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Interconvertible Forms of Glycogen Phosphorylase in *Neurospora crassa**

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Abstract. Glycogen phosphorylase in extracts of *N. crassa* mycelia has two interconvertible forms: one active (*a* form) and the other inactive (*b* form) in the absence of 5′-AMP. The conversion of the *b* to the *a* form requires ATP-Mg^{2+} and proceeds at higher rate in the presence of 3′,5′-cyclic AMP.

It has been suggested that the regulation of glycogen metabolism in some eucaryotic microorganisms such as *Saccharomyces cerevisiae*,[1–3] *Blastocladiella emersonii*,[4] and *Neurospora crassa*[5, 6] proceeds through modulation in the enzymatic activity of glycogen synthetase (UDP-glucose:glycogen α-4 glucosyltransferase, EC 2.4.1.11) and phosphorylase (α-1,4-glucan:orthophosphate glucosyltransferase, EC 2.4.1.1) mediated by allosteric interactions with intracellular metabolites.

On the other hand, interconversions between phosphorylated and dephosphorylated forms of glycogen synthetase and phosphorylase are a prominent feature in the regulation of glycogen metabolism in mammalian tissues.[7–10]

In a previous paper, it was reported that glycogen synthetase in *N. crassa* has two interconvertible forms, one independent and the other dependent on glucose-6-phosphate.[11] This result suggests that the regulation of glycogen metabolism in this ascomycete fungus occurs in a fashion similar to that found in higher organisms.

The present paper reports evidence showing that glycogen phosphorylase of *N. crassa* also exists under two interconvertible forms. In addition, the results obtained in experiments designed to investigate the effect of 3′,5′-cyclic AMP on these phosphorylase conversions are reported.

Experimental Procedure. A wild-type strain of *N. crassa* (St. L. 74) was grown, harvested, and stored as described previously.[11] Extracts were prepared by homogenizing the liophylized mycelia in 15 vol of 50 mM Tris-HCl buffer, pH 7.4, containing 1 mM EDTA and 5 mM mercaptoethanol, with a Teflon-glass homogenizer. The crude extract was centrifuged for 15 min at 10,000 × *g* and the supernatant fluid was precipitated by the addition of 2 vol of cold saturated ammonium sulfate solution. The pellet obtained after centrifugation for 15 min at 10,000 × *g* was resuspended in a small volume of Tris-mercaptoethanol-EDTA buffer and 0.2-ml aliquots of this suspension were filtered through G-25 Sephadex columns (0.7 × 10 cm) equilibrated with the same buffer. The turbid eluates were pooled and used as a source of enzyme.

In order to study enzyme conversions, 0.04 ml of the enzyme preparation was incubated with different additions in a final volume of 0.06 ml. The reaction was stopped

by adding 0.2 ml of ice-cold 50 mM Tris-HCl buffer, pH 7.4, containing 50 mM NaF, 20 mM EDTA, and 10 mM mercaptoethanol. The samples were further diluted with 0.3 ml of ice-cold Tris-mercaptoethanol-EDTA buffer solution and aliquots were assayed for glycogen phosphorylase activity. Unless otherwise indicated this assay was performed in the absence of 5'-AMP. The assay mixture contained: 1% glycogen, 2 mM ^{14}C-glucose-1-phosphate (spec. act. 300,000 cpm/μmole) and 0.02 ml of the enzyme (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 0.05 ml. After incubation for 20 min at 30°C, the reaction was stopped by the addition of 0.05 ml of 20% trichloroacetc acid, and the glycogen was isolated and measured for radioactivity as indicated previously.[11] Protein was assayed by the method of Lowry *et al.*[12]

Results and Discussion. Figure 1 shows that incubation of the enzyme with ATP-Mg^{2+} (plus an ATP-generating system)leads to a 10-fold enhancement of

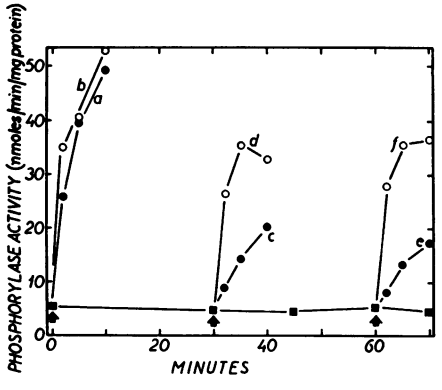


FIG. 1.—Activation of glycogen phosphorylase. The enzyme fraction was incubated at 30°C for the indicated periods without any addition, and after dilution it was assayed for phosphorylase activity (■). At the times indicated by the arrows, aliquots of the incubated fractions were further incubated at 30°C for different times with 3.3 mM ATP, 15 mM $MgCl_2$, 8.3 mM phosphoenolpyruvate, and 50 mM KCl in the presence (○) or absence (●) of 0.01 mM 3',5'-cyclic AMP. Reactions were stopped as indicated in the text, and the diluted samples were assayed for phosphorylase activity.

