Regulation of Glycogen Synthase in *Sclerotium rolfsii*

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Changes in glycogen, extracellular polysaccharides, glycogen synthase (EC 2.4.1.11) and cAMP were followed during growth of submerged mycelium of *Sclerotium rolfsii*. While glucose was present in the medium glycogen and extracellular polysaccharides accumulated to maximum levels of 40 µg (mg dry wt)⁻¹ and 0.3 mg (mg dry wt)⁻¹ respectively; they were degraded following glucose exhaustion. Neither glycogen nor extracellular polysaccharide concentrations were affected by increasing the glucose concentration in the growth medium from 0.5 to 1.5% (w/v). Following inoculation, both glucose-6-phosphate-dependent and glucose-6-phosphate-independent glycogen synthase activities increased during exponential growth, remained constant during the stationary phase as long as glucose was present in the medium, and declined rapidly after the exhaustion of glucose. The cAMP content of submerged cultures of *S. rolfsii* was 5 pmol (mg protein)⁻¹; however, upon glucose exhaustion there was a sharp increase followed by a decrease to the initial basal level. The activity of glycogen synthase in crude extracts was increased by glucose 6-phosphate and inhibited by cAMP.

INTRODUCTION

Submerged mycelium of the phytopathogenic fungus *Sclerotium rolfsii* Sacc. cannot produce sclerotia, but it develops a potential for sclerotium formation as a response to glucose depletion in liquid medium (Hadar *et al.*, 1981). Concomitantly, the intracellular level of cAMP sharply increases upon glucose removal (Hadar *et al.*, 1983). In many biological systems cAMP plays a role in stimulating the catabolism of stored carbon reserves such as glycogen and triglycerides (Pall, 1981). Glycogen, the principal cellular polysaccharide in fungi, often approaches 5% of the mycelial dry weight (Cochrane, 1958). Unlike bacteria, yeasts and filamentous fungi resemble mammals in utilizing UDP-glucose as the donor for glycogen synthesis. Furthermore, glycogen synthesis is regulated by the enzyme glycogen synthase (Dawes & Senior, 1973). The role of this enzyme in glycogen metabolism during the life cycle of filamentous fungi has been extensively studied in the water mould *Blastocladiella emersonii* (Camargo *et al.*, 1969), in *Neurospora crassa* (Tellez-Inon *et al.*, 1969) and in *Coprinus macrorhizus* (Uno & Ishikawa 1978). In this paper we present data concerning glycogen metabolism and glycogen synthase activity and regulation in *S. rolfsii*.

METHODS

Strain and growth medium. *Sclerotium rolfsii* type A ATCC 26325 (Chet & Henis, 1972) was grown in 250 ml Erlenmeyer flasks containing 50 ml synthetic medium (Okon *et al.*, 1973). Flasks were inoculated with 1 ml of a suspension containing 10 mg dry weight of mycelium and incubated at 30 °C in a rotary shaker (New Brunswick Scientific) at 120 r.p.m. for 3 d. Inoculum was prepared by homogenizing mycelium in culture flasks with an Ultra-Turrax homogenizer (Janke & Kunkel KG) operated at one-third of maximum speed (Zweck *et al.*, 1978).

Determinations of biomass, carbohydrates and proteins. Mycelium was separated from extracellular polysaccharide by centrifugation at 12000 g for 20 min at 4 °C. Polysaccharides were precipitated from the supernatant with

2 vols ethanol at 0 °C overnight. The precipitate was collected on a glass rod, washed three times with cold ethanol, freeze-dried, and weighed. To determine mycelial dry weight, washed samples were dried at 70 °C to constant weight. Glucose was determined using glucose oxidase reagent (Sigma) according to the manufacturer's directions. Protein was assayed by the Lowry method using bovine serum albumin as a standard. Determination of glycogen was based on the method described by Johnson & Fusaro (1963). Samples (50–100 mg) of freeze-dried mycelium were homogenized for 3 min in 5 ml KOH (33%, w/v) using a high-speed homogenizer and kept in a boiling water bath for 1 h. After cooling, the insoluble material was collected by centrifugation at 3000 g for 10 min; 2 vols ice-cold ethanol were added to the clear supernatant and it was incubated at 0 °C for 1 h. After centrifugation the sediments were dissolved in 2 ml water and treated again with cold ethanol as described above. This procedure was repeated three times and samples from the final solution were used for glycogen determination. Glycogen was hydrolysed with amyloglucosidase [EC 3.2.1.3; 1.4 U ml⁻¹; Sigma, 5000–10000 U (g solids)⁻¹] at 37 °C for 1 h. The liberated glucose was determined by the glucose oxidase reagent (Sigma); the amount of glycogen is expressed as µg anhydrous glucose equivalents.

