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PRESENCE OF MULTIPLE PROTEIN KINASE ACTIVITIES IN *COPRINUS MACRORHIZUS*

ISAO UNO and TATSUO ISHIKAWA

Institute of Applied Microbiology, University of Tokyo, Bunkyo-ku, Tokyo (Japan)

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SUMMARY

Protein kinase (ATP:protein phosphotransferase, EC 2.7.1.37) activity was found in crude extracts from monokaryotic mycelia of a basidiomycete, *Coprinus macrorhizus*. The enzyme activity of crude extracts was significantly inhibited by the addition of cyclic AMP and was influenced by the culture age of mycelia.

Sepharose 6B chromatography resolved the protein kinase activity into four peaks designated as Peak I, II, III and IV. The activity of Peak II was stimulated in vitro by cyclic AMP, and that of Peaks I and III was inhibited by cyclic AMP. The activity of Peak IV was independent of cyclic AMP. The pH optima of these enzymes were found to be 6.5–7.0. The activities of these enzymes were promoted by Mg^{2+} and Mn^{2+}, and were partially inhibited by Cu^{2+} and Zn^{2+}.

INTRODUCTION

The phosphorylation of nuclear proteins has been suggested to play a role in the control of various cellular functions [1–3]. The occurrence of protein kinase (ATP:protein phosphotransferase, EC 2.7.1.37) which is activated by adenosine 3′:5′-monophosphate (cyclic AMP) has been found by Walsh et al.[4] in rabbit skeletal muscle and similar enzymes have since been reported to be present in many different eukaryotic and prokaryotic cells [5–11]. It is now believed that cyclic AMP-activated protein kinases are receptors for cyclic AMP in several tissues and are responsible for various biological effects of this nucleotide [12–15]. On the other hand, protein kinases which were independent of cyclic AMP [16] or inhibited by cyclic AMP [11] were also found, although the biological roles of these are virtually unknown.

We have demonstrated that the strains of *Coprinus macrorhizus* which are able to form fruiting bodies produced adenyl cyclase and cyclic AMP [17, 18]. It was therefore inferred that cyclic AMP acts as an intracellular inducer to form fruiting bodies. The investigation of protein kinases which may occur in the course of fruiting body formation is important for understanding the control of this process in this fungus. This communication describes that there are three types of protein kinases in crude extracts of monokaryotic mycelia of *C. macrorhizus*.

MATERIALS AND METHODS

Organism and culture medium

The monokaryotic mycelia of the *fis*^{c} *A8B7* strain of *C. macrorhizus* Rea f. *microsporus* Hongo were used. The origin and characteristics of this strain have been described elsewhere [19]. All cultures were grown on a malt-yeast medium which contained 10 g of malt extract, 4 g of yeast extract and 4 g of glucose per l of deionized water.

Preparation of cell-free extracts

Mycelial suspensions were inoculated and incubated in 100-ml Erlenmeyer flasks containing 20 ml of malt–yeast medium at 30 °C for 5–14 days under continuous illumination (2000 ergs·cm^{-2}·s^{-1}). Mycelia, fruiting body primordia, and fruiting bodies were harvested on a filter paper, washed well with distilled water, and macerated in 0.05 M Tris–HCl buffer (pH 7.4) with a Waring blender for 5 min. The suspension obtained was further homogenized with a Braun homogenizer for 3 min. The resulting homogenate was centrifuged at 1000 × *g* for 20 min. The supernatant fluid was used as the crude extract.

Fractionation procedures

All operations were performed at 4 °C. A 100-ml amount of crude extract was further centrifuged at 15 000 × *g* for 20 min and 72 g of $(NH_4)_2SO_4$ was added to the supernatant fluid. The precipitate was collected by centrifugation and dissolved in 10 ml of 0.05 M Tris–HCl buffer (pH 7.4). After removing the precipitate by centrifugation, a 5-ml amount of the supernatant fluid was applied on a Sepharose 6B column (2.5 cm × 40 cm) and eluted with the same buffer into 3-ml fractions. The flow rate of buffer was 12–15 ml/h.

