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INCREASE OF ENZYME ACTIVITIES IN *NEUROSPORA CRASSA* DURING INCUBATION AT LOW TEMPERATURES

MICHAEL J NORTH *

Department of Biochemistry, University of Newcastle upon Tyne, Newcastle upon Tyne, NE1 7RU (U K)

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Summary

The effect of lowering the incubation temperature of sucrose-grown cultures of *Neurospora crassa* on the level of various enzyme activities was investigated. Of twelve inducible/derepressible activities studied, three, in addition to glycerol kinase, were found to increase during 48 h of incubation at 4—6°C trehalase (increase in specific activity of 3—10-fold), β-glucosidase (6—12-fold) and β-*N*-acetylglucosaminidase (4 to 6-fold) The maximum increases occurred at 6°C and no increases took place in mycelia incubated at 0°C The kinetics of the changes in activity were markedly different from those observed previously with glycerol kinase. The increases were inhibited by cycloheximide Trehalase, β-glucosidase and β-*N*-acetylglucosaminidase activities were not rapidly lost when cultures incubated at 6°C were returned to 26°C

Introduction

In *Neurospora crassa*, as in many microorganisms, changes in the levels of particular enzyme activities occur in response to alterations to the composition of the medium [1], and also as cultures age [2,3]. It has recently been demonstrated [4] that the glycerol kinase (ATP. glycerol-3-phosphotransferase, EC 2 7 1.30) activity in this organism is sensitive to an additional factor, namely the growth temperature When cultures growing in sucrose medium at 26°C are transferred to temperatures below 12°C there is a large increase in the level of glycerol kinase activity which is greater the lower the temperature This activity is rapidly lost when cultures are returned to 26°C [5]

At normal growth temperatures the level of glycerol kinase activity is sensitive to changes of carbon source, with high levels of activity induced not only by glycerol [4,6,7], but also by compounds that are not substrates, such as

* Present address Biochemistry Department, University of Stirling, Stirling, FK9 4LA, Scotland

galactose and deoxyribose [8] Certain similarities exist between glycerol kinase and other enzymes involved in carbon catabolism with respect to their response to different carbon sources [8] In the light of these findings it was of interest to determine whether other enzymes were affected by cold incubation in a similar manner to glycerol kinase The study was extended to include a number of other inducible/derepressible enzymes that are not directly related to carbon source utilization In most cases significant changes in activity were not observed, but cold-induced increases in three activities are reported here· trehalase (trehalose 1-glucohydrolase, EC 3 2 1 28), β-glucosidase (β-D-glucoside glucohydrolase, EC 3 2 1 21) and β-*N*-acetylglucosaminidase (β-2-acetamido-2-deoxy-D-glucoside acetamidodeoxyglucosaminidase, EC 3 2 1 30) It is shown that none of these responds to low temperature in the same way as glycerol kinase

Materials and Methods

Strain N crassa strain 74A (wild-type) was used throughout

Growth and extract preparation Cultures were grown in 50- or 250-ml flasks containing either 20 or 100 ml of Fries minimal medium [9], respectively, with 2% (w/v) sucrose as sole carbon source Inoculation and incubation were carried out exactly as described previously [4] Extracts were prepared from mycelia from whole 20-ml cultures or from portions (10 ml) of 100-ml cultures by the method described previously [4], depending on the activity to be assayed either 0 083 M tris(hydroxymethyl)aminomethane buffer (pH 8 0) or 0 05 M sodium phosphate buffer (pH 6 0) was used For glycerol kinase assays Tris buffer was used, and for β-glucosidase, β-*N*-acetylglucosaminidase, invertase and trehalase assays phosphate buffer was used

Assays β-*N*-Acetylglucosaminidase and β-glucosidase activities were both assayed at pH 5 0 and 37°C using the corresponding *p*-nitrophenyl glycoside as substrate β-Glucosidase was assayed essentially by the method of Eberhart [10], but using sodium acetate/acetic acid buffer (0 1 M) β-*N*-Acetylglucosaminidase was assayed by the following discontinuous method The reaction mixture contained 1 5 ml of 0 1 M sodium acetate/acetic acid buffer (pH 5 0), 0 3 ml of cell extract (diluted with 0.05 M sodium phosphate buffer (pH 6 0) when necessary) and 0 3 ml of 0 01 M *p*-nitrophenyl-2-acetamido-2-deoxy-β-D-glucopyranoside. After a suitable incubation period (5—10 min) the reaction was stopped by the addition of 1.0 ml of saturated Na_2CO_3, and the absorbance measured at 420 nm The activities of both enzymes are expressed as nmol of *p*-nitrophenol released/min per mg protein.

