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# Genetic and Enzymatic Characterization of the Inducible Glycerol Dissimilatory System of *Neurospora crassa*

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The glycerol dissimilatory system in *Neurospora crassa* was analyzed through the characterization of 18 Glp$^-$ mutants which were isolated after inositol-less death and filtration enrichment. All mutants obtained by this procedure could be assigned to one of three complementation groups. The subsequent genetic characterization of these *glp* mutations revealed lesions on the I, II, and VI chromosomes at the *glp-1*, *glp-2*, and *glp-4* loci, each of which was subjected to fine-structure analysis. Evidence from the enzymatic characterization of these mutants indicated that *glp-2* and *glp-4* were the structural genes encoding the mitochondrial glycerol-3-phosphate dehydrogenase and cytosolic glycerokinase, respectively. Additional evidence, obtained from studies of the inducibility of glycerokinase by glycerol, cold treatment, or deoxyribose, suggests that *glp-1* is involved in controlling the expression of *glp-4*.

The inducible enzyme system required for the dissimilation of glycerol in *Neurospora crassa* consists of cytosolic glycerol kinase (GK) (ATP:glycerol 3-phosphotransferase, EC 2.7.1.30) and a mitochondrial glycerol-3-phosphate (G3P) dehydrogenase [*sn*-G3P:(acceptor) oxidoreductase, EC 1.1.99.5] (5). Induction of GK and G3P dehydrogenase is glycerol specific, but the synthesis of these two enzymes is noncoordinate (6). These observations not only differ from those found for glycerophosphate (Glp) systems in procaryotes, such as *Escherichia coli* (10), but they also contrast with those found for *Saccharomyces cerevisiae*, in which induction of GK and G3P dehydrogenase can be effected by ethanol and lactate as well as by glycerol (22).

In addition to GK and G3P dehydrogenase, several other enzymatic activities involved with triose and glyceraldehyde metabolism have been detected in sucrose-grown *N. crassa*, leading to the suggestion that there are several alternative pathways of glycerol metabolism in this ascomycete (23). However, the activities of these enzymes are relatively low in comparison to GK and G3P dehydrogenase, and none of these other enzymes is apparently induced by growth on glycerol, dihydroxyacetone, or glyceraldehyde.

As a means of elucidating in *N. crassa* both the glycerol metabolic pathways and the relevant genetic regulatory mechanisms, glycerol-nonutilizing mutants were isolated from a strain previously derived for its ability to grow on glycerol without accompanying conidiation (7). In this report, we describe the biochemical and genetic properties of 18 independently isolated Glp$^-$ strains.

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## MATERIALS AND METHODS

***Neurospora* strains.** Strains of *N. crassa* employed in this study were obtained from the Fungal Genetics Stock Center, Arcata, Calif. The wild-type strain was St. Lawrence 74-OR23-1A (74A). The genotype of strain 33, used for the isolation of Glp$^-$ derivatives, was *glp-3 inl*(Ts) *ylo-1*. Other strains used in this analysis have been listed in an earlier publication (9).

**Growth conditions and enzymatic assays.** Throughout this investigation, cells were routinely grown on the minimal medium of Vogel (25) containing either 40 mM sodium acetate, 100 mM glycerol, or 100 mM respective sugar as the sole carbon source. Linear growth rates were measured in tubes (1.2 [inside diameter] by 32 cm) containing 0.85% Ionagar (Oxoid) plus glycerol. Cell extracts were prepared, and GK and G3P dehydrogenase activities were measured, as previously described (9). Glyceraldehyde kinase (EC 2.7.1.28) and NADP-dependent glycerol dehydrogenase (EC 1.1.1.6) were assayed by using the procedure of Viswanath-Reddy et al. (24). Enzymatic activities are expressed as nanomoles of product formed per minute per milligram of protein. Protein concentrations were determined by the method of Lowry et al. (15).

**Biochemical determinations.** The method of Kinsey (13) was adapted for the determination of GK cross-reacting material (CRM) levels present in glycerol-grown extracts, using antibodies directed against 40-fold purified wild-type GK (6). The $K_m$ for ATP of GK in crude cytosolic preparations was calculated from

duplicate determinations by plotting 1/v versus 1/S by the method of Lineweaver and Burk (14).

