REQUIREMENT FOR SPECIFIC CARBON SOURCES IN THE LOW TEMPERATURE INDUCTION OF GLYCEROL KINASE IN *NEUROSPORA CRASSA*

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1. Introduction

The synthesis of glycerol kinase (ATP:glycerol phosphotransferase), an inducible enzyme in *Neurospora crassa*, has recently been shown to occur at low temperatures during growth on sucrose media [1]. The cause of this low temperature synthesis is not known, but it may be the result of unique themal dependent properties of the glycerol repressor(s) in this organism. Alternatively, the increase in activity may be the result of metabolically generated inducer(s) at depressed temperatures. Since previous work on glycerol kinase induction has indicated that at normal growth temperatures (20-35°C) glycerol is the only known effector for this system [2], these studies have been undertaken to elucidate further the mechanism involved in induction. The results indicate that the induction of GK at low temperatures is highly specific, and is dependent on the carbon source used during the low temperature incubation. It was also found that low temperatures do not cause induction of a second enzyme involved in glycerol utilization, glycerol-3- phosphate (G3P) dehydrogenase (glycerol (acceptor) oxidoreductase), wich normally is synthesized after growth in glycerol-containing media [2].

2. Materials and methods

Cultures were started using strain 74-4, a strain derived in this laboratory from the wild type strain 74-OR23-1A and characterized by the greater yields it gives when grown on glycerol. Conidia were inoculated into a 250 ml Erlenmeyer flask containing 50 ml of the minimal salts medium of Bonner and Vogel [3] plus 1% sucrose. After growth (16 hr) on a reciprocating shaker at 30°C, the cultures were filtered, washed with cold water, and resuspended in four liter Erlenmeyer flasks containing two liters of minimal salts medium plus the desired carbon source. The cultures were shaken at the designated temperatures on a gyrotory shaker, and were harvested by filtration onto filter paper in a Buchner funnel. After several washes with cold water the mycelial pad was pressed dry and ground with twice its weight of sea sand, using a mortar and pestle. At the start, one volume (v/w) of grinding buffer (0.25 M sucrose, 0.1 M KCl, 1 mM β-mercaptoethanol (BME), and 10 mM glycerol) was added, and grinding continued until a homogenous mixture was obtained. Two additional volumes (v/w) of grinding buffer were than added and mixed with the extract. The crude extract was then spun in a Sorval RC2-B Superspeed centrifuge at 1085 *g* for 10 min to remove sand and cellular debris. The supernatant was spun at 27 000 *g* for 15 min, and the mitochondrial pellet obtained was resuspended in one tenth the original volume of grinding buffer and was used to assay for G3P dehydrogenase and succinate dehydrogenase (succinate (acceptor) oxidoreductase). The high speed supernatant was used to assay glycerol kinase and isocitrate lyase (L_S-isocitrate glyoxylate lyase) activity.

Glycerol kinase was assayed by determining the amount of G3P formed in a 1 ml assay containing 5 μmol ATP, 10 μmol $MgCl_2$, 100 μmol glycerol, 1 mg bovine serum albumin (BSA), and 100 μmol Tris–HCl (pH 8.0). The assay mix, with extract, was incubated at 37°C for 15 min, and the amount of G3P formed was determined by addition of 0.325 μmol phenazine methosulfate (PMS), 0.6 μmol 3(4,5 dimethylthiazoyl 1-2) 2,5 diphenyl tetrazolium bromide (MTT), 1μmol NAD, and 6.0 units of glycerol phosphate dehydrogenase. The amount of G3P present was determined from a standard

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curve constructed by use of a known amount of G3P [4].

The G3P dehydrogenase assay, a modification of the previous procedure of Lin et al. [5], contained 200 μmol G3P, 25 μmol NaCN, 0.012 μmol MTT, 1 mg BSA, 170 μmol K_2HPO_4 (pH 7.8) and extract in 2.1 ml. The reaction was started by the addition of 0.1 ml of 6.5 mM PMS and the rate of MTT formazan formation was measured as the increase in absorbancy at 547 nm.

The succinate dehydrogenase assay was essentially that of Courtright and Henning [6]. Samples were preincubated in 2 ml of the assay mix (40 μmol sodium succinate, 25 μmol NaCN, 0.012 μmol MTT, and 170 μmol K_2HPO_4 (pH 7.8)) for 1 min before the reaction was started with 0.1 ml of 6.5 mM PMS. The rate of MTT formazan formation was followed at 547 nm.

Isocitrate lyase was assayed by the procedure of Dixon and Kornberg [7].

Protein was estimated by the method of Lowry et al. [8], using bovine serum albumin as the standard.

α-glycerol phosphate dehydrogenase was a product of the Sigma Chemical Company.