Invertase (β-D-fructofuranoside fructohydrolase, EC 3 2 1 26) and trehalase were both assayed at pH 6 0 and 37°C by the method described by Sussman et al [11], and the activity expressed as nmol of glucose released/min per mg protein

Glycerol kinase was assayed by the method described previously [4] and the activity expressed as nmol of glycerol phosphate bound (to DE-81 filter)/min per mg protein

Protein was assayed by the method of Lowry et al. [12] using bovine serum albumin as standard

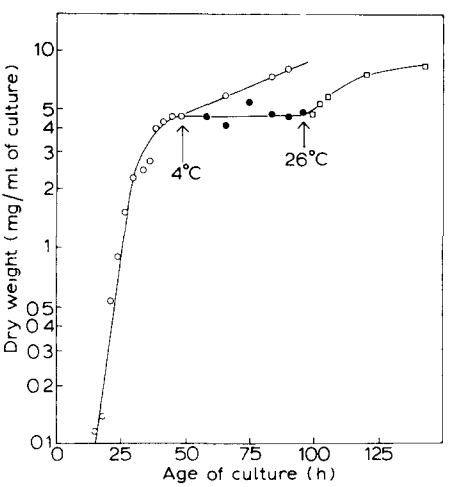
Sources Cycloheximide was supplied by Sigma London Chemical Co (King-

ston upon Thames, U K.), *p*-nitrophenyl-β-D-glucopyranoside and *p*-nitrophenyl-2-acetamido-2-deoxy-β-D-glucopyranoside by Koch Light Laboratories Ltd (Colnbrook, U K.), [1-^{14}C]glycerol by the Radiochemical Centre (Amersham, U K) and all other chemicals by B.D H Chemicals Ltd (Poole, U K.)

Results and Discussion

Growth of *N crassa* is severely restricted at low temperatures [13] Fig 1 shows the effect of lowering the growth temperature from 26 to 4°C of cultures for which sucrose was the sole carbon source For a period at least 48 h at the lower temperature there was no change in mycelial dry weight, but once cultures were restored to the original growth temperature an increase in the dry weight began almost immediately. The lack of change in dry weight at 4°C is parallelled by a constant cellular protein level, and so alterations in the specific activity of enzymes such as glycerol kinase [4] represent changes in the actual level of enzyme protein present in the mycelia.

A number of enzyme activities were assayed in extracts prepared from cultures incubated at 4—6°C for up to 96 h, but a significant change in the level of activity was not found with most of these. Amylase, β-galactosidase (pH 4.2 and pH 7 5), α-glucosidase, invertase, isocitrate lyase, acid phosphatase, alkaline phosphatase (repressible and constitutive), aryl-sulphatase and tyrosinase were all unaffected by cold incubation. The extraction and assay procedures used



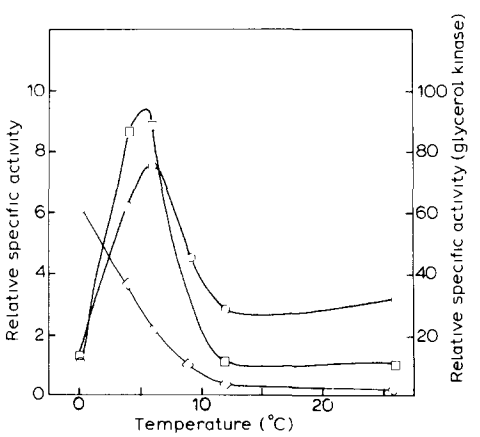


Fig 1 Growth of *Neurospora* at 26 and 4°C 100-ml cultures were inoculated and incubated at 26°C (○———○) as described in Materials and Methods After 48 h of incubation some cultures were transferred 4 C (●———●) and the remainder retained at 26°C After a further 48 h of incubation at 4°C the former cultures were returned to 26°C (□———□) At intervals 2-ml samples were removed and the dry weight of mycelium determined Values represent an average of four determinations using two samples each from duplicate cultures