The procedure for measuring [$^{14}$C]glycerol metabolism was essentially that of Sprague and Cronan (22). Conidia, inoculated into minimal acetate medium, were adjusted in the same medium to a concentration of 7 × $10^5$/ml after 12 h of incubation. [$^{14}$C]glycerol (19.9 μCi/mmol) was added at a concentration of 50 nCi/ml, and 1.0-ml samples were collected at hourly intervals on filter paper and washed successively with 10 ml of minimal acetate-glycerol medium, 10 ml of 5% trichloroacetic acid–1% glycerol, 6 ml of 1:1 ethyl ether-methanol, and 4 ml of ethyl ether. The filters containing the samples were then placed in 10 ml of scintillation cocktail and counted. The uptake of glycerol under these conditions was linear with time.

**Electrophoresis of G3P dehydrogenase.** Solubilized mitochondrial extracts, obtained after lysis of the mitochondria with 1% Nonidet P-40, were layered onto gels containing 1.8 ml of 4% acrylamide, 0.05 M glycine (pH 9.5), 10% glycerol, 0.08% ammonium persulfate, and 0.02% Nonidet P-40 and electrophoresed in 0.05 M glycine (pH 9.5) at 2 mA per gel for 3 h. The position of the G3P dehydrogenase in the gels was determined by immersing the gels for 10 min in enzyme stain (0.083 M $K_2HPO_4$ [pH 7.8], 1% bovine serum albumin, 0.1% phenazine methosulfate, and 0.2% nitro blue tetrazolium [2,2′-di-*p*-nitrophenyl-5-5′-diphenyl-3,3′-(3,3′-dimethoxy-4,4′-diphenylene) ditetrazolium chloride]), containing 0.2 M G3P as the substrate. Protein bands were detected by staining with 0.05% Coomassie blue in 7% acetic acid for 3 h and destaining with 7% acetic acid–10% methanol for 48 h.

**Mutant isolation.** Ace$^+$ Glp$^-$ mutants were derived from strain 33 after UV light or nitrosoguanidine mutagenesis. After a period (approximately 120 h) of inositol-less death and filtration enrichment at 37°C, surviving cells were plated onto minimal acetate medium, and colonies which formed on these plates after incubation at 25°C were transferred to sucrose slants and saved for subsequent screening. Mutant phenotypes were determined by comparison of growth on minimal acetate or glycerol medium. Mutants obtained by this procedure were able to grow on minimal mannitol, sorbitol, and xylitol media and therefore were not *polyol*$^-$ protoperithecial-type mutants (24). Isolates which yielded a mycelial mass on acetate at least four times greater than that on glycerol were used in this study. Revertants were selected as growing mycelial masses in minimal glycerol medium inoculated with parental or UV-treated conidia.

**Genetic analysis.** Genetic crosses and complementation analyses were carried out according to standard procedures (8). The Glp$^+$ phenotype was routinely checked by determining growth in minimal liquid glycerol medium. For fine structure analysis, heat-shocked ascospores, at a concentration of 100 to 200 per tube, were inoculated into 2 ml of liquid minimal glycerol medium, as used for Qa$^+$ detection (2). Poisson analysis of the number of tubes yielding Glp$^+$ recombinants indicated that growth in all tubes scored as positive was the result of a single recombinant spore. Total viable ascospores were determined by plating samples on complete medium containing glycerol and sorbose (9).

## RESULTS

**Enzymatic deficiencies in Glp$^-$ strains.** As a result of the filtration enrichment and inositol-less death selection, 18 Glp$^-$ strains were isolated. Cultures of each were grown to stationary phase on glucose minimal medium and transferred to either glycerol- or acetate-containing minimal medium. Homogenates were prepared from each culture, and GK and G3P dehydrogenase activities were determined (Table 1). The data in Table 1 are grouped according to the enzymatic phenotype: class I mutants with demonstrable amounts of glycerokinase and near-normal G3P dehydrogenase activities, class II mutants with no detectable G3P dehydrogenase, and class III mutants with no detectable glycerokinase activity.

TABLE 1. Growth properties and enzymatic activities of Glp$^-$ mutants

| Strain | Growth[a] | | Sp act[b] | | | |
|---|---|---|---|---|---|---|
| | | | GK | | G3P dehydrogenase | |
| | Acetate | Glycerol | Acetate | Glycerol | Acetate | Glycerol |
| Wild type | 16.0 | 107.3 | 0.9 | 32.6 | 62.3 | 342.2 |
| Class I[c] | 13.4 | 0 | 0.3 | 5.8[d] | 45.1 | 198.5[e] |
| Class II | 13.6 | 0 | 0.3 | 9.7 | <0.1 | <0.1 |
| Class III | 13.1 | 0 | <0.1 | <0.1 | 55.1 | 290.9 |

[a] Weight in milligrams of dried mycelial pad after 4 days of growth at 25°C in 20 ml of minimal medium containing the indicated carbon source.