cAMP assay. Mycelium was rapidly filtered and washed with cold water on a Buchner funnel. The wet mycelium was removed, placed in 5 ml cold 7% (v/v) HClO₄, homogenized with an Ultra-Turrax homogenizer, and centrifuged at 12000 g for 10 min at 4 °C. The supernatant was neutralized with 2 M-KHCO₃. After a second centrifugation, samples were acetylated and assayed by radioimmunoassay according to the modifications of Gardner *et al.* (1978) and Brown *et al.* (1977) of the method of Harper & Brooker (1975). The antibody-bound fraction was separated from the free ligand using *Staphylococcus aureus* (Bar & Hurwitz, 1979).

Preparation of cell-free extracts. Submerged mycelium was harvested by centrifugation at 10000 g for 10 min at 4 °C and washed twice with cold 100 mM-Tris/HCl pH 7.4. Mycelium (2 g) was homogenized with 5 ml 40 mM-Tris/HCl pH 7.4 containing 4 mM-NaEDTA and 5 mM-DTT, using the Ultra-Turrax homogenizer at maximum speed for 2 min. The homogenate was further homogenized with a Braun homogenizer for 3 min. Cell debris were removed by centrifugation at 20000 g for 20 min at 4 °C, and the supernatant fluid was the crude extract. The crude extract was saturated with (NH₄)₂SO₄ at 4 °C and after 3 h the precipitate was collected by centrifugation at 20000 g for 20 min. The precipitate was dissolved in 2 ml 40 mM-Tris/HCl pH 7.4, and put on a Sephadex G-25 column (2 × 45 cm) equilibrated with the same buffer. The protein fractions were collected and used as enzyme source (G-25 fraction).

Enzyme assay. Glycogen synthase (EC 2.4.1.11) activity was determined from the rate of incorporation of [¹⁴C]glucose from UDP-[¹⁴C]glucose into glycogen (Uno & Ishikawa, 1976). The assay mixture (150 µl) consisted of 50 µl 400 mM-Tris/HCl pH 7.4, 50 mM-NaF, 30 mM-DTT, 10 mM-MgCl₂, 1% (w/v) glycogen and unlabelled UDP-glucose to the desired final concentration; when indicated, 10 mM-glucose 6-phosphate and 50 µM-cAMP together with 0.1 mM-3-isobutyl-1-methylxanthine were included. To start the reaction, 50 µl UDP-[¹⁴C]glucose [$1-2 \times 10^5$ c.p.m., 50–100 nmol, specific activity > 200 mCi nmol⁻¹ (> 7.4 GBq nmol⁻¹)] and 50 µl of the enzyme source were added to the reaction mixture. After incubation for 90 min at 30 °C, samples (50 µl) of the reaction mixture were dried on a Whatman no. 3 filter paper (2 × 2 cm) and rinsed four times with cold 66% (v/v) ethanol (Thomas *et al.*, 1968). Radioactivity of the precipitated glycogen was measured in a liquid scintillation spectrometer with Bray's solution (Bray, 1960).

RESULTS

Effect of glucose 6-phosphate and cAMP on glycogen synthase activity in S. rolfssii

Glycogen synthase in mammalian cells and in some fungi has two interconvertible forms, one glucose 6-phosphate independent (*a*) and the other (*b*) dependent on this phosphoric ester (Friedman & Lerner, 1963; Tellez-Inon *et al.*, 1969). To examine the existence of the two forms of the enzyme in *S. rolfssii*, the effect of UDP-glucose concentration on glycogen synthase activity was measured in the presence and absence of glucose 6-phosphate. Submerged culture at the middle of exponential growth phase was used as the enzyme source. Glycogen synthase as a crude extract or as G-25 fraction showed typical Michaelis-Menten behaviour. Glucose 6-phosphate increased the reaction velocity in a cooperative manner, increasing $V_{\max}$ from 20 nmol min⁻¹ (mg protein)⁻¹ to 30 nmol min⁻¹ (mg protein)⁻¹, but not affecting the K_m (2.5 mM-UDP-glucose).