Fig 2 Temperature dependence of increases in β-glucosidase, trehalase and glycerol kinase activities 20-ml cultures were incubated in sucrose medium for 48 h at 26°C, then for 48 h at the temperature indicated and were then extracted The specific activity is given relative to that present after 48 h of incubation at 26°C β-glucosidase (1 48 units/mg protein, ○———○), trehalase (3 31, □———□), glycerol kinase (0 04, ◇———◇)

were sufficiently sensitive to allow the detection of changes in these activities at 26°C In addition to these inducible/derepressible activities, the activity of a second kinase, hexokinase, was assayed, but the level was also unaffected at 4°C However, increases in three activities, namely trehalase, β-glucosidase and β-*N*-acetylglucosaminidase, were observed An increase in β-glucosidase activity was detected by assaying both at pH 5 0 and at pH 7 0, but since the increase at the lower pH was larger, subsequent assays were all performed at pH 5 0

Unlike glycerol kinase, none of the other three cold-induced activities increased at 0°C, and as Fig 2 demonstrates for β-glucosidase and trehalase there was a considerable difference between the temperature dependence of these increases and that of glycerol kinase maximum increases were observed at approx 6°C The rates of increase were all very much slower than that for glycerol kinase (Fig 3), and the relative increase achieved over the 48-h period studied were much smaller.

It is interesting to note that the activity of invertase failed to increase at a time when that of trehalase was rising (Fig 3) Whilst they are not coordinately controlled [3], these two activities are often elevated by the same conditions [3,14,15] The lack of increase of invertase activity may be explained, not necessarily by a lack of the appropriate conditions at 6°C, but by the inability of the mycelia to respond to those conditions and produce invertase activity Mycelia grown in galactose medium at normal growth temperatures produce high levels of invertase [15], but the provision of galactose at 4°C failed to result in any increase in activity (Table I) In addition to invertase, many others of the enzyme activities tested, including amylase, β-galactosidase, acid and alkaline phosphatases and aryl-sulphatase, failed to respond at 4°C when mycelia were provided with specific conditions which elevated the activities at 26°C Incubation of *Neurospora* at 4°C in glycerol medium fails to induce an increase in glycerol phosphate dehydrogenase activity [16]. In contrast, the levels of both

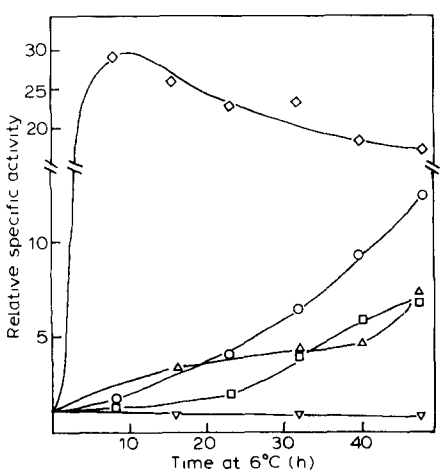


Fig 3 Time course of cold-induced increases in enzyme activities 20-ml cultures were incubated for 48 h at 26°C, and then transferred to 6 C Extracts were prepared at intervals from whole cultures The specific activity is given relative to that present after 48 h of incubation at 26°C β-*N*-acetylglucosaminidase (17 7 units/mg protein, △———△), β-glucosidase (1 18, ○———○), invertase (759, ▽———▽), trehalase 3 70, □———□), glycerol kinase (0 05, ◇———◇)

TABLE I

EFFECT OF CHANGING THE CARBON SOURCE ON THE LEVEL OF ENZYME ACTIVITY DURING INCUBATION AT 4°C

20-ml cultures were grown on sucrose medium for 48 h at 26°C, and then for 2 h at 4°C Mycelia from one culture were extracted, and the mycelia from others washed and resuspended in 20 ml of fresh medium (pre-cooled to 4°C) containing either no carbon source or the carbon source specified at a concentration of 5 mg/ml One remaining culture was kept intact and together with the cultures of resuspended mycelia was incubated for a further 46 h at 4°C before extraction The initial specific activity refers to that present in the mycelia extracted after 2 h at 4°C, no change refers to the culture kept intact, no carbon to the culture resuspended in fresh medium with no carbon source and specific medium to that with the specified carbon source

Enzyme	Carbon source	Initial specific activity	Specific activity after 48 h at 4°C		
			No change	No carbon	Specific medium
β-Glucosidase	Cellobiose	1 05	9 75	14 25	37 9
Invertase	Galactose	603	563	471	470
Trehalase	Galactose	6 48	17 6	15 7	15 7
Trehalase	Trehalose	—	—	—	31 5