3. Results and discussion

Although strains of other organisms with a temparature sensitive repressor have been known for some time [9,10], these have all proven to be sensitive to elevated rather than to depressed temperatures. Nevertheless, the unique possibility remains that low temperature induction of glycerol kinase may be the result of a cold sensitive repressor, which could be identical to the glycerol repressor. Therefore, to determine if the glycerol repressor could be cold sensitive, cultures were grown at 4°C on either glucose or glycerol. If indeed a cold sensitive repressor is involved, and if it is different from the normal glycerol repressor, then the inducing effects of glycerol and low temperatures should be additive, and result in a higher glycerol kinase specific activity for the glycerol grown culture. However, a cold sensitive glycerol repressor should result in equal activities for the two cultures. Table 1 shows that the glycerol kinase activities of the glycerol and glucose grown cultures are essentially the same, thereby eliminating the possibility of a cold sensitive repressor which is distinct from the glycerol repressor. Although the possibility of complex genetic regulation cannot be excluded, the results found imply that if a cold sensitive repressor is involved, it is likely to be the glycerol repressor.

Table 1
Effects of different carbon sources on induction

Carbon Source	Temp (°C)	Specific Activity[a] GK	G3PDH	SDH
Acetate	4	1.2	–	–
	26	1.3	–	–
Glucose	4	22.8	38.0	100.0
	26	2.5	91.0	143.0
Glycerol	4	21.3	39.0	104.0
	26	64.7	359.0	95.0
Xylose	4	2.9	–	–
	26	0.7	–	–

[a]Specific activity in terms of nanomoes/min/mg protein in in extracts prepared from cells grown 17–21 hr at the indicated temperature.

If the glycerol repressor actually is cold sensitive, there should be induction of glycerol kinase activity at low temparatures, regardless of the carbon source on which growth occurs. Cultures were therefore grown at 4°C on either acetate or xylose, two carbon sources which enter the glycolytic pathway at a point different from glucose. There is no significant difference in glycerol kinase activity when comparing the acetate cultures grown at 4°C or 26°C, but comparing the 4°C and 26°C xylose cultures shows a slight increase in the activity of the culture grown at the lower temperature (table 1). The fact that growth on neither acetate nor xylose at low temperatures results in measurable synthesis of glycerol kinase rules out the possibility of a cold sensitive glycerol repressor and suggests that the induction is dependent on the formation of either glycerol or a phosphorylated intermediate. Although this inducer has not yet been positively identified for *N. crassa*, it is likely to be glycerol-3-phosphate which has previously been characterized as the effector for the *glp* (glycerol phosphate) regulon in *Escherichia coli* [11]. The failure to observe the induced synthesis of glycerol kinase after growth on either acetate or xylose therefore implies that the intracellular concentration of the presumed phosphorylated intermediate(s) is not sufficient to cause glycerol kinase induction.

An alternative explanation is that the increase in glycerol kinase levels is the result of a generalized induction of cytosol enzymes in response to growth on glucose at low temperatures. To test this possibility, isocitrate

Table 2
Specific induction of isocitrate lyase

Carbon Source	Temp (°C)	Specific Activity[a]
Acetate	26	10.7
Glucose	4	1.8
	26	1.1
Glycerol	4	1.8
	26	1.6

[a]Specific activity in terms of nanomoles glyoxylate formed/min/mg protein in extracts prepared from cells grown 17 hr at the indicated temperature.

lyase, a glyoxylate cycle enzyme wich is normally fully induced in acetate grown [12], but uninduced in glucose grown cultures, was assayed. As can be seen from table 2, growth on either glucose or on glycerol at 4°C does not result in an appreciable induction of isocitrate lyase activity, thus ruling out any possibility of a generalized induction.

Finally, G3P dehydrogenase levels were also examined to see if this enzyme was subject to the same low temperature induction as glycerol kinase. The results show (table 1) that the enzyme is uninduced in glucose cultures grown at 4°C, and in fact is also uninduced under similar conditions in glycerol grown cultures. Succinate dehydrogense activities, a constitutive mitochondrial enzyme, are given for reference. Since in this case G3P dehydrogenase remains uninduced under conditions which do result in glycerol kinase induction, the mechanisms of induction of the two enzymes at low temperatures must be different. The finding that the two enzymes known to be involved in glycerol utilization by *N. crassa* are not subject to the same metabolic regulation, implies that the genes for these two enzymes probably do not comprise an operon.

The results of these studies are consistent with the fact that low temperature induction of glycerol kinase is not a response solely to the cold temperature, but is also dependent on the carbon source used for growth. It appears that growth at low temperatures, leads to the metabolic generation of an internal inducer, and results in net synthesis of the enzyme glycerol kinase.

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