Glycogen synthase activity in the G-25 fraction was inhibited by exogenous cAMP added to the incubation mixture (Table 1). The activity (1 h incubation at 30 °C) decreased with increasing concentrations of cAMP in the range 10⁻⁸–10⁻⁴ M; half-maximal inhibition occurred with approximately 0.08 µM-cAMP (not shown). On the other hand, addition of glucose 6-phosphate to the incubation mixture increased glycogen synthase activity by about 40% in the

Table 1. *Effect of cAMP, ATP and glucose 6-phosphate, added to the reaction mixture, on glycogen synthase activity of S. rolfii*

The results are the means of three samples examined in duplicate. Variation among samples was within 5% of the mean. 100% glycogen synthase activity = 24 nmol min⁻¹ (mg protein)⁻¹.

cAMP (5 µM)	ATP (3.3 mM)	Glucose 6-P (10 mM)	Glycogen synthase activity (%)
—	—	—	70
—	—	+	100
—	+	—	72
—	+	+	98
+	—	—	15
+	—	+	70
+	+	—	2
+	+	+	60

presence or absence of ATP. The inhibition of enzyme activity by cAMP was partially reversed in the presence of glucose 6-phosphate. Addition of ATP alone did not affect enzyme activity but ATP amplified the inhibition caused by cAMP (Table 1).

Changes in biomass, cAMP concentrations and glycogen synthase activity during growth of S. rolfii in submerged culture

When *S. rolfii* was grown in liquid medium containing 0.5% or 1.5% (w/v) glucose, it reached the stationary phase within 60 h and 120 h respectively. Biomass production in the latter medium was threefold greater than that in the former (Fig. 1*a, b*). However, glucose concentration in the growth medium had little effect on the glycogen content of the submerged mycelium. Glycogen accumulated during the exponential phase, reached the same maximal level of 40 µg (mg dry wt)⁻¹, and was degraded immediately after the exhaustion of glucose in the medium (Fig. 1*c, d*). *S. rolfii* produces an extracellular, highly viscous polysaccharide (Kritzman *et al.*, 1979). This polysaccharide responded to glucose in a similar pattern to the glycogen: a maximum amount of 33% (dry weight basis) was obtained at the end of the exponential growth phase of the fungus grown with either 0.5% or 1.5% (w/v) glucose (Fig. 1*c, d*). A similar low level of intracellular cAMP was found in *S. rolfii* at the two glucose concentrations. A sharp increase in cAMP content to about eight times the initial level, followed by a decrease to the initial level, was observed as soon as the mycelium (in both glucose concentrations) reached the stationary phase (Fig. 1*e, f*). Glycogen synthase specific activity was measured in the presence of glucose 6-phosphate (assaying forms *a* plus *b*) and in its absence (assaying form *a*) during growth (Fig. 1*e, f*). Maximum activity was reached after 30 h incubation and was maintained until the end of the exponential phase. During this period glycogen synthase *a* activity comprised 70% of the total activity (*a* plus *b*). A sharp decline in glycogen synthase *a* activity was observed as soon as the fungus entered the stationary phase, followed about 40 h later by a decline in the total enzyme activity. The enzyme activities and behaviour patterns during growth were similar at the two glucose concentrations used.

Glycogen synthase activity in mycelium grown in glucose-free medium

S. rolfii develops the potential for sclerotium formation only when glucose is exhausted in the growth medium (Hadar *et al.*, 1983). Since the activity of glycogen synthase *a* was more sensitive to environmental change than the total glycogen synthase activity, we followed the former activity during exposure of the mycelium to starvation conditions. Samples of submerged mycelium grown in liquid medium containing 1.5% (w/v) glucose were taken at the middle of the exponential growth phase and washed twice by centrifugation and resuspension in sterile, fresh, glucose-free medium. The washed mycelium was finally suspended in 50 ml glucose-free medium in 250 ml Erlenmeyer flasks and incubated at 30 °C in the shaker. Samples were taken at intervals for enzyme assay and dry weight determinations. Enzyme activity decreased during

Fig. 1. Effect of glucose concentration on *Sclerotium rolsii* grown in submerged culture. Left, 0.5% (w/v) glucose; right, 1.5% (w/v) glucose. (a, b) Glucose utilization (△) and biomass accumulation (○); (c, d) production of glycogen (△) and extracellular polysaccharides (○); (e, f) cAMP content (○), total activity of glycogen synthase (a plus b) (▲) and activity of glycogen synthase a (glucose 6-phosphate independent) (△). Each point represents the mean of four samples, variation among which was within 5% of the mean.

