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Morphology-Associated Expression of Nicotinamide Adenine Dinucleotide-Dependent Glutamate Dehydrogenase in *Mucor racemosus*

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The in vivo regulation of glutamate dehydrogenase (GDH) was studied in *Mucor racemosus* as a function of nutritional conditions and morphological state. Both nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP)-dependent GDH activities were found. The effect of carbon and nitrogen source on the specific activity of the NAD-dependent GDH suggests that its role is primarily catabolic. The NAD-dependent activity was generally an order of magnitude greater in mycelial cells than in yeast-phase cells grown on the same medium. During yeast-to-hyphal morphogenesis the increase in NAD-dependent activity preceded the appearance of hyphal cells both under aerobic and anaerobic conditions. Exogenous dibutyryl-cyclic AMP prevented the increase in NAD-dependent GDH concomitantly with the suppression of morphological differentiation. The NADP-dependent activity did not change appreciably during morphogenesis.

Mucor racemosus is a dimorphic phycomycete which grows vegetatively either in a filamentous or in a yeast phase. The pathway of morphological development is controlled primarily by the incubation atmosphere of the culture. In general, under aerobic conditions development is in the mycelial phase whereas under CO_2 growth is in the yeast phase (1). Under anaerobiosis in the absence of CO_2 either morphology can be produced depending on culture conditions (14). Our studies suggest that dimorphism is controlled by multiple metabolic signals. Within a range of growth conditions the nucleotide, cyclic 3',5'-AMP (cAMP), is involved in the control of morphology (10, 16), whereas under another set of conditions development is controlled by a volatile morphogen (14, 16). Our aim is to define the morphological differentiation in terms of biochemical correlates and to determine the molecular events which control gene expression during the process.

Mucor is more fastidious in the yeast phase than in the mycelial phase with respect to both carbon and nitrogen sources. Cells in the yeast phase have an absolute requirement for a fermentable hexose whereas in the mycelial phase a wide variety of carbon sources can be used (2). Complex nitrogen sources, e.g., yeast extract and peptone or an amino acid mixture, are required for optimal growth of yeast cells, whereas rapid growth of mycelial cells occurs with an ammonium salt as the sole nitrogen source (5). The more stringent growth requirements of yeast cells as compared to mycelial cells suggested that some key enzymes of carbon and nitrogen metabolism might be differentially expressed in the two morphological forms.

The reductive amination of α-ketoglutarate, forming glutamate, and the dissimilation of glutamate, releasing ammonia and permitting the entry of glutamate carbon into the tricarboxylic acid cycle, are the reactions catalyzed by glutamate dehydrogenase (GDH). Since this enzyme is at a key point in the juncture of carbon and nitrogen metabolism, we examined the expression of NAD-dependent GDH (EC 1.4.1.2; L-glutamate:NAD oxidoreductase, deaminating) and NADP-dependent GDH (EC 1.4.1.4; L-glutamate:NADP oxidoreductase, deaminating) in yeast and mycelial cultures and during the dimorphic transition in *M. racemosus*.

MATERIALS AND METHODS

Organism. *M. racemosus* (*M. lusitanicus*) ATCC 1216 B was used throughout.

Media. Yeast extract/peptone (YP) broth, supplemented with 2.0% (wt/vol) glucose unless otherwise noted, was the complex medium (1). The minimal medium used, which supports the growth of both yeast- and mycelial-phase cells, has been previously described (17). For one set of experiments, various

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components were omitted from the minimal medium as indicated in Table 1. Media were adjusted to pH 4.5 with sulfuric acid.

Culture conditions. Growth media inoculated with sporangiospores at 5×10^5/ml were incubated with vigorous shaking at 20 to 22°C. Aerobic mycelial cultures (100 ml) were grown in 2,800-ml Fernbach flasks for 13 to 14 h. Yeast-phase cultures (50 or 100 ml) were grown to 40 Klett units in 500-ml flasks by sparging the medium with CO_2 at a flow rate of 0.20 volume of gas/min per volume of culture. In CO_2-to-air shifts, exponentially growing yeast-phase cultures were transferred to 2,800-ml flasks for subsequent aerobic growth. In anaerobic shifts, 80-ml cultures were grown in 500-ml side-arm flasks. The sparging gas was changed from CO_2 to N_2, the flow rate remaining constant at 0.20 volume of gas/min per volume of culture. At the time of the shift, 1 ml of 7% wt/vol KOH solution was put into the side arm to trap residual and metabolically generated CO_2.

