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EFFECT OF CYCLIC AMP ON GLYCOGEN PHOSPHORYLASE IN *COPRINUS MACRORHIZUS*

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Summary

Glycogen phosphorylase (1,4-α-D-glucan:orthophosphate α-glucosyltransferase, EC 2.4.1.1) activity was found in mycelial extracts of *Coprinus macrorhizus* concurrently with decrease of glycogen content in mycelial cells. Incubation of the enzyme sample with cyclic AMP and ATP leads to a 3-fold activation of the glycogen phosphorylase activity. Activation of the enzyme partially purified through Sepharose 6B required a cellular fraction containing cyclic AMP-dependent protein kinase.

Introduction

It has been shown that glycogen metabolism in some eukaryotic microorganisms such as *Neurospora crassa* [1,2] and *Saccharomyces cerevisiae* [3—5] is regulated through interconversions of active and inactive forms of glycogen synthase (UDP-glucose:glycogen 4-α-glucosyltransferase, EC 2.4.1.11) and glycogen phosphorylase (1,4-α-D-glucan:orthophosphate α-glucosyltransferase, EC 2.4.1.1).

Experiments with mammalian tissues indicate that cyclic adenosine 3′:5′-monophosphate (cyclic AMP) exerts a regulatory role in interconversions of two forms of these enzymes [6—11]. In previous reports [12,13], we showed that the strains of a basidiomycete, *Coprinus macrorhizus*, that are able to form fruiting bodies produce cyclic AMP. The present report describes evidence showing that the glycogen phosphorylase of *C. macrorhizus* is activated in the presence of cyclic AMP. In addition, experiment is described in which a cellular fraction responsible for an apparent regulatory role of cyclic AMP was studied.

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Materials and Methods

Organism. The following strains of *C. macrorhizus* Rea *f. microsporus* Hongo were used: 708-15 *A8B7 fis*^{c} and 708-15 *A8B7 fis*$^{-}$. The origin and characterization of these strains have been described elsewhere [14].

Medium and cultivation. Production of mycelia, fruiting body primordia and fruiting bodies, and culture maintenance, were carried out 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. Mycelial suspensions were inoculated and incubated in 100-ml Erlenmeyer flasks containing 20 ml of malt/yeast medium at 30°C for 8 days under continuous illumination (1000—6000 ergs/cm^2 per s) or in the dark unless otherwise stated.

Preparation of cell-free extract. Mycelia, fruiting body primordia, and fruiting bodies were harvested on a filter paper, washed well with distilled water, and macerated in 50 mM tris(hydroxymethyl)aminomethane hydrochloride Tris · HCl) buffer, pH 7.4, with a Waring blendor for 5 min. The suspension obtained was further homogenized with a Braun homogenizer for 3 min. The resulting homogenate was centrifuged at 20 000 × *g* for 20 min. The supernatant fluid was used as the crude extract. Crude extract (100 ml) was saturated with $(NH_4)_2SO_4$ (71 g) and after 2 h centrifuged at 20 000 × *g* for 20 min. The precipitate was dissolved in 10 ml of 50 mM Tris · HCl buffer, pH 7.4, put on a Sephadex G-25 column (2 × 40 cm), and eluted with the same buffer. The turbid fractions were collected and used as the enzyme preparation (G-25 fraction).

Glycogen measurement. Mycelia and fruiting bodies were homogenized in ethanol (96%) in a Braun homogenizer for 1 min. The homogenate was made up to 50 ml by adding ethanol to give a concentration of 1 g of mycelia per 50 ml ethanol. A 10-ml fraction of the homogenate was centrifuged and the sediment was washed once with water. The sediment was then suspended in water, heated at 100°C for 10 min, and washed three times with water to remove viscous water-soluble materials. The sediment was then treated with 1 M KOH for 18 h at 25°C. The insoluble material was removed by centrifugation, and the clear supernatant fluid was adjusted to pH 5.0 with acetic acid. The precipitate was removed by centrifugation, and the glycogen content in the supernatant fluid was determined by the anthrone method [15].

