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Nitrogen Source Regulates Glutamate Dehydrogenase NADP Synthesis in *Neurospora crassa*

GEORGINA HERNÁNDEZ, RAY SÁNCHEZ-PESCADOR, RAFAEL PALACIOS, AND JAIME MORA*
Centro de Investigación sobre Fijación de Nitrógeno, UNAM, Cuernavaca, Mor., Mexico

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Neurospora crassa glutamate dehydrogenase-NADP (EC 1.3.1.3) has a higher activity when mycelium is grown on ammonium or nitrate as nitrogen source than when grown on glutamate or glutamine. Quantitative immunoelectrophoresis established that, under all conditions, enzyme activity corresponded to enzyme concentration. Isotope incorporation studies demonstrated that the nitrogen source exerts its regulation at the level of de novo enzyme synthesis.

Different species of yeasts and filamentous fungi such as *Neurospora crassa* and *Aspergillus nidulans* possess two glutamate dehydrogenases (4, 20, 23). One depends on NADP and has a biosynthetic role, whereas the other depends on NAD and catabolizes glutamate. There have been reports on the metabolic regulation of glutamate dehydrogenase (GDH)-NADP in some of these microorganisms (6, 7, 11, 19, 27). Pateman has studied the changes in activity of this enzyme in *A. nidulans* and claims that *N. crassa* exhibits a similar pattern of regulation (17). Although there have been some reports on the effect of nitrogen nutrition on the activity of *N. crassa* GDH-NADP (5, 9, 24), there is not much information about the regulation of this enzyme (18). On the other hand, the detailed genetic and structural studies of Fincham and colleagues (3, 10, 12, 25, 26) have established the oligomeric structure and the amino acid sequence of the enzyme and have demonstrated the colinearity between the mutational sites and the amino acid positions in the polypeptide.

Our studies of nitrogen assimilation in *N. crassa* have indicated the existence of two different pathways that operate under high or low ammonium concentration. Under ammonium excess, GDH-NADP and octameric glutamine synthetase (GS), formed by β monomers, participate in ammonium assimilation. In contrast, when ammonium is limiting, a tetrameric GS formed by α monomers and an NADH-dependent glutamate synthase (GOGAT) are responsible for nitrogen assimilation (13, 17). We have reported the metabolic regulation of GS (28) and GOGAT (14) and have established that in the case of GS the nitrogen source regulates enzyme activity through changes in the rate of synthesis that correspond to specific mRNA levels (21, 22). Central in understanding ammonium assimilation is to establish the levels at which regulatory controls operate in the corresponding pathways. In this paper we report that the regulation of GDH-NADP by the nitrogen source is exerted at the level of specific enzyme synthesis.

N. crassa wild-type strain 74-A was grown after inoculating conidia on Vogel minimal medium (28, 29) with 1.5% sucrose in the presence of different nitrogen sources. The inorganic nitrogen source used was nitrate or ammonium; the latter is one of the substrates of GDH-NADP. The organic nitrogen sources used were glutamate or glutamine, the products of the two enzymes that can fix ammonium: GDH-NADP and GS, respectively. Doubling times for the growth of *N. crassa* wild-type strain 74-A on nitrate, ammonium, glutamate, or glutamine as the nitrogen source were 4, 3, 3.25, and 2 h, respectively (Fig. 1A). GDH-NADP activity, measured as described by Fincham (8), was low in spores and increased during outgrowth on either culture. Enzyme activity reached higher levels when grown on ammonium or nitrate, especially after 12 h of growth. The levels reached in the inorganic nitrogen sources were about threefold higher than those present when grown on glutamate or glutamine (Fig. 1B). GDH-NADP specific activity does not reach a steady state during exponential growth (Fig. 2B); we have also reported this for GS concentration (28). As we have proposed (28), the absence of a steady state in GS and GDH-NADP specific activities may be related to the fact that *Neurospora* spp. are filamentous organisms in which developmental processes are not excluded.