Preparation of cell extracts. Cells were collected on membrane filters (type HA, pore size 0.45 μm, Millipore Corp.), washed with chilled potassium phosphate buffer (200 mM, pH 8.0), and broken in a chilled French pressure cell. The extract was centrifuged at 5,000 × *g* for 15 min, and the supernatant fluid was used as the source of the enzyme. After samples were withdrawn for protein determination, β-mercaptoethanol was added to a final concentration of 1 mM in the extracts.

GDH assays. GDH activity was measured at 20 to 22°C in the direction of reductive amination of α-ketoglutarate by following the decline of absorbance of NADH or NADPH at 340 nm in an Hitachi Perkin Elmer model 124 double-beam spectrophotometer. Both the standard and sample cuvettes contained the enzyme extract, NH_4Cl, and α-ketoglutarate at a final concentration of 200 mM and 30 mM, respectively, in 100 mM, pH 8.0 potassium phosphate buffer. The reaction was started by the addition of 0.125 mM NADH or NADPH to the sample cuvette. A control was run after each assay in which the substrates α-ketoglutarate and/or NH_4Cl were omitted from the reaction mixture. The difference in the rate with and without substrates was taken as the measure of NAD- or NADP-dependent GDH activity. This correction was generally small except in the case of cultures grown aerobically in the presence of dibutyryl-cAMP which had low NAD-dependent GDH activity and relatively high NADH dehydrogenase activity. Specific activity was expressed as micromoles of NADH or NADPH oxidized per minute per milligram of protein.

Determination of morphology. Just prior to harvesting for GDH assays, an aliquot from each culture was examined microscopically on a hemocytometer grid. Single spherical cells or spherical cells with unabscissed buds were counted as one yeast morphological unit; cells with one or more germ tube were counted as one hyphal morphological unit. For each experimental point, 1×10^2 to 3×10^2 cells were counted. The morphological determinations and enzyme assays shown in Fig. 1 to 4 were done using two or more independent cultures.

RESULTS

The specific activity of the NAD and NADP-dependent GDH was measured as a function of the glucose concentration in extracts from CO_2-grown yeasts and from aerobically grown mycelial cultures. Increasing the concentration of glucose in YP medium resulted in lowering both the NAD and NADP-dependent activities for both morphological types (Fig. 1A and B); largest glucose repression was found in the case of the NAD-dependent activity from mycelial cultures. Since yeast cells have an absolute requirement for a hexose carbon source (2), the lowest glucose concentration at which yeast cultures were grown was 1.0% (wt/vol). The specific activity of the NAD-dependent GDH was 25- to 35-fold lower in yeast cultures than in mycelial cultures over the range of glucose concentration from 1.0 to 10% (wt/vol) in YP medium (Fig. 1A). In contrast, the specific activity of the NADP-dependent GDH was two- to three-fold higher in yeast cultures than in mycelial cultures over the same range of glucose concentration.

NAD and NADP-dependent GDH activities were also determined in mycelial and yeast cultures grown in complete minimal medium containing amino acids, NH_4Cl, and glucose and on

TABLE 1. *Effect of carbon and nitrogen source on the specific activity of NAD- and NADP-GDH in mycelial- and yeast-phase cultures*

Medium	Sp act[a]			
	NAD-GDH		NADP-GDH	
	Mycelia	Yeast	Mycelia	Yeast
Amino acids	4.3		1.1×10^{-2}	
Amino acids + NH_4Cl	5.6		2.1×10^{-2}	
NH_4Cl + glucose	2.7×10^{-1}		3.5×10^{-3}	
Amino acids + glucose	5.3×10^{-1}	3.0×10^{-2}	3.4×10^{-3}	2.6×10^{-2}
Amino acids + NH_4Cl + glucose	1.6×10^{-1}	2.8×10^{-2}	0.8×10^{-3}	2.2×10^{-2}

[a] In micromoles of NADH or NADPH oxidized per minute per milligram of protein.