Enzyme assay. Glycogen phosphorylase activity was measured as follows: The assay mixture contained 1% glycogen, 2 mM [^{14}C]glucose 1-phosphate (specific activity, 300 000 cpm per μmol), 15 mM $MgCl_2$, and 20 μl of the enzyme sample (diluted in 50 mM Tris · HCl buffer, pH 7.4, containing 20 mM NaF, 8.6 mM EDTA, and 7 mM mercaptoethanol). The total volume was 50 μl. After incubation for 20 min at 30°C, the reaction was stopped by addition of 50 μl of 20% trichloroacetic acid. The assay mixture was then precipitated by 2 ml of 66% ethanol. The precipitate was isolated by centrifugation and washed with 66% ethanol. The radioactivity of the precipitated glycogen was measured in a liquid scintillation spectrometer with Bray's solution [16]. One unit of glycogen phosphorylase activity is defined as the amount of enzyme which catalyzes the incorporation of 1 nmol of [^{14}C]glucose 1-phosphate into glycogen in 1 min. Glycogen-phosphorylase-activating activity was measured by the follow-

ing method. The fractions which showed high glycogen phosphorylase activity in Sepharose 6B chromatography (Fractions Nos. 14—18 in Fig. 5) were pooled and used to test activation of glycogen phosphorylase. The incubation mixture contained 50 μM cyclic AMP, 3.3 mM ATP, 15 mM $MgCl_2$, 50 mM KCl, 50 mM Tris · HCl buffer (pH 7.4), 20 μl of glycogen phosphorylase fraction (1.0 unit), and 10 μl of fraction to be tested for the activating activity. The total volume was 50 μl. After incubation for 30 min at 30° C an aliquot (10 μl) of the mixture was assayed for glycogen phosphorylase activity. One unit of glycogen-phosphorylase-activating activity is defined as the amount of enzyme which caused an increase of one unit of glycogen phosphorylase activity under these conditions. Cyclic AMP-dependent protein kinase activity was measured by the method described previously [17]. Cyclic AMP-binding activity was measured by the millipore filter method as described previously [12].

Protein measurement. Protein concentration was determined by the method of Lowry et al. [18].

Chemicals. Special chemicals used and their sources were as follows: malt extract, Difco; yeast extract, Kyokuto; Sephadex G-25, Sepharose 6B, and Dextran Blue, Pharmacia; cyclic AMP, glucose 1-phosphate and glycogen, Boehringer Mannheim; and [^{14}C]glucose 1-phosphate, the Radiochemical Centre.

Results

Glycogen content and glycogen phosphorylase in mycelia and fruiting bodies

The amount of glycogen in mycelia of this strain was at the maximum level 6 days after inoculation (Fig. 1). The amount of glycogen in fruiting bodies continued to increase until 10 days after inoculation, but that in mycelia decreased significantly (Fig. 1). Glycogen content of *fis*^{c} mycelia grown in the dark and *fis*$^-$ mycelia grown in the light, both of which are unable to form a fruiting body, was similar to that in *fis*^{c} mycelia grown in the light (Fig. 1).

As shown in Fig. 2, glycogen phosphorylase activity in crude extracts of *fis*^{c} mycelia grown in the light was at the maximum level 5 or 6 days after inoculation and then decreased gradually. Levels of the activity in *fis*^{c} mycelia grown in the dark and *fis*$^-$ mycelia grown in the light were lower than that in *fis*^{c} mycelia grown in the light.

Effect of cyclic AMP on glycogen phosphorylase activity

The addition of cyclic AMP (50 μM) to crude extracts showed no significant effect on glycogen phosphorylase activity as indicated in Fig. 2. However, as shown in Table I, incubation of the G-25 fraction obtained from *fis*^{c} mycelia grown in the light with cyclic AMP lead to an activation of glycogen phosphorylase. No stimulating effect of preincubation with ATP alone was observed, but preincubation with cyclic AMP and ATP results in a 3-fold activation of the enzyme (Table I). The maximum activation of the enzyme was observed after preincubation for 30 min in the presence of cyclic AMP (50 μM) and ATP (3.3 mM). The effect of incubation with cyclic AMP and ATP was not observed in G-25 fractions from *fis*^{c} mycelia grown in the dark and *fis*$^-$ mycelia grown in the light (Table II). No interference of these enzyme samples with that of *fis*^{c} mycelia grown in the light was observed in the mixing experiments (Table II).

