Nitrogen Regulation of Glutamine Synthetase in *Neurospora crassa*

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A higher activity of glutamine synthetase (EC 6.3.1.2) was found in *Neurospora crassa* when NH_4^+ was limiting as nitrogen source than when glutamate was limiting. When glutamate, glutamine or NH_4^+ were in excess, a lower activity was found. Immunological titration and sucrose gradient sedimentation of the enzyme established that under all these conditions enzyme activity corresponded to enzyme concentration and that the octamer was the predominant oligomeric form. When *N. crassa* was shifted from nitrogen-limiting substrates to excess product as nitrogen source, the concentration of glutamine synthetase was adjusted with kinetics that closely followed dilution by growth. When grown on limiting amounts of glutamate, a lower oligomer was present in addition to the octameric form of the enzyme. When the culture was shifted to excess NH_4^+ , glutamine accumulated at a high rate; nevertheless, there was only a slow decrease in enzyme activity and no modification of the oligomeric pattern.

INTRODUCTION

Numerous reports have established in prokaryotes a role of glutamine synthetase in the regulation of nitrogen metabolism, including its own synthesis (Foor, Janssen & Magasanik, 1975; Stadtman & Ginsburg, 1974; Streicher, Bender & Magasanik, 1975; Tyler, De Leo & Magasanik, 1974; Magasanik *et al.*, 1974; Weglenski & Tyler, 1977; Wohlhueter, Schutt & Holzer, 1973). This enzyme has also been thoroughly characterized from eukaryotic organisms (Meister, 1974; McParland *et al.*, 1976; Sims, Toone & Box, 1974 *a, b*; Tiemeier & Milman, 1972) and there have been reports on its regulation in *Saccharomyces* (Kohlaw, Dragert & Holzer, 1965), *Candida* (Ferguson & Sims, 1974 *a, b*), filamentous fungi (Pateman, 1970), cultured mammalian cells (Kulka & Cohen, 1973; Milman, Portnoff & Tiemeier, 1975) and chick retina (Moscona, Frankel & Moscona, 1972; Sarkar & Griffith, 1976).

Studies in our laboratory have established in *Neurospora crassa* a metabolic link of glutamine biosynthesis with its distribution to several amino acids, mainly arginine, as well as with nitrogen catabolism. If growth is restricted, nitrogen accumulates, mainly as glutamine and arginine, and catabolism of the latter amino acid is impaired (Mora *et al.*, 1978; Espín & Mora, 1978). Since glutamine is the nitrogen donor for the synthesis of arginine, we have studied the regulation of glutamine synthesis. This paper reports on the nitrogen regulation of glutamine synthetase in *N. crassa*. We have previously purified and partially characterized the enzyme (Palacios, 1976) and specific antibodies against it have been obtained (Palacios, Campomanes & Quinto, 1977).

METHODS

Organism and growth. *Neurospora crassa* wild type 74-A was grown on Vogel's minimal medium N (Vogel, 1964) supplemented with 1.5 % (w/v) sucrose; NH_4NO_3 was replaced by other nitrogen sources as indicated in the text and figure legends. Mycelium was grown from a conidia inoculum (Sánchez, Martínez & Mora, 1972) in liquid medium sparged with moist air at 25 °C. In certain experiments, nitrogen limitation was achieved by supplying different nitrogen compounds at a constant rate using a syringe attached to a pump (Limón-Lason *et al.*, 1977).

Glutamine synthetase activity. Mycelium was filtered (Whatman no. 41 paper) and washed with distilled water. Acetone powders were prepared from the mycelium, ground with dry ice and homogenized at 4 °C in extraction buffer (5 mM-phosphate, 0.5 mM-EDTA, 50 mM- K_2SO_4 , pH 7.2). The homogenate was centrifuged for 15 min at 12500 *g* and the supernatant was used as the source of enzyme. Glutamine synthetase [EC 6.3.1.2; L-glutamate:ammonia ligase (ADP-forming)] was measured by its synthetase and transferase activities as described by Ferguson & Sims (1974*a*). Protein was determined by the method of Lowry *et al.* (1951).

Glutamate and glutamine pools. Mycelium was harvested by filtration, washed with distilled water and homogenized in 20 ml 80 % (v/v) ethanol containing 0.5 μCi of L-[U- ^{14}C]glutamine. The homogenates were boiled for 10 min, cooled and filtered. The alcohol-insoluble material was re-extracted in 20 ml 80 % (v/v) ethanol. The filtrates were lyophilized and the dried samples were resuspended in 2 ml deionized water plus 0.2 μCi of L-[U- ^{14}C]glutamic acid. Glutamic acid and glutamine were separated and measured using the method of Yemm & Cocking (1955) as modified by Ferguson & Sims (1974*a*). Glutamic acid and glutamine concentrations were calculated by dilution of the specific radioactivity of the labelled compounds.