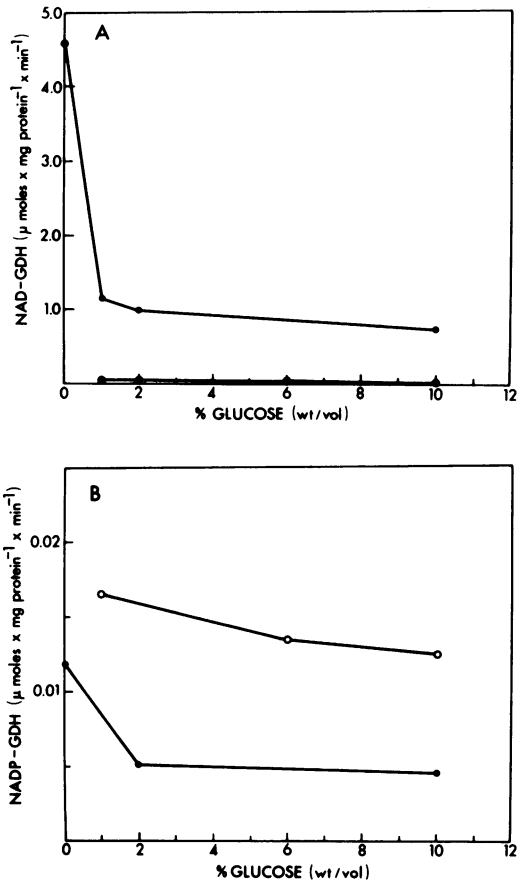

FIG. 1. *Effect of glucose concentration in YP medium on the specific activity of (A) NAD-dependent GDH and (B) NADP-dependent GDH of mycelial* (●) *and yeast-phase cells* (○).

media from which one or more of these components had been omitted (Table 1). Very high NAD-dependent activity was found in mycelial cells when the amino acid mixture was the sole carbon source either with or without NH_4Cl. The addition of glucose to the amino acid medium resulted in an eightfold decrease of the NAD-dependent activity in the medium without NH_4Cl and a 34-fold decrease in the medium with NH_4Cl. The NADP-dependent activity was also repressed 3- to 26-fold by the addition of glucose to the amino acid medium in mycelial cultures. The omission of components from the complete minimal medium did not affect the rate of aerobic germination, whereas the omission of glucose completely prevented germination and growth under CO_2. The NAD-dependent activity was depressed 6- to 17-fold in yeast cells as compared to mycelial cells grown on the same medium whereas the NADP-dependent activity was 8- to 27-fold higher in yeast cells than in mycelial cells grown on the same medium. In mycelial cells, the NADP-dependent activity constituted only a minor fraction of the total GDH activity, even when NH_4Cl was provided as the sole nitrogen source. In yeast cells the two activities were present at comparable levels.

From the foregoing results, it appeared that GDH activity was regulated at two distinct levels: one in response to changes in nutritional conditions and the second in response to morphological signals. To assess the morphology-associated component of the regulation, yeast cultures growing in YP-glucose medium were shifted from CO_2 to air. Growth was allowed to continue in the same medium after the shift while morphological differentiation and GDH activities were monitored. The cells continued to grow in the yeast phase for about 2 h after the shift. Germ tubes began to appear after 3 h of aerobic growth. The proportion of hyphal cells continued to increase during the next 3 h with the most rapid increase occurring between the fourth and fifth hours after the shift (Fig. 2A). An increase in NAD-dependent activity preceded the morphological change by about an hour, the pattern of increase of enzymatic activity closely paralleling the pattern of increase of hyphal cells (Fig. 2A). The NADP-dependent activity did not change significantly for 6 h following the shift (Fig. 2B). Cycloheximide (200 μg/ml), which completely inhibits protein synthesis in this organism (15), was added to the culture at the time of the shift or 2 h after the shift. The inhibitor prevented both the increase in NAD-dependent GDH activity and the emergence of germ tubes (data not shown).