[b] Specific activities of GK and G3P dehydrogenase were determined in the cytosolic and mitochondrial fractions, respectively, of homogenates prepared from cultures grown for 16 h in minimal medium containing the indicated carbon source. Specific activity was determined as described in reference 9 and expressed as nanomoles per minute per milligram of protein.

[c] Class designation is based on enzymatic properties. Class I: *B-32*, *2-4*, and *2-49* strains; class II: *A-25*, *A-55*, *A-74*, *B-7*, *B-95*, *D-1*, *D-14*, and *1-9* strains; class III: *A-71*, *A-73*, *D-41*, *1-43*, *2-42*, *2-92*, and *2-100* strains.

[d] Range in GK activities was 2.2 to 7.7 for class I, 8.0 to 11.5 for class II, and less than 0.1 for class III strains.

[e] Range in G3P dehydrogenase activities was 176.5 to 210.3 for class I, less than 0.1 for class II, and 223.5 to 336.2 for class III strains.

**Genetic analysis.** The genetic analysis of these mutants was initiated by inoculating conidia onto minimal sucrose slants in pairwise combinations and testing the resultant heterokaryons for their ability to use glycerol. This complementation analysis revealed three distinct complementation classes identical to the three phenotypic classes described in Table 1. To ensure that heterokaryons had been formed under these nonforcing conditions, $pan^- \, glp^-$ or $inl(Ts) \, glp^-$ derivatives of all mutants were constructed, and [$pan^- \, glp^- + inl(Ts) \, glp^-$] heterokaryons were selected by growth on minimal sucrose medium at 37°C. Upon retesting of these forced heterokaryons on minimal glycerol medium, no additional instances of $Glp^+$ complementation were detected among the intraclass heterokaryons.

The class I mutants were crossed to a strain containing mutations at the chromosome I loci *al-2* and *arg-13*. Recombination frequencies between these markers and the affected *glp* locus revealed the gene order to be centromere–*glp*–*al-2*–*arg-13*, suggesting that the mutations in these strains are allelic to the previously described *glp-1* locus (12, 16, 24). The mutants in class II were analyzed by crosses to a strain with chromosome II markers *arg-5* and *fl*. These crosses showed the position of the class II *glp* locus to be approximately 9.5 centimorgans to the right of *arg-5*, a position previously reported for the *glp-2* mutation in the *33-14* and *33-17* strains (9). Finally, mutants of class III were crossed to a strain with *cys-1* and *ylo-1* mutations on the left arm of chromosome VI. All class III mutations mapped to a position 6.6 centimorgans to the right of *ylo-1*, very near the centromere, and apparently are allelic to *glp-4* (24).

**Fine-structure analysis.** In the course of localizing the *glp-1*, *glp-2*, and *glp-4* genes, some variation in the map distances, amounting to 1 to 2 centimorgans, was encountered in several crosses. To examine the genetic structure of these loci more closely, several pairwise crosses were set up for representative alleles of each locus. The data from these crosses are summarized in Table 2, which lists a minimum genetic distance for each locus studied. The fine-structure map for each locus was a linear, internally consistent structure. No recombination was detected between the *glp-1* alleles *B-32* and *2-4*. Likewise, alleles *17*, *A-25*, *A-74*, and *B-95* of the *glp-2* locus must be located at or near the same site, since no $Glp^+$ recombinants were obtained among a cumulative total of 50,400 ascospores analyzed. At the *glp-4* locus, there was no detectable recombination among alleles *A-71*, *1-43*, *2-42*, and *2-92*, with 18,300 ascospores analyzed.

To determine whether any of these loci represent the structural gene for either GK or G3P dehydrogenase, attempts were made to isolate revertants. Despite repeated attempts, only two revertants of class III mutations were obtained. One of these, $Glp\text{-}4^+$, the *A-73* revertant, grew at wild-type rates and possessed a GK with normal properties. However, the partial *A-71* revertant grew at wild-type rates on minimal sucrose or acetate medium, but at only 60% of the wild-type rate on minimal glycerol medium as measured in growth tubes (1.2 versus 2.0 cm/day). No differences in the electrophoretic mobility or thermal stability of the GK from the wild type and revertant strain were detected. However, the GK from the *A-71* revertant had a $K_m$ for ATP of $3.68 \times 10^{-4}$ M, which was approximately twice the value of $1.87 \times 10^{-4}$ M found for the wild-type enzyme.