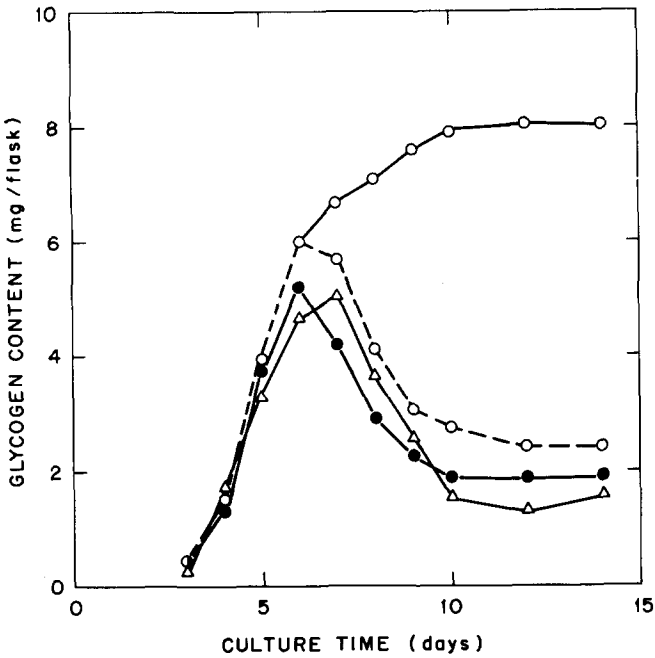

Fig. 1. Changes of glycogen content in strains *fis*^{C} and *fis*$^{-}$ grown in the light or dark. The amount of glycogen was measured. ○———○, mycelia and fruiting bodies of *fis*^{C} strain grown in the light; ○- - - - -○, mycelia of *fis*^{C} strain grown in the light; ●———●, mycelia of *fis*^{C} strain grown in the dark; △———△, mycelia of *fis*$^{-}$ strain grown in the light.

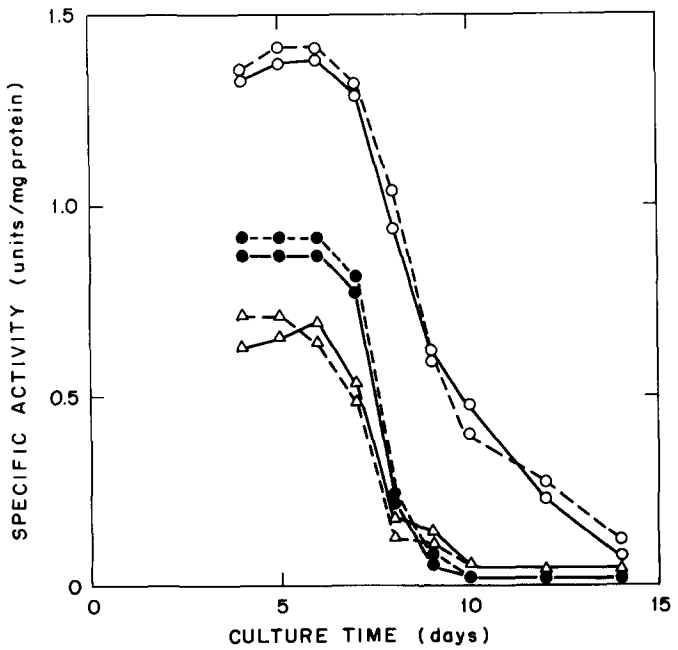

Fig. 2. Glycogen phosphorylase activity of crude extracts obtained from mycelia (including fruiting body primordia and fruiting bodies) of various ages in strains *fis*^{C} and *fis*$^{-}$ grown in the light or dark. Glycogen phosphorylase activity in crude extracts was assayed in the presence (solid line) or absence (broken line) of 50 μM cyclic AMP. ○, Strain *fis*^{C} grown in the light; ●, strain *fis*^{C} grown in the dark; △, strain *fis*$^{-}$ grown in the light.

