the phosphorylation of some acceptor proteins but decreasing that of other proteins in variable ratios (27). However, no substrate other than histone and protamine has been identified for cyclic GMP-dependent enzyme. Nevertheless, based on such variation of specificity for substrate proteins, coupled with relative differences of divalent cation requirements, it has been postulated that these two classes of protein kinase are probably different and, therefore, serve different physiological roles in biological systems (25). The present studies support this proposition and clearly establish that cyclic GMP-dependent and cyclic AMP-dependent protein kinases obtained from a species like silkworm appears to belong to an entirely different entity. However, species and tissue specificities of the latter class of enzyme as well as its natural substrate proteins intimately involved in the regulation of biological processes are inevitable for further investigations.

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