Assay for protein kinase

The standard reaction mixture (0.2 ml) containing 10 μmoles sodium acetate buffer (pH 6.5), 1.5 nmole [γ-^{32}P]ATP, 2 μmoles $MgSO_4$, 0.4 mg histone, 0.2 nmole cyclic AMP, and enzyme sample was incubated at 30 °C for 5 min. The reaction was initiated by addition of [γ-^{32}P]ATP and terminated by addition of 4 ml of 4% trichloroacetic acid. As a carrier protein 0.2 ml of 0.63% bovine serum albumin was added to the reaction mixture. The reaction mixture was allowed to stand for 5 min at 4 °C, and was then centrifuged. The precipitate was dissolved in 0.1 ml of 1 M NaOH, and the solution was reprecipitated by addition of 2 ml of 5% trichloroacetic acid. This procedure was repeated twice. The protein-bound ^{32}P was determined by a liquid scintillation spectrometer. One unit of protein kinase activity is defined as the amount of enzyme which catalyzes the incorporation of 1 pmole of ^{32}P into protein in 1 min. The activity was corrected for the phosphorylation of endogenous substrates.

Protein measurement

Protein concentration was determined by the method of Lowry et al. [20].

Chemicals

Malt extract was a product of Difco, and yeast extract was purchased from Kyokuto. Calf thymus histone (Type II-A) and bovine serum albumin were obtained from Sigma Chemical Co., and casein was purchased from Merk. The [γ-^{32}P]ATP was prepared by the method of Glynn and Chappell [21]. Other special chemicals used were as follows: Sepharose 6B and Blue Dextran (Pharmacia), cyclic AMP and ATP (Sigma Chemical Co.).

RESULTS

Effects of cyclic AMP on the protein kinase activity in crude extracts

The phosphorylation of histone, bovine serum albumin and casein by a crude extract of *fis*^{c} *A8B7* mycelia grown for 8 days was observed (Table I). Cyclic AMP (1 μM) had an inhibitory effect on the reaction. The phosphorylation measured in the absence of a particular substrate was at a significant level, so that the results in the following experiments were corrected for the phosphorylation of proteins contained in the enzymatic preparation.

TABLE I

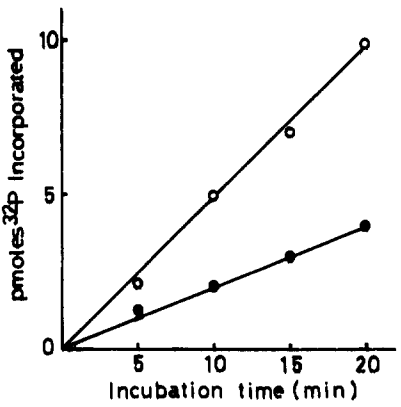
PROTEIN PHOSPHORYLATION BY CRUDE EXTRACT UNDER THE DIFFERENT SUBSTRATE CONDITIONS

Each protein substrate and the no-substrate control were assayed for protein kinase activity with crude extract from *fis*^{c} *A8B7* mycelia grown for 8 days in the absence and presence of 1 μM cyclic AMP.

Substrate	Substrate concn (mg/ml)	Protein kinase activity (units)	
		Without cyclic AMP	With cyclic AMP
None	—	10.3	8.5
Calf thymus histone	2	29.6	25.0
Bovine serum albumin	2	19.5	15.2
Casein	2	20.5	16.3

The initial rate of histone phosphorylation remained approximately constant under the conditions of incubation for 20 min in both the presence and absence of cyclic AMP (Fig. 1). The inhibition of the protein kinase activity was dependent on the concentration of cyclic AMP, but high concentrations of cyclic AMP did not result in the complete inhibition (Fig. 2), suggesting the existence of cyclic AMP-stimulated or independent protein kinases.

The protein kinase activity in crude extracts was lower at the earlier stages of growth and increased in the course of fruiting body formation. The activity of the same enzyme samples was lower in the presence of 1 μM cyclic AMP than in the absence of it (Fig. 3).