In addition to the shift from CO_2 to air, the yeast-to-mycelial phase transition can be induced entirely under anaerobic conditions by changing the sparging gas from 100% CO_2 to 100% N_2 at low flow rates (14). The NAD-dependent GDH activity also increased in the anaerobic shift, and the pattern of increase was similar to that found in the aerobic shift (Fig. 3). The two major differences were that the lag between the increase in enzymatic activity and the increase in the proportion of hyphal cells was shorter under anaerobic conditions, and the plateau of activity under anaerobic conditions was about one-third that attained in the aerobic shift. These studies showed that the synthesis of NAD-dependent GDH did not require oxygen.

The addition of dibutyryl-cAMP (dbcAMP) to the culture at the time of the shift from CO_2 to air suppresses hyphal development and maintains the culture in the yeast phase during aero-

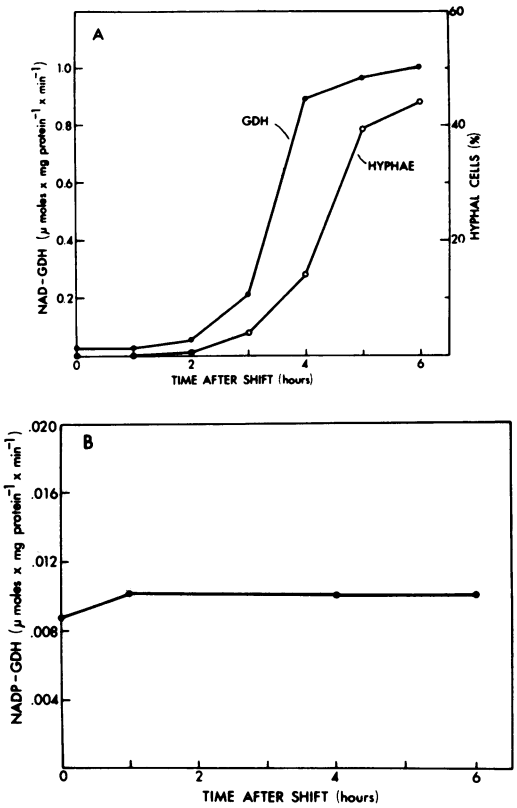

FIG. 2. *Specific activity of (A) NAD-dependent GDH and (B) NADP-dependent GDH during yeast-to-hyphal transition in a CO_2-to-air shift. Specific activity of NAD(P)-dependent GDH* (●) *and percent hyphal cells* (○).

bic growth (10). We determined whether, in a shift from CO_2 to air in the presence of dbcAMP, the increase in NAD-dependent GDH activity is prevented concomitantly with hyphal development. These experiments were done in minimal medium containing amino acids, NH_4Cl, and glucose since the morphological effect of dbcAMP is evident at about threefold-lower concentration in minimal medium than in YP-glucose broth (10). After 6 h of aerobic growth, 57% of the cells in the control culture had hyphal projections whereas over 98% of the cells in the culture with 3 mM and 6 mM dbcAMP remained yeastlike (Fig. 4A). During this period there was a 14-fold increase in the specific activity of the NAD-dependent GDH in the control culture. In the presence of 3 mM dbcAMP, there was a fourfold increase in the specific activity of the enzyme and in the presence of 6 mM dbcAMP no increase occurred over the level found in CO_2-grown yeast cultures. There was a transient decrease in the specific activity of NAD-dependent GDH to very low levels between 2 and 4 h of aerobic growth in the presence of either 3 or 6 mM dbcAMP (Fig. 4B).

DISCUSSION

In a survey of the properties of fungal GDHs, all higher fungi (deuteromycetes, ascomycetes, and basidiomycetes) were found to have two distinct enzymes: one NAD-dependent and one NADP-dependent, whereas more than 40 species of lower fungi (myxomycetes and phycomycetes) had only the NAD-dependent enzyme (11). It has been argued that an ancestral phycomycete must have arisen which had both NAD and NADP-dependent GDH (12). Among the lower fungi, oomycetes and hypochytridiomycetes have an unusual NAD-dependent GDH which may represent a transitional form (12). This type of NAD-dependent GDH is allosterically regulated by NADP and is kinetically similar to the NADP-dependent GDH of higher fungi (21). In this study we found that the phycomycete *M. racemosus* has both NAD- and NADP-dependent GDH activity. In mycelial-phase cells, the NADP-dependent GDH constitutes only a minor fraction of the total GDH activity, whereas in yeast-phase cells the specific activities of NAD- and NADP-dependent enzymes are comparable.