TABLE I

EFFECT OF CYCLIC AMP AND ATP ON GLYCOGEN PHOSPHORYLASE ACTIVITY IN *C. MACRORHIZUS*

G-25 fractions obtained from *fis*^{C} mycelia grown in the light for 8 days were preincubated in the presence of 15 mM $MgCl_2$ with substances as indicated at 30° C for 10 min and cooled. After preincubation another G-25 fraction was obtained from preincubated G-25 fraction as described in Materials and Methods. Glycogen phosphorylase activity was assayed for the G-25 fraction thus prepared with or without 50 μM cyclic AMP.

Addition in preincubation mixture	Addition of cyclic AMP (50 μM) in reaction mixture	Glycogen phosphorylase activity (units/mg protein)
None	—	1.10
	+	1.65
ATP (3.3 mM)	—	0.97
	+	1.67
Cyclic AMP (50 μM)	—	1.38
	+	1.39
Cyclic AMP (50 μM) and ATP (3.3 mM)	—	3.11
	+	3.22

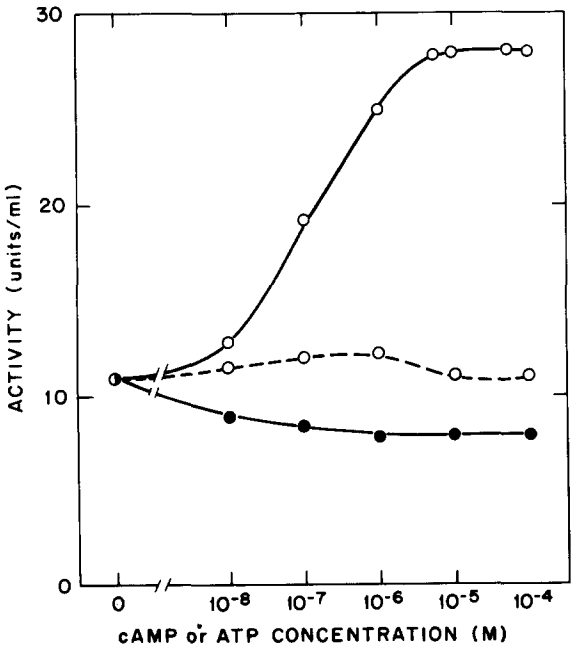

Fig. 3. Effect of cyclic AMP and ATP on glycogen phosphorylase activity. Glycogen phosphorylase activity of G-25 fraction prepared from *fis*^{C} mycelia grown in the light was assayed with the following additives: o———o, the indicated amount of cyclic AMP and 3.3 mM ATP; o- - - - -o, the indicated amount of cyclic AMP; •———•, the indicated amount of ATP.

TABLE II

GLYCOGEN PHOSPHORYLASE ACTIVITY IN MYCELIA OF *fis*^{C} AND *fis*$^{-}$ STRAIN GROWN IN THE LIGHT OR DARK

G-25 fraction was prepared from mycelia of each strain grown in the light or dark. Glycogen phosphorylase activity was assayed with or without 50 μM cyclic AMP in the presence of 3.3 mM ATP and 15 mM $MgCl_2$.

Enzyme source	Culture condition	Addition of cyclic AMP (50 μM) in reaction mixture	Glycogen phosphorylase activity (units/mg protein)
fis^{C}	Light	—	1.2
		+	3.3
fis^{C}	Dark	—	0.50
		+	0.50
fis^{-}	Light	—	0.35
		+	0.36
fis^{C} *	Light and dark *	—	0.70
		+	1.7
fis^{C} and *fis*$^{-}$ **	Light **	—	0.80
		+	1.9

* Mixture of G-25 fractions (10 μl each) from *fis*^{C} mycelia grown in the light and dark was used.
** Mixture of G-25 fractions (10 μl each) from *fis*^{C} and *fis*$^{-}$ mycelia grown in the light was used.