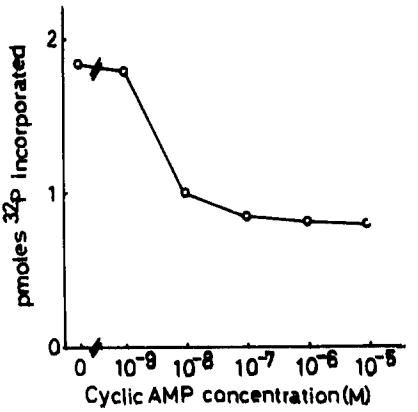


Fig. 1. Effect of cyclic AMP on the time course of phosphorylation reactions catalyzed by a crude extract of *fis*^{c} *A8B7* mycelia grown for 8 days. The reactions were carried out with histone as substrate in the presence (●—●) and absence (○—○) of 1 μM cyclic AMP.

Fig. 2. Effect of varying the concentration of cyclic AMP on the assay of protein kinase in a crude extract of *fis*^{c} *A8B7* mycelia grown for 8 days. The reactions were carried out as described in Materials and Methods except for a variation in the cyclic AMP concentration.

Fractionation of different protein kinase activities in mycelial extracts

The crude extract of *fis*^{c} *A8B7* mycelia grown for 8 days was chromatographed on a Sepharose 6B column. When fractions were assayed for protein kinase activity in the presence and absence of 1 μM cyclic AMP, at least four peaks of the enzyme

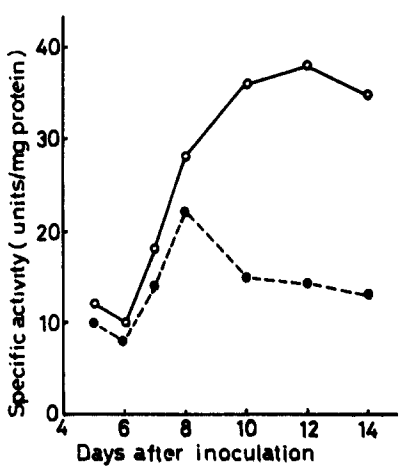


Fig. 3. Protein kinase activity in crude extracts of *fis*^{c} *A8B7* mycelia grown for 5–14 days. The reactions were carried out with histone as substrate in the presence (●---●) and absence (○—○) of 1 μM cyclic AMP.

activity appeared (Fig. 4). The enzyme in the first peak (Peak I) was partially inhibited by the addition of cyclic AMP; a large amount of activity was observed in the absence of cyclic AMP. The enzyme in the second peak (Peak II) was active only in the presence of cyclic AMP. In contrast, the enzyme in the third peak (Peak III) was

significantly inhibited by the addition of cyclic AMP. The enzyme in the last peak (Peak IV) showed no significant response to cyclic AMP; essentially similar activity was observed whether or not cyclic AMP was present.

The effect of various concentrations of cyclic AMP was examined on the protein kinase activity of four peaks shown in Fig. 4 (Fig .5). A low concentration of

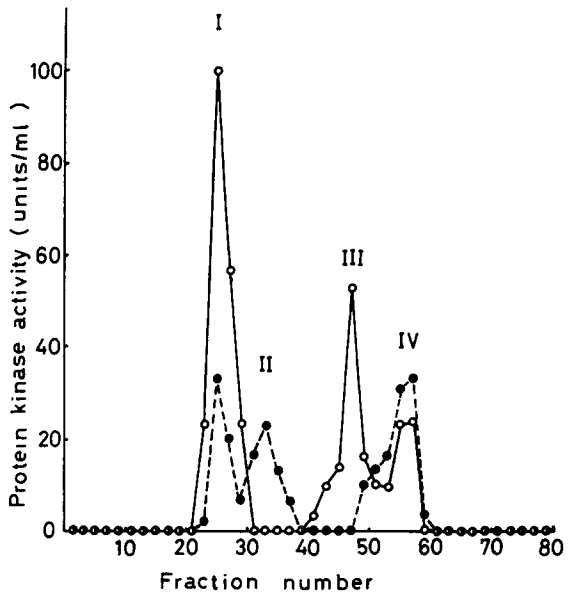


Fig. 4 Sepharose 6B chromatography of protein kinases in the extract of *fis*^{c} *A8B7* mycelia grown for 8 days. The $(NH_4)_2SO_4$ fraction was applied to the column as described in Materials and Methods. Each fraction was assayed for protein kinase activity with histone as substrate in the presence (●---●) and absence (○—○) of 1 μM cyclic AMP.

cyclic AMP (10 nM) was effective in stimulating the protein kinase activity in Peak II and the level of stimulation was 7-fold above that of the control lacking cyclic AMP. However, the same concentration of cyclic AMP was effective in inhibiting the activities in Peaks I and III and the levels of inhibition varied from 70 to 80% of the control, lacking cyclic AMP. The uninhibitable activity may be contaminating activity from the cyclic AMP-stimulated or independent enzymes. The protein kinase activity of Peak IV was independent of the presence of cyclic AMP.