To quantify enzyme concentration and in vivo synthesis, immunological approaches were followed. The enzyme was purified according to Blumenthal and Smith (1), with the following modifications: mycelia were obtained from cultures grown for 12 h on Vogel minimal medium

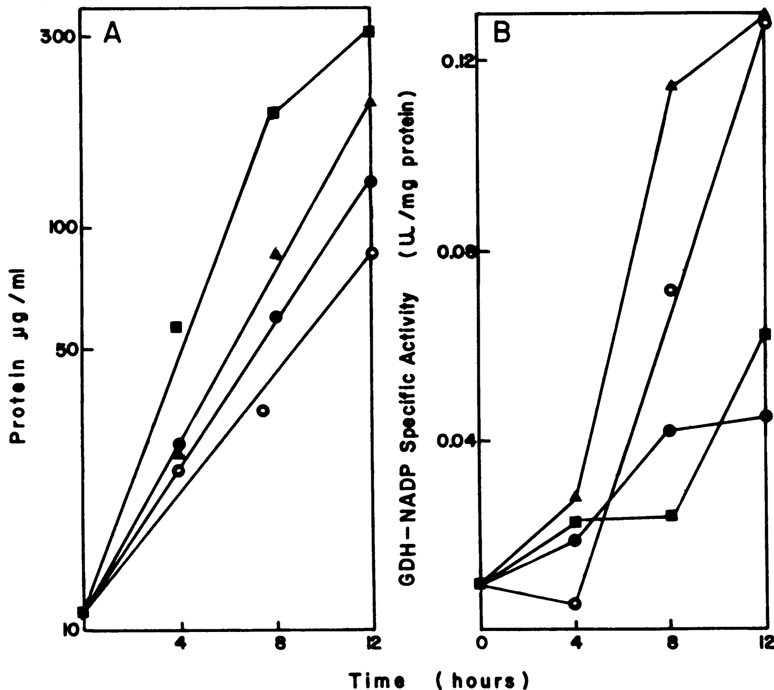

FIG. 1. Growth and GDH-NADP activity of *N. crassa* grown on different nitrogen sources. Strain 74-A was grown on Vogel minimal medium containing 25 mM NH_4Cl, 25 mM KNO_3, 5 mM glutamate, or 5 mM glutamine as nitrogen source. (A) Protein content per milliliter of culture; (B) GDH-NADP specific activity expressed as units per milligram of culture protein (1 U represents 1 µmol of substrate transformed per min). Cultures were grown on NH_4Cl (▲), KNO_3 (○), glutamate (●), or glutamine (■).

(29) with 25 mM NH_4Cl as the nitrogen source; the buffer used to equilibrate the DEAE-cellulose and Sephadex G-200 columns contained 0.1 M Tris-hydrochloride–7 mM EDTA, pH 8. Finally, after filtration on Sephadex G-200, the sample was subjected to preparative electrofocusing in a Sephadex G-75 gel, using a pH gradient from 4 to 6, and run at 8 W for 16 h at room temperature. Using this purification procedure, we obtained an 83-fold purification with a 25% recovery of enzyme activity. Acrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (16) showed a single main protein band (Fig. 2A) with a molecular weight of 48,000, which is in accordance with that reported for this enzyme (1).

Anti-GDH-NADP antibodies were prepared by injecting rabbits with purified enzyme in Freund complete adjuvant. Serum was fractionated with ammonium sulfate to obtain the total gamma globulin fraction. The specificity of this antibody fraction was demonstrated by a double immunodiffusion study (Fig. 2B), which shows a single precipitation band with crude extract of either the *N. crassa* wild-type strain 74-A or the am-1 strain, which has a missense mutation in the GDH-NADP structural gene (3). Strain am-132 (kindly provided by J. A. Kinsey), which has a deletion in the GDH-NADP structural gene and therefore lacks cross-reacting material (15), does not show a precipitation band. When quantitative rocket immunoelectrophoresis (30) was performed (Fig. 2C), the immunoprecipitate area found with pure enzyme was the same as that found with a cell-free extract containing the same enzyme activity units. This indicates that activity per enzyme molecule is the same in the crude extract and in the purified preparation.

To determine the relative activity per enzyme molecule of GDH-NADP from *N. crassa* grown on different nitrogen sources, samples of cell extracts containing the same amount of enzyme activity were subjected to quantitative rocket immunoelectrophoresis (Fig. 3). The immunoprecipitate areas found in extracts from cultures grown on ammonium, nitrate, glutamate, or glutamine were similar, indicating that the activity per enzyme molecule was the same under all conditions. This result indicates that the differences in specific activity of GDH-NADP presented in Fig. 1 correspond to differences in enzyme concentration.