the glycogen phosphorylase activity. No effect of cyclic AMP was observed under these conditions (curves *a* and *b*). On the other hand, if the enzyme preparation was preincubated at 30°C without any addition, and afterwards it was further incubated with ATP-Mg^{2+} (plus the ATP-generating system), the rate of conversion of the glycogen phosphorylase decreased (curves *c* and *e*). However, the inclusion of cyclic AMP in the activating mixture nearly restored the initial rate of conversion (compare curve *a* with *d* and *f*). This experiment suggests that the activity of the enzyme(s) responsible for the conversion of the inactive to the active form of glycogen phosphorylase is regulated by a cyclic AMP-dependent reaction as has been found in muscle.[13–15] In a similar experiment, the enzyme preparation was preincubated for 60 min without any addition, and then it was converted with ATP-Mg^{2+} (plus the ATP-generating system) and different concentrations of cyclic AMP (Fig. 2*a*). As can be seen, the maximal effect was observed at 10^{-5} *M* but a significant response to the cyclic adenylate was obtained at concentrations of this metabolite between 1.7×10^{-8} *M* and 8.3×10^{-7} *M* (Fig. 2*b*). The dependence of the phosphorylase conversion to the active form on the concentration of cyclic AMP was roughly similar to that found for the stimulation and inhibition of the conversion of muscle phosphorylase kinase (ATP:phosphorylase phosphotransferase, EC 2.7.1.38) and phosphatase (phosphorylase phosphohydrolase, EC 3.1.3.17), respectively.[13–15]

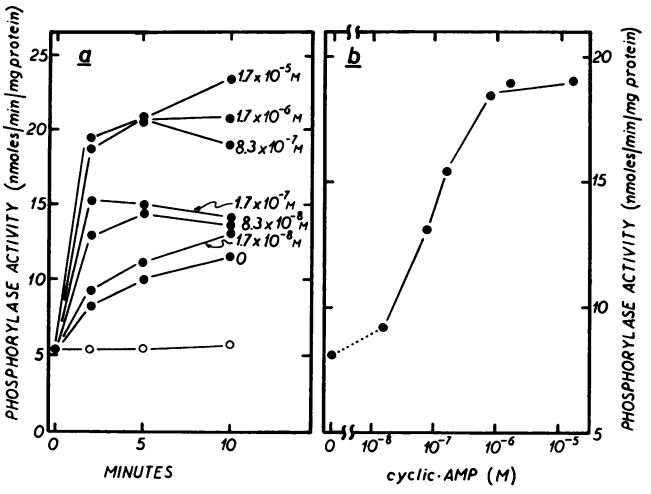
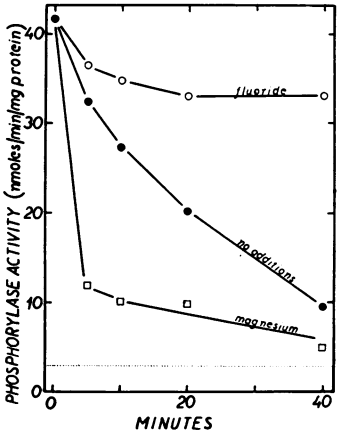


FIG. 2.—Effect of varying the concentration of cyclic AMP on phosphorylase activation. (*a*) The enzyme fraction was incubated at 30°C for 60 min. Then aliquots of this extract were further incubated for different periods in the presence of ATP-Mg^{2+}-phosphoenolpyruvate-K^+ and the indicated concentrations of 3′,5′-cyclic AMP (●). In the control curve (○), the enzyme was incubated without any addition. (*b*) Concentration dependence of cyclic AMP effect. Phosphorylase activities corresponding to the incubations performed for 2 min in the presence of the cyclic adenylate, were plotted as a function of the concentration of this metabolite. Other conditions were those described in Fig. 1.

The enzymatic extract obtained from a large-scale incubation performed in the presence of ATP-Mg^{2+} was purified by precipitation with ammonium sulfate and passage through a G-25 Sephadex column. The enzyme preparation was then further incubated for conversion to the inactive form. Figure 3 shows that

FIG. 3.—Inactivation of glycogen phosphorylase. The preparation of the enzyme used in this experiment was as follows: the enzyme fraction (0.8 ml) was incubated for 10 min at 30°C in the presence of 3.3 mM ATP, 15 mM $MgCl_2$, 8.3 mM phosphoenolpyruvate, 50 mM KCl, and 0.01 mM 3′,5′-cyclic AMP. The total volume was 1.2 ml. The reaction was stopped by the addition of 1.2 ml ice-cold 50 mM Tris-HEl buffer, pH 7.4, containing 50 mM NaF, 20 mM EDTA, and 10 mM mercaptoethanol, plus 4.8 ml of an ice-cold saturated ammonium sulfate solution. The precipitate was collected by centrifugation at 10,000 × *g* for 10 min, and was resuspended in 0.3 ml of 50 mM Tris-HCl buffer, pH 7.4, containing 1 mM EDTA and 5 mM mercaptoethanol. Aliquots of 0.2 ml of this suspension were passed through G-25 Sephadex columns (0.7 × 10 cm) equilibrated with the same buffer solution. The turbid eluates were pooled and then aliquots were incubated for the indicated periods at 30°C with the following additions: none (●), 50 mM NaF (○), and 5 mM $MgCl_2$ (□). The dotted line indicates the activity of the enzyme before the incubation with ATP-Mg^{2+}-phosphoenolpyruvate-K^+-cyclic AMP. Other conditions were as those described in Fig. 1.