A large body of evidence from the study of in vivo regulation of GDH (4, 7, 8, 18, 20) and the isolation of mutants deficient in NAD- or NADP-dependent GDH (6, 9, 19) in higher fungi indicates that the NAD-dependent GDH functions primarily as a catabolic enzyme in the direction of oxidative deamination of glutamate and the NADP-dependent GDH functions primarily as a biosynthetic enzyme in the direction of reductive amination of α-ketoglutarate. In *M. racemosus* the highest NAD-dependent activity was found in mycelial cells grown in YP broth without glucose or in minimal medium in which a mixture of amino acids was the sole carbon source. The addition of glucose to these media resulted in the repression of the NAD-dependent enzyme by an order of magnitude. Preferential utilization of glucose as the carbon source lessens the need for enzymes of amino acid catabolism and such enzymes are commonly regulated by carbon catabolite repression (3, 13). A threefold nitrogen catabolite repression of the NAD-dependent enzyme was found in mycelial cells upon the addition of NH_4Cl to the defined medium containing glucose and amino acids. Thus the pattern of regulation of NAD-dependent GDH by carbon and nitrogen catabolite repression is consistent with a primarily catabolic role of this enzyme in mycelial cells. In yeast-phase

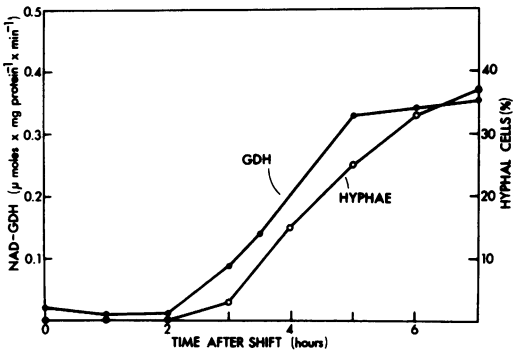

FIG. 3. *Specific activity of NAD-dependent GDH during yeast-to-hyphal transition in a CO_2-to-N_2 shift. Specific activity of NAD-dependent GDH* (●) *and percent hyphal cells* (○).

cells the specific activity of the NAD-dependent GDH was low under all conditions tested, and no significant changes occurred in response to changes of glucose concentration of the medium or upon the addition of NH_4Cl.

The regulation of the NADP-dependent GDH of *M. racemosus* is atypical in several respects in comparison to the NADP-dependent GDH of higher fungi. The NADP-dependent activity was repressed by glucose although the amplitude of repression was not as large as that of the NAD-dependent enzyme. In contrast, the two GDH activities in higher fungi are regulated reciprocally by glucose, the NADP-dependent enzyme being glucose inducible and the NAD-dependent enzyme glucose being repressible (4, 7, 8). The specific activity of the NADP-dependent GDH is high in cells grown in media with an inorganic nitrogen source in those fungi where this enzyme constitutes the main pathway of ammonia assimilation (18, 23). This was not found to be the case with *M. racemosus*; the specific activity of the NADP-dependent enzyme remained low in mycelial cells whether NH_4Cl or a mixture of amino acids was provided as the nitrogen source. Since mycelial cells can use inorganic nitrogen salts efficiently but yeast cells require a complex nitrogen source for rapid growth, it may be expected that the NADP-dependent GDH is higher in mycelial- than in yeast-phase cells. However, in both complex and defined media, yeast-phase cells had severalfold greater NADP-dependent activity than mycelial-phase cells grown in the same medium. Thus it appears that GDH is not the main enzyme of ammonia assimilation in *M. racemosus*. These considerations prompted us to look for an alternate pathway and we will present evidence elsewhere that ammonia fixation in this organism occurs by the glutamine synthetase/glutamate synthase pathway.