**Immunochemical properties of glycerokinase.** To measure the amount of glycerokinase CRM, extracts of glycerol-induced cultures were examined for the ability to neutralize an antibody directed against glycerokinase. After correcting for the antigenic contribution of any glycerokinase activity present in mutants, the levels of enzymatically inactive CRM were calculated. No CRM was detected in class I mutants, but 5.4, 9.8, 2.0, and 12.4% of wild-type CRM levels

TABLE 2. Genetic maps of *glp* loci

| Locus | Linkage group | Gene map[a] |
|---|---|---|
| *glp-1* | IR | 0.12   0.05<br>B32 —— 234 —— 2-49 |
| *glp-2* | IIR | 0.084   0.08   0.14   0.09<br>A55 —— B7 —— 17 —— D1 —— D14 |
| *glp-4* | VIR | 0.074   0.026   0.015<br>A71 —— A73 —— D41 —— 2-100 |

[a] Genetic maps are derived from crosses involving the indicated alleles. The total number of ascospores analyzed was 20,000 for *glp-1*, 167,000 for *glp-2*, and 96,000 for *glp-4*. Genetic distances in centimorgans are given above the line; alleles mapped are given below the line. See text for other details.

were found in the class III *A-71*, *A-73*, *D-41*, and *2-92* strains, respectively.

**Induction of GK by low temperature and by deoxyribose.** To ascertain whether the inducibility of glycerokinase was impaired in class I or III mutants having decreased levels of GK, cultures from each were subjected to low-temperature incubation or to incubation in the presence of deoxyribose, conditions previously shown to be adequate for the induction of the GK in wild-type cells (17–19). These alternative inducing procedures resulted in the full induction of GK in wild-type cells, as well as in the Glp-1 *B-32*, *2-4*, and *2-49* strains (Table 3). In contrast to these results, Glp-4 mutants remained uninducible under these alternative inducing conditions.

**Glycerol metabolism in Glp-1 mutants.** Although GK can be induced in Glp-1 strains by deoxyribose, the presence of this enzyme in gratuitously induced mutants is not sufficient to allow growth of these strains in minimal glycerol medium (data not shown). Consequently, a possible explanation for the Glp-1 phenotype is that the product of this locus might be analogous to the facilitator protein for glycerol discovered in *E. coli* (11, 20). To measure glycerol metabolism, wild-type and the *B-32*, *A-71*, and *A-74* strains were grown in acetate medium with [$^{14}$C]glycerol as described above. Under these conditions, the rate of glycerol uptake by Glp-1$^-$ and Glp-2$^-$ cells was reduced to approximately one-third the rate of wild-type cells (94, 114, and 356 cpm incorporated per $10^6$ hyphae/h, respectively). However, no detectable glycerol metabolism could be demonstrated in Glp-4 cells, which suggests a need for phosphorylation of glycerol to be retained during the collection and washing of the hyphae.

**Effect of *glp-2* mutations on mitochondrial G3P dehydrogenase.** Two mutations at the *glp-2* locus have been previously found to be lacking demonstrable G3P dehydrogenase (9). To date, we have not been able to achieve a satisfactory purification of this enzyme from *Neurospora*; therefore, a specific antibody preparation to test for G3P dehydrogenase CRM was not available. As an alternative means of determining the effect of mutations at this locus on the enzyme, mitochondria obtained from glucose-grown wild-type, *D-14*, and *A-25* strains were lysed with 1% Nonidet P-40 and electrophoresed on polyacrylamide gels, which were stained for G3P dehydrogenase. Although a single zone of activity could be visualized in gels containing wild-type extracts, no G3P dehydrogenase activity was detected in the gels containing mutant extracts. Duplicate gels were stained for protein, and the results of the densitometric scans of these gels revealed that no protein component could be detected at a position in the gel which corresponds to the location of G3P dehydrogenase in wild-type extracts (data not shown).

TABLE 3. Inducibility of GK in Glp-1 and wild-type cells

| Strain | Sp act of GK | | |
|---|---|---|---|
| | Glucose 26°C | Glucose[a] 4°C | Deoxyribose[b] 26°C |
| *33* | 1.6 | 32.1 | 36.1 |
| *B-32* | 0.9 | 36.8 | 39.2 |
| *2-4* | 1.1 | 25.8 | 33.8 |
| *2-49* | ND[c] | 27.3 | 32.6 |
| *234*[d] | 1.5 | ND | 36.8 |

[a] Cells were grown for 48 h at 4°C. Specific activity is given as in Table 1.