To study the role of enzyme synthesis on the regulation of enzyme concentration, the experi-

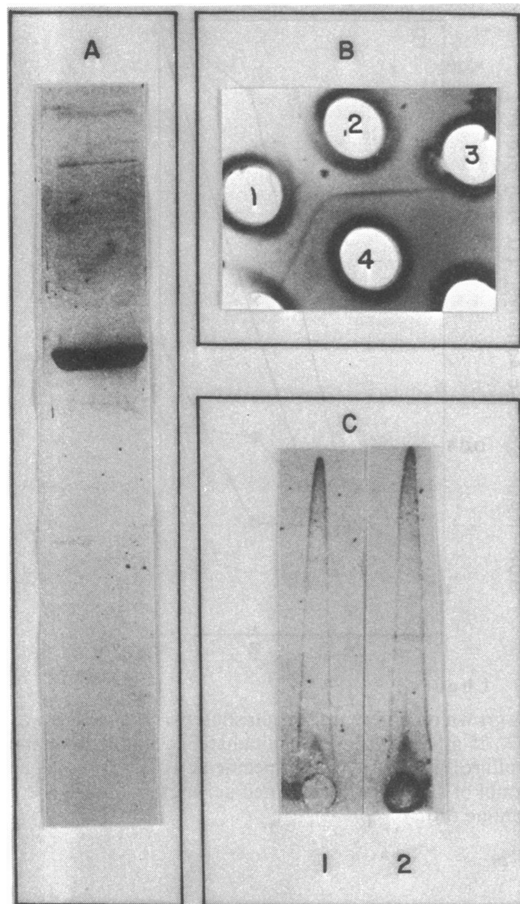

FIG. 2. Sodium dodecyl sulfate-acrylamide gel electrophoresis of purified GDH-NADP (A) and double immunodiffusion of GDH-NADP and specific antibody (B). *N. crassa* crude extract from: 1, wild-type strain 74-A; 2, mutant strain am-1; 3, mutant strain am-132; 4, anti-GDH-NADP antibodies. (C) Rocket quantitative immunoelectrophoresis of GDH-NADP. The procedure was performed according to Weeke (29). 1, 20 mU of purified GDH-NADP; 2, 10 mU of GDH-NADP from an extract of mycelium grown on NH_4Cl as nitrogen source mixed with 10 mU of purified enzyme.

ment presented in Fig. 4 was performed. The relative rate of GDH-NADP synthesis between cultures grown on either ammonium or glutamate was measured by isotope incorporation followed by specific immunoprecipitation of enzyme protein. Both cultures received a 30-min pulse with [^{3}H]leucine (10 μCi/ml) at 7.5 h of growth; extracts were processed to determine the radioactivity incorporated in total protein and in GDH-NADP by trichloroacetic acid precipitation or specific immunoprecipitation, respectively. In the specific immunoprecipitation procedure used, the same amount of radioactivity incorporated into total proteins (3.0×10^6 cpm) was adjusted for each extract, and both of them were mixed with 300 mU of enzyme activity, present in a nonlabeled extrat which served as a carrier for the immunoprecipitation. The immunoprecipitates were subjected to acrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (16), stained with Coomassie blue, and treated for fluorography (2). After fluorography, gels were sliced and radioactivity was counted in each slice. As indicated by the stained pattern, the immunoprecipitate contains four major polypeptides which migrate with the molecular weights of albumin (used in the immunoprecipitation procedure [21]), the heavy chain of gamma globulin, *N. crassa* GDH-NADP, and the light chain of gamma globulin. The fluorography pattern indicates that the only labeled polypeptide in the immunoprecipitate corresponds to *N. crassa* GDH-NADP. The radioactivity incorporated in GDH-NADP quantifies the relative rate of synthesis of the enzyme in the two growth conditions. Figure 4A shows that when ammonium is the nitrogen source, the radioactivity incorporated in glutamate dehydrogenase-NADP is threefold higher than when glutamate is the nitrogen source (Fig. 4B). These values correspond to the differences found in specific enzyme activity and indicate that the regulation of this enzyme by the nitrogen source is exerted at the level of specific enzyme synthesis.