Fig. 2. Glycogen synthase activity in *Sclerotium rolsii* after washing and resuspension of the mycelium in fresh medium without glucose (A, ●) or fresh medium supplemented with 1.5% (w/v) glucose (B, △). ○, Mycelium in glucose-supplemented medium throughout. Each point represents the mean of four samples, variation among which was within 5% of the mean.

the first 30 h of incubation from $16 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$ to approximately $5 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$ (Fig. 2). Thereafter, fungal dry weight and enzyme activity remained constant for a further 50 h in the glucose-free medium. However, resuspending the mycelium in fresh glucose-containing medium caused a sharp increase in enzyme activity, maximum activity being restored within 10 h (Fig. 2).

DISCUSSION

Glycogen metabolism in submerged cultures of *S. rolfii* is strongly affected by the presence of glucose in the growth medium. Glycogen is degraded as soon as exogenous glucose is either fully consumed or removed. On the other hand, the presence of glucose enhances the synthesis of both glycogen and extracellular polysaccharides. This is in contrast to the reported behaviour of the slime mould *Physarum polycephalum*, in which degradation of glycogen was coupled to slime production as indicated by increase in viscosity (McCormick *et al.*, 1970). *S. rolfii* responded to increased glucose concentration by increasing its biomass; however, the amounts of glycogen and extracellular polysaccharides remained constant relative to dry weight. Similarly, total lipids in *S. rolfii* did not change when the glucose concentration in the medium was increased (Shapira *et al.*, 1984).

Glycogen synthase activity in fungi can be controlled by allosteric effectors, such as glucose 6-phosphate and cAMP (Smith & Berry, 1975). In addition, glycogen synthase can exist in either of two interconvertible forms: glucose 6-phosphate dependent (*b*) and independent (*a*) (Tellez-Inon *et al.*, 1969). In crude extracts of *S. rolfii* glycogen synthase showed Michaelis-Menten kinetics in the presence or absence of glucose 6-phosphate. Our results generally corroborate those of Camargo *et al.* (1969) obtained with the water mould *Blastocladiella emersonii*. *S. rolfii* grown in the presence of glucose showed a relatively high activity of glycogen synthase. Following glucose exhaustion the contribution of glycogen synthase *a* to the total glycogen synthase activity fell rapidly from about 70% to about 30%, followed by a later decline in the total activity, leading to a decline in glycogen content 10 h later. Thus, *S. rolfii* responded immediately to glucose depletion by regulating its glycogen content, in contrast to the slow response reported for *Penicillium chrysogenum*, which showed a drop in the total macromolecular carbohydrate content only after 2 d starvation (Trinci & Righelato, 1970).

Glycogen synthase is interconvertible between an active and an inactive form by a phosphorylation-dephosphorylation process. Conversion of the active to the inactive form is stimulated by cAMP in the presence of ATP in mammalian crude tissue extracts (Soderling & Park, 1974; Rubin & Rosen, 1975). Glycogen synthase activity in *S. rolfii* was inhibited by cAMP and this inhibition was partially reversed by glucose 6-phosphate, as has been described for other eukaryotes. Half-maximal inhibition of the *S. rolfii* enzyme occurred with approximately $0.08 \mu\text{M}$ -cAMP, similar to the situation found in *Coprinus macrorhizus* (Uno & Ishikawa, 1978). However, in *Saccharomyces cerevisiae* no correlation was observed between glycogen degradation and endogenous cAMP levels (Fonzi *et al.*, 1979). It is, therefore, possible that cAMP is not the only metabolite influencing glycogen metabolism (Pall, 1981). Our observation that glycogen synthase activity was inhibited by cAMP in the absence of exogenous ATP suggests that phosphorylation at the protein level may not be involved. However, more work is required to measure the internal levels of ATP and to elucidate the manner by which cAMP stimulates the conversion of the enzyme from its active to its inactive form. Moreover, although glucose 6-phosphate and cAMP have effects *in vitro* there is no clear indication that these substances are crucial in the metabolic changes measured.

In addition to its effect on glycogen metabolism, glucose depletion triggers morphogenetic events such as sclerotium initiation in *S. rolfii*, which are probably related to cAMP levels (Hadar *et al.*, 1983). Watkinson (1979) suggested that in the early stages of all types of vegetative aggregations morphogenesis takes place at the expense of endogenous substrates. Indeed, glycogen accumulation just before differentiation and its dramatic decrease following glucose depletion indicates its role in the morphogenetic process.

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