Preliminary surveys on the pH optimum and on the effects of divalent cations on the protein kinase activities of four enzymes were performed. The activities of all four enzymes were most active at pH 6.5–7.0, and no significant difference in the pH optimum was observed among them. The basal activities of four enzymes obtained in the absence of added ions increased up to 230–270% by adding Mg^{2+} or Mn^{2+} (2 μM) to the reaction mixtures and up to 150–170% by adding Fe^{2+} or Ca^{2+} (2 μM). In contrast, Cu^{2+} and Zn^{2+} (2 μM) inhibited the basal activities of four enzymes by 50–70%. No significant difference in the requirement for divalent cations was observed among four enzymes.

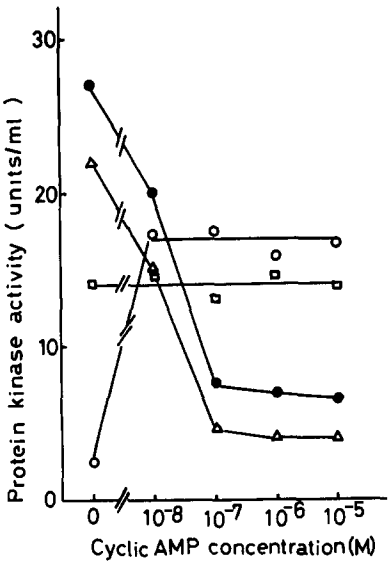


Fig. 5. Response of the Sepharose fractions to various concentrations of cyclic AMP. The fractions representing Peaks I–IV described in Fig. 4 were used as the enzyme samples. The reactions were carried out as described in Materials and Methods except for varying the cyclic AMP concentration. ●—●, Peak I (Tubes 22–28); ○—○, Peak II (Tubes 31–34); △—△, Peak III (Tubes 45–48); □—□, Peak IV (Tubes 55–58).

DISCUSSION

The results obtained in this report confirm that protein kinase activity is present in the crude extracts of monokaryotic mycelia of *C. macrorhizus*. The levels of protein kinase activity of crude extracts were dependent on the culture age of mycelia. The enzyme activity was significantly inhibited by the addition of cyclic AMP, but no complete inhibition was attained with high concentrations of cyclic AMP. The results suggested that the crude mycelial extract contains multiple forms of protein kinase. The fractionation of crude mycelial extract by Sepharose 6B chromatography indicated the existence of at least three types of protein kinase, which have an identical catalytic function but are differentially regulated by cyclic AMP. The type of protein kinase which is stimulated by cyclic AMP was found in various cell types and species [4–11]. The occurrence of both cyclic AMP-activated and cyclic AMP-inhibited protein kinases has been observed in the acellular slime mold [11]. Cyclic AMP-independent protein kinase was found in mammalian tissues [16], but there is a possibility that this type of enzyme may be the catalytic unit of cyclic AMP-dependent protein kinase from the same mycelial extract.

The simultaneous occurrence in the mycelial cells of these three types of protein kinase may be connected with the formation of fruiting bodies. Mycelia of strains of *C. macrorhizus* which form fruiting bodies produced the adenyl cyclase [18], and there is evidence to show that an active component of fruiting-inducing substances is cyclic AMP [17]. The present results indicated that the protein kinases were stimulated or inhibited by concentrations of cyclic AMP in the range of intracellular concentration (Uno, I. and Ishikawa, T., unpublished). All this evidence raises the

possibility that the cyclic AMP-mediated steps in fruiting body formation imply phosphorylation reactions. However, since the intracellular substrates of the protein kinases have not been determined, it is not at present possible to conclude that the protein kinase activity detected is involved in the process of fruiting body formation.

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