[b] Cells were grown for 2 h at 26°C in liquid minimal medium containing only 5 mg of D-deoxyribose per ml as sole carbon source.

[c] ND, Not determined.

[d] Strain *234* was obtained from H. G. Kølmark and has a mutation at the *glp-1* locus (12).

**Glyceraldehyde kinase and glycerol dehydrogenase.** A mutant strain with a lesion at the *glp-5* locus has been described as lacking glyceraldehyde kinase activity (24). In an attempt to determine whether any of the mutants in our collection lacked this enzyme, extracts were assayed for glyceraldehyde kinase. No detectable activity was found in either wild-type or mutant extracts under any of the conditions of growth used, and further attempts to demonstrate this activity were discontinued. Glycerol dehydrogenase activities were examined in extracts of representatives of each of the different complementation groups. All mutants examined possessed a specific activity comparable to that of the parental strain.

## DISCUSSION

The experimental approach undertaken in the analysis of the glycerophosphate system in *Neurospora* has relied upon the characterization of the enzymatic or genetic defect in mutants which are unable to use glycerol as a sole source of carbon and energy. All Glp-4 mutants are totally lacking glycerokinase activity, and four of these strains possess detectable glycerokinase CRM, indicating a structural alteration in this enzyme. In addition, the *A-71* revertant, derived from the CRM$^+$ *A-71* strain, possessed a GK with an altered $K_m$ for ATP. These results support the conclusion that the *glp-4* gene represents the structural gene for GK.

The *glp-2* locus appears to be the structural gene for the mitochondrial glycerol-3-phosphate dehydrogenase. This conclusion is based on the fact that no G3P dehydrogenase activity can be detected in the mitochondria under all conditions of growth tested, and that the characteris-

tic mitochondrial protein component associated with this enzyme is apparently absent in the *D-14* and *1-25* strains. Although this evidence does not rule out a regulatory role for the product of the *glp-2* locus, the effect of mutations at this locus appears to be limited to the G3P dehydrogenase, since GK is normal and can be induced in these strains (9). In addition, the *glp-2* locus contains only a single complementation group and therefore may encode only a single polypeptide, as found for the rabbit enzyme (4). The genetic map of this locus is larger than most loci which have been analyzed in *Neurospora* (2, 3, 21), including an approximate distance of 0.11 centimorgan for the entire *aro* cluster for five complementation groups. A comparable map size of 0.4 centimorgan has been obtained for the *pan-2* locus, which contains three distinct complementation groups (1).

The role of the *glp-1* locus is still not fully understood. The product of this locus could be a regulatory protein defective in its ability to bind glycerol or one of its derivatives, resulting in decreased expression of the *glp-4* gene. Alternatively, the product of the *glp-1* locus may be involved in the formation of a facilitator protein similar to that described in the *E. coli* system (11). At present, the data are not sufficient to distinguish between these alternatives.

Although conclusive evidence for the chemical structure of the true inducer for the enzymes of the glycerophosphate system could not be determined in this study, it is worthwhile to note that a four- to fivefold increase in the activity of the mitochondrial G3P dehydrogenase could be found in both Glp-1 and Glp-4 mutants after growth on glycerol. This evidence strongly suggests that glycerol or a non-phosphorylated derivative is capable of acting as an inducer for this enzyme. Interestingly, deoxyribose did not serve as an inducer for G3P dehydrogenase (unpublished data), in contrast to the pattern found in the *glp* system of *E. coli*, where G3P acts as the inducer for both GK and G3P dehydrogenase.

It has been proposed that there are alternative pathways for the dissimilation of glycerol in *Neurospora* (23, 24) resulting in the formation of dihydroxyacetone or glyceraldehyde and converging with the formation of 3-phosphoglycerate. However, none of the enzymes catalyzing these reactions is induced after growth on glycerol, dihydroxyacetone, or glyceraldehyde, and some are actually reduced in several instances (23). It should be pointed out, moreover, that the activities of these enzymes are up to 15-fold lower than those of the GK and the mitochondrial G3P dehydrogenase. If these alternative pathways for the dissimilation of glycerol do exist, the fact that glycerol-nonutilizing mutants in the present study could be derived in a single step suggests that the primary pathway for glycerol utilization is by means of phosphorylation and subsequent oxidation of the resultant G3P. In addition, despite the extensive search for glycerol-defective strains, mutations at loci other than *glp-1*, *glp-2*, and *glp-4* were not detected. It therefore seems likely that these other enzymes catalyzing reactions with triose phosphates have primary metabolic roles other than the dissimilation of glycerol in *Neurospora*.


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