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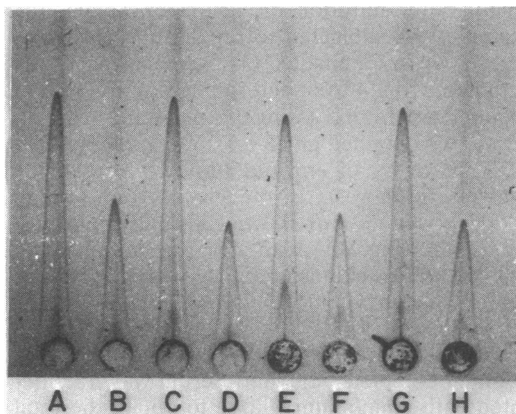

FIG. 3. Rocket quantitative immunoelectrophoresis of GDH-NADP from crude extracts of wild-type *N. crassa* grown for 12 h on different nitrogen sources. Extracts from cultures grown on 25 mM NH_4Cl (A, B); 25 mM KNO_3 (C, D); 5 mM glutamate (E, F); or 5 mM glutamine (G, H). Enzyme activity in each well was either 10 (A, C, E, G) or 5 (B, D, F, H) mU.

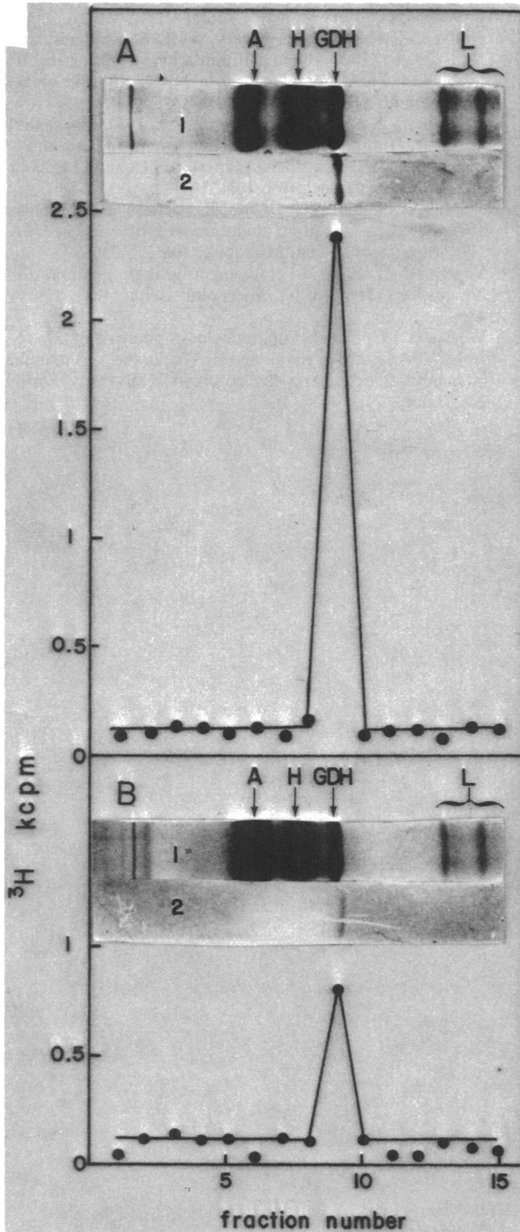

FIG. 4. Relative rate of GDH-NADP synthesis in *N. crassa* cultures grown on ammonium or glutamate as the sole nitrogen source. Parallel cultures of *N. crassa* were grown on either 25 mM NH_4Cl or 5 mM glutamate as the sole nitrogen source. After 7.5 h, each culture received a 30-min pulse with [^{3}H]leucine (10 μCi/ml). Mycelium from both cultures was collected and extracts were prepared. GDH-NADP was immunoprecipitated (see text) and subjected to slab gel electrophoresis, and after fluorography the gels were sliced and [^{3}H]radioactivity was measured in each slice. (A) Extract from culture grown on 25 mM NH_4Cl; (B) extract from culture grown on 5 mM glutamate. 1, Gel stained with Coomassie blue; arrows indicate the position of albumin (A), heavy chain of gamma globulin (H), glutamate dehydrogenase-NADP (GDH), and light chain of gamma globulin (L). 2, Gel after fluorography.

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