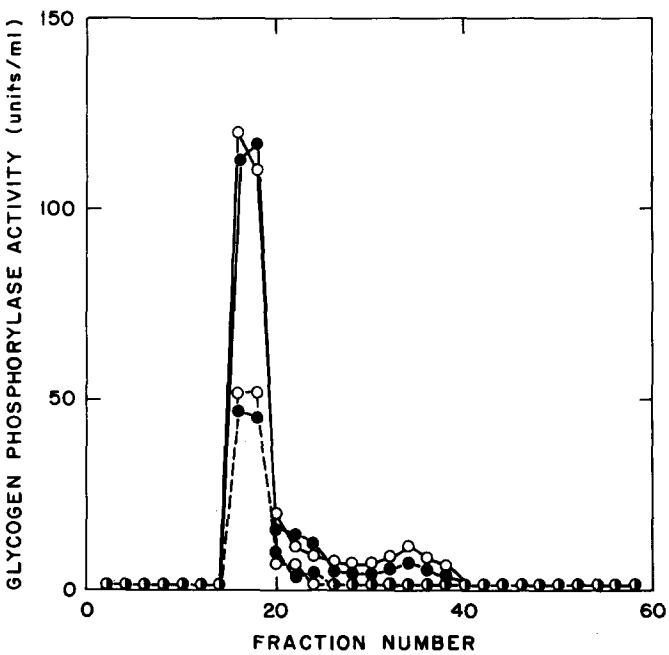

Fig. 4. Sepharose 6B chromatography of glycogen phosphorylase in G-25 fraction from *fis*^{C} mycelia grown in the light. 4 ml of G-25 fraction containing 3.3 mM ATP and 15 mM $MgCl_2$ were preincubated with (solid line) or without (broken line) 50 μM cyclic AMP at 30° C for 10 min. The enzyme sample thus prepared was applied to a Sepharose 6B column (2.5 × 45 cm) and eluted with 50 mM Tris · HCl buffer (pH 7.4). Fractions (4.5 ml) were collected and glycogen phosphorylase activity in each fraction was measured with (●) or without (○) 50 μM cyclic AMP.

The glycogen phosphorylase activity in the G-25 fraction increased as influenced by concentration of cyclic AMP in the preincubation mixture with ATP. Half maximum stimulation of this enzyme occurred with approximately 0.1 μM cyclic AMP in the presence of 3.3 mM ATP (Fig. 3).

A cellular factor responsible for activation of glycogen phosphorylase

The enzyme sample (G-25 fraction) obtained from *fis*^{c} mycelia grown in the light was eluted from Sepharose 6B mainly as a single activity peak (Fig. 4). Preincubation of the same enzyme sample with cyclic AMP and ATP lead to a significant activation of the enzyme, but did not influence the elution position of the enzyme on the profile (Fig. 4). Further addition of cyclic AMP into each