β-glucosidase and trehalase activities did respond at low temperatures to the provision of carbon sources which at normal growth temperatures induce high levels of activity (Table I), i.e. cellobiose for β-glucosidase [17] and trehalose for trehalase [15]

The increases in trehalase, β-glucosidase and β-*N*-acetylglucosaminidase activities were all inhibited by the addition of cycloheximide to the medium at the time of the temperature change (Table II), this indicates a requirement for de novo protein synthesis [18]

Cold-induced glycerol kinase activity is rapidly lost when cultures are returned at 26°C [5], and, although some of the elevated trehalase and β-glucosidase activities was lost at 26°C, the decrease in specific activity was much slower (Table III), indicating that these enzyme activities were more stable The addition of cycloheximide had no significant effect on these changes of activity

At normal growth temperatures (20—30°C), with the exception of glycerol kinase, elevated levels of the activities which increased at low temperatures are associated with the starvation or ageing of cultures The level of trehalase activity is inversely related to the ability of the carbon source provided to support growth [19], and the activity increases with ageing in both standing [3,20] and

TABLE II

EFFECT OF CYCLOHEXIMIDE ON THE INCREASES IN ENZYME ACTIVITIES AT 6°C

20-ml sucrose cultures were incubated at 26°C for 48 h and then at 6°C for 48 h To one culture 1 4 μg of cycloheximide were added per ml at the time of the temperature change The initial specific activity was that of a culture extracted after 48 h of incubation at 26°C only

Enzyme	Specific activity			Inhibition by cycloheximide (%)
	Initial	No cycloheximide	With cycloheximide	
β *N*-Acetylglucosaminidase	19 7	72 8	24 0	92
β-Glucosidase	1 51	9 72	2 00	94
Trehalase	3 70	38 9	2 78	100

TABLE III

EFFECT OF RETURN TO 26° C ON ELEVATED ENZYME ACTIVITIES IN CULTURES INCUBATED AT 6°C

A 100-ml sucrose culture was incubated at 26°C for 48 h, and then at 6° C for 48 h The culture was returned to 26°C, and samples removed and extracted at intervals An additional sample (10 ml) was removed at the time of the temperature increase, and 1 4 μg of cycloheximide added per ml This sample was incubated separately in a 50-ml Erlenmeyer flask at 26°C and extracted after 7 5 h

Enzyme	Specific activity			
	0 h	5 h	7 5 h	7 5 h with cycloheximide
β *N*-Acetylglucosaminidase	70 8	74 7	74 3	80 6
β-Glucosidase	9 5	8 0	7 3	9 0
Trehalase	44 4	31 5	26 8	28 7

shaken [14] cultures Two enzymes possessing activity on *p*-nitrophenyl-β-D-glucopyranoside are known in *Neurospora* [21] these have been named cellobiase and aryl-β-glucosidase in accordance with their substrate preference [22] Aryl-β-glucosidase is also produced in ageing cultures just prior to conidiation [17] Less is known about β-*N*-acetylglucosaminidase activity in *Neurospora*, but elevated activities have been found in poorly growing cultures [23] The increases in activity at low temperature suggest that cold incubation may have mimiced starvation and/or ageing, despite the presence of sufficient nutrients in the medium to permit further growth when the temperature was elevated (Fig 1) Such premature ageing would also explain the appearance of carotenoids in cold-incubated cultures [23] which was also found to occur optimally at 6°C

Cold-induced increases in enzyme activities in some higher organisms have been shown to have a physiological role [24,25], but a role for the cold-induced changes reported here is not yet apparent It has been proposed [8] that glycerol kinase could have a role during the adaption of *Neurospora* mycelia to certain new growth conditions, and it is noteworthy that significant changes in the levels of other cold-induced activities occurred only after glycerol kinase activity had reached, and passed a maximum (Fig 3). During the initial 24 h of incubation at 4°C the mycelia utilize exogenous sucrose and glucose with a decreasing efficiency [23] the proportion of the carbon from any sucrose or glucose taken up which is incorporated into $HClO_4$-insoluble material is smaller the longer the mycelia have been incubated at 4°C However, during the second 24-h period this efficiency increases Throughout the whole 48-h period the efficiency of utilization of exogenous glycerol increases [23] It is possible that there is an initial period during which the increase in glycerol kinase activity permits the cells to carry out subsequently processes required for the new conditions. Among such processes at low temperatures may be the production of trehalase, β-glucosidase and β-*N*-acetylglucosaminidase activities

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