We used three criteria to determine whether the differential expression of GDH activities in the yeast and mycelial phases of *M. racemosus* is a biochemical correlate of the morphological differentiation. First, a biochemical correlate should precede the morphological outcome. The pattern of increase in the NAD-dependent GDH during the transition from the yeast to mycelial phase paralleled the appearance of hyphal cells very closely under two different sets of conditions. The increase in NAD-dependent activity preceded the morphological change by about 1 h in CO_2-to-air shifts and by a shorter interval in CO_2-to-N_2 shifts. Secondly, the biochemical correlate should be sensitive to signals which affect the morphological outcome. The signal inducing the transition from yeastlike to hyphal growth appears to be the removal of CO_2. The superimposition of a second morphological signal, i.e., the addition of dbcAMP at the time of the shift from CO_2 to air which has been shown to suppress the emergence of germ tubes (10), concomitantly prevented the increase in NAD-dependent GDH. In contrast, the development of respiratory capacity is associated directly with

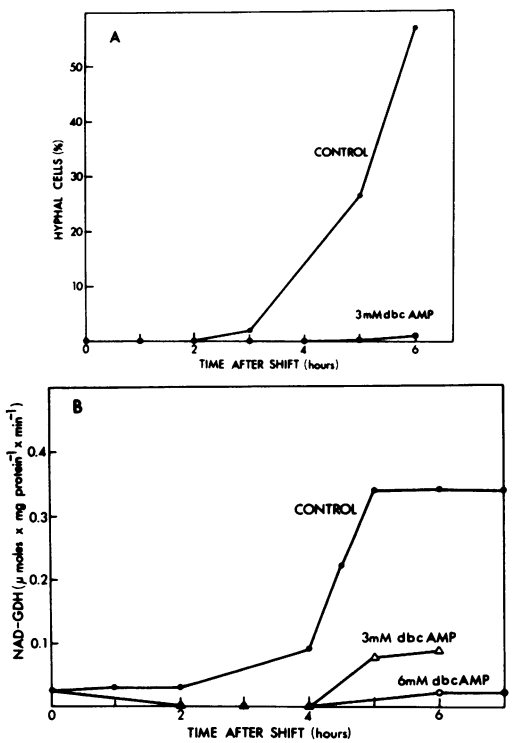

FIG. 4. *Effect of dbcAMP on (A) morphology and (B) specific activity of NAD-dependent GDH during CO_2-to-air shifts. Percent hyphal cells without dbcAMP* (●) *and with 3 mM dbcAMP* (○). *Specific activity of NAD-dependent GDH without dbcAMP* (●), *with 3 mM dbcAMP* (△) *and with 6 mM dbcAMP* (○).

the shift to aerobic conditions and is not affected by the addition of dbcAMP; it was therefore concluded that respiratory capacity is not an obligatory correlate of differentiation (16). The third criteria of a morphological correlate was that the biochemical change should occur under different nutritional conditions where the morphological change also occurs. The time course of the increase in the NAD-dependent activity was similar in complex and minimal medium in CO_2-to-air shifts; only the magnitude of the change was dependent on the medium indicating some measure of adaptive physiological control. One pathway of yeast-to-mycelial morphogenesis includes the following sequence of events: a drop in the intracellular cAMP concentration which occurs within 30 min after the removal of CO_2 (10), the cessation of budding after about 2 h, an increase in the NAD-dependent GDH beginning at about 3 h after the shift, and the appearance of hyphal cells after 4 h under aerobic conditions or after a shorter lag under N_2.

The data presented here show that there is a close correlation in the morphological transition of *M. racemosus* and in the expression of NAD-dependent GDH. The possible causal involvement of this enzyme, or the consequences of its activity, in morphogenesis will have to await thorough characterization of appropriate mutants. We do have preliminary information from mutant studies. One morphology mutant of *M. racemosus* fails to form hyphae in air in the absence of exogenous methionine. Under these conditions the mutant fails to respond as the wild-type organism does with a large increase in the rate of NAD-GDH synthesis. We have also studied the morphology mutant of *M. bacilliformis* isolated by Storck and Morrill (22). This mutant, which is respiratory deficient, does not increase its rate of NAD-GDH synthesis when exposed to air, although the wild-type organism does.

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