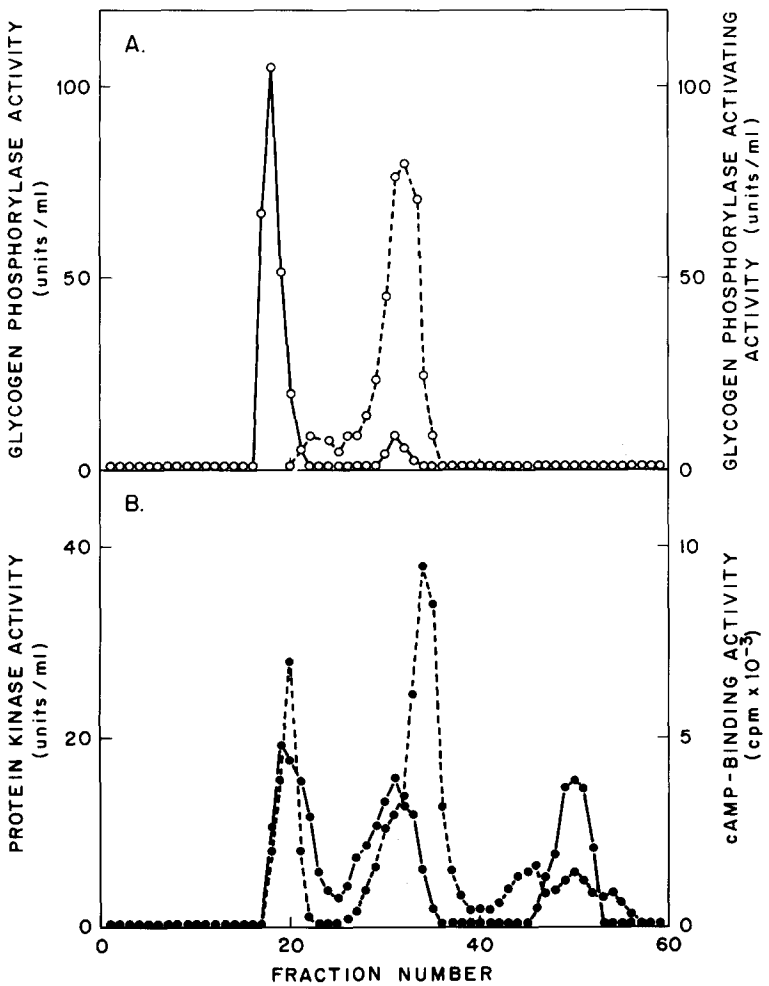

Fig. 5. Elution profile of glycogen phosphorylase, glycogen phosphorylase activating activity, cyclic AMP-dependent protein kinase and cyclic AMP-binding activity in Sepharose 6B chromatography. Sepharose 6B chromatography of G-25 fraction of *fis*^{c} mycelia grown in the light was performed as described in Fig. 4. A: ○———○, glycogen phosphorylase activity; ○- - - - -○, glycogen phosphorylase activating activity; B: ●———●, cyclic AMP-dependent protein kinase activity; ●- - - - -●, cyclic AMP-binding activity.

fraction showed no activation of the enzyme (Fig. 4). The fractions containing glycogen phosphorylase activity in Sepharose 6B chromatogram were collected and incubated with fractions containing no glycogen phosphorylase activity in the presence of cyclic AMP and ATP. Some fractions (Fractions Nos. 22—35) were effective in enhancing the glycogen phosphorylase activity (Fig. 5). In the same chromatogram at least five peaks of cyclic AMP-binding activity were found and the second one located at the same position as that stimulating glycogen phosphorylase activity (Fig. 5). Likewise, three main peaks of cyclic AMP-dependent protein kinase activity were observed and the second one coincided with the position of that stimulating glycogen phosphorylase activity (Fig. 5).

Discussion

We have previously reported the existence of cyclic AMP and cyclic AMP-dependent protein kinase in this fungus [12,13,17], and the requirement of cyclic AMP and cyclic AMP-binding activity for fruiting body formation [12]. A significant activation of glycogen phosphorylase was observed only when cyclic AMP was added to partially purified enzyme samples. This may be due to the fact that crude extracts of mycelia which are able to form fruiting bodies contain cyclic AMP [13]. Loss of activation of glycogen phosphorylase even with cyclic AMP and ATP in enzyme samples obtained from mycelia which are unable to form a fruiting body may be due to the lack of cyclic AMP-binding activity [12] or proper protein kinase in these mycelia as discussed previously [17].

The activation of glycogen phosphorylase required preincubation of enzyme sample with cyclic AMP and ATP, and a particular fraction that is essential for the activation of partially purified enzyme fraction was found in Sepharose 6B chromatography. Such fraction had cyclic AMP-dependent protein kinase activity as well as cyclic AMP-binding activity. The results suggest that the activation of glycogen phosphorylase may be regulated by a cyclic AMP-dependent protein kinase. Thus, the regulation of glycogen phosphorylase activity in *C. macrorhizus* resembles that of mammalian cells [6—11]. More works are, however, required to show phosphorylation of glycogen phosphorylase protein as a mechanism of activation of this enzyme.

A constant level of glycogen content in mycelial cells which form fruiting bodies may be ensured by activation of glycogen phosphorylase by cyclic AMP synthesized in the course of fruiting body formation. Mycelia which are unable to form a fruiting body may keep a constant level of glycogen content by production of low levels of glycogen phosphorylase and glycogen synthetase.

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