Mg^{2+} enhanced the inactivation of the phosphorylase. On the other hand, fluoride inhibited this conversion, as occurs in mammalian tissues.[16]

As shown in Figure 4, the activation of glycogen phosphorylase by ATP-Mg^{2+} leads to an increase in the maximal velocity for glucose-1-phosphate, but no appreciable change in the apparent K_m (0.7 mM) was observed. This K_m value is about 10 to 20 times higher when the glycogen phophorylase activity is assayed in the presence of 80 mM glycerophosphate-HCl buffer, pH 6.8 and 100 mM NaF, according to the method of Shepherd and Segel.[5] In addition, as was found in muscle, 5′-AMP stimulates the inactive form of the enzyme, but not the

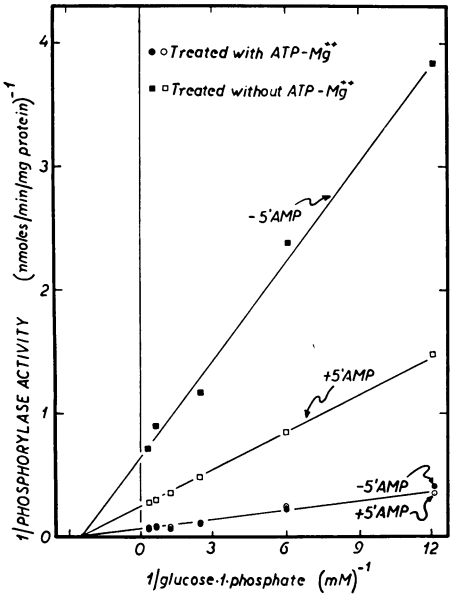


FIG. 4.—Effect of glucose-1-phosphate concentration on the activity of glycogen phosphorylase. Lineweaver-Burk plots of the resuls obtained with the enzymes prepared by incubation of 0.8 ml of the enzyme fraction in the presence (○ ●) or absence (□ ■) of 3.3 mM ATP, 15 mM $MgCl_2$, 8.3 mM phosphoenolpyruvate, 50 mM KCl, and 0.01 mM 3′,5′-cyclic AMP. After incubation for 10 min at 30°C in a total volume of 1.2 ml, the reactions were stopped by the addition of 1.2 ml of an ice-cold solution containing 50 mM Tris-HCl buffer, pH 7.4, 50 mM NaF, 10 mM EDTA, and 20 mM mercaptoethanol, plus 4.8 ml of an ice-cold saturated ammonium sulfate solution. The precipitates were collected by centrifugation at 10,000 × g for 10 min and resuspened in 0.2 ml of the Tris-F^--EDTA-mercaptoethanol buffer solution. After that each sample was passed through a G-25 Sephadex column (0.7 × 10 cm) equilibrated with the same solution, and the eluates were further diluted in Tris-mercaptoethanol-EDTA buffer solution as indicated under *Experimental Procedure*. Glycogen phosphorylase activity was assayed at different concentrations of glycose-1-phosphate in the presence (○, □) or absence (● ■) of 2.5 mM 5′-AMP as described in the text.

active one. The nucleotide increased the maximal velocity but never to the value obtained after the conversion with ATP-Mg^{2+}. In addition, 5′-AMP does not modify to a great extent the apparent K_m for glucose-1-phosphate.

The results reported in this paper indicate that glycogen phosphorylase in *N. crassa* has two interconvertible forms: one active (*a* form) and the other almost inactive (*b* form) under the assay conditions. The enzymatic activity of the *a* form is not influenced by the presence of 5′-AMP but that of the *b* form is stimulated by this nucleotide. The conversion of the *b* to the *a* form requires ATP-Mg^{2+} and proceeds at higher rate in the presence of 3′,5′-AMP.

In conclusion, the regulation of glycogen metabolism in *N. crassa* resembles that of mammalian cells. The evidence obtained in experiments performed *in vitro* indicates that, as it occurs in higher organisms, the conditions for activation of glycogen phosphorylase appear to be roughly similar to those required

for glycogen synthetase inactivation and *vice versa*. Moreover, a membrane-bound adenyl-cyclase activity was detected in *N. crassa* mycelia.[17] Therefore, it appears that the system designed to amplificate hormonal signals at the membrane level of mammalian cells is quite "old" in the evolution scale.

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