Preparation and characterization of anti-glutamine synthetase antibodies. Glutamine synthetase was purified to homogeneity by DEAE-cellulose chromatography followed by affinity chromatography on anthranilate-bound Sepharose (Palacios, 1976). Rabbits were immunized as previously described (Palacios *et al.*, 1977) and the serum was fractionated with ammonium sulphate to obtain the total γ -globulin fraction. This fraction was further purified by affinity chromatography using a matrix of *N. crassa* glutamine synthetase bound to Sepharose. Different experimental criteria such as double immunodiffusion, immunoelectrophoresis, immunoprecipitation of enzyme activity and immunoprecipitation of 'in vivo' labelled *N. crassa* extract indicated that this antibody fraction was specific for *N. crassa* glutamine synthetase (Palacios *et al.*, 1977).

Immunochemical titration of glutamine synthetase activity. Both direct and indirect immunological titrations were done. In the direct procedure, samples of *N. crassa* extracts containing a constant amount of glutamine synthetase activity were incubated for 90 min at 4 °C in the presence of extraction buffer (as above, but also containing 2 mg bovine serum albumin ml^{-1}) with various amounts of anti-glutamine synthetase total γ -globulin fraction. The final volume was 0.5 ml. After incubation, the reaction mixture was centrifuged for 5 min at 10000 *g* and glutamine synthetase activity (transferase) was measured in the supernatant. In the indirect procedure, *N. crassa* extracts containing a constant amount of glutamine synthetase activity were incubated for 90 min with 25 μg purified anti-glutamine synthetase antibody. Anti-rabbit γ -globulin was then added at different concentrations and incubation was continued for another 90 min. The reaction mixture was centrifuged and enzyme activity (transferase) was measured in the supernatant.

Preparation of glutamine synthetase labelled in vivo. To a culture of *N. crassa* grown on 5 mM-glutamate as the sole nitrogen source was added [^3H]methionine (5 $\mu\text{Ci ml}^{-1}$; sp. act. 10 Ci mmol^{-1}) for 60 min. Radioactive enzyme was then purified as previously described (Palacios, 1976) but using two consecutive steps of affinity chromatography on anthranilate-bound Sepharose. The specific radioactivity of the purified preparation was 1.2×10^6 c.p.m. (mg protein) $^{-1}$.

Sedimentation of glutamine synthetase. Samples of *N. crassa* extracts were layered on to 5 to 20 % (w/v) continuous sucrose gradients prepared in extraction buffer (see above) and centrifuged for 12 h at 284000 *g* in a SW40 Beckman rotor. The gradients were then fractionated and glutamine synthetase activity (transferase) was measured in each fraction. The molecular weight was calculated as previously described using globular proteins as markers (Palacios, 1976). In some experiments, radioactive glutamine synthetase was included as an internal marker.

RESULTS

Substrates and product of glutamine synthetase as nitrogen source

Doubling times for the growth of *N. crassa* strain 74-A on glutamate, glutamine or NH_4^+ as nitrogen source were 3, 2 and 2.5 h, respectively (Fig. 1*a*). When both glutamate and NH_4^+ were present in excess, the doubling time was similar to that with only NH_4^+ in

Fig. 1. Growth, glutamine synthetase activity and amino acid pools of *N. crassa* grown on different nitrogen sources. Strain 74-A was grown in Vogel's minimal medium (containing NH_4NO_3) or with 5 mM-glutamine or 5 mM-glutamate as nitrogen source instead of NH_4NO_3 . (a) Protein content per ml culture grown on NH_4NO_3 ($\circ$), glutamine ($\bullet$) or glutamate ($\square$). (b) Glutamine synthetase specific activity measured by the transferase (open symbols) or synthetase (closed symbols) assays: $\circ, \bullet$, grown on NH_4NO_3 ; $\square, \blacksquare$, grown on glutamine; $\triangle, \blacktriangle$, grown on glutamate. (c) Glutamine pool, symbols as in (a). (d) Glutamate pool, symbols as in (a).

Fig. 2. Immunological titration of glutamine synthetase activity. *Neurospora crassa* strain 74-A was grown for 12 h on different nitrogen sources. (a) Direct immunological titration (see Methods) of glutamine synthetase from cultures grown on glutamine ($\circ$) or glutamate ($\bullet$). The titre was obtained by dividing the amount of antibody by the number of enzyme units in the supernatant at 50% decrease in enzyme activity and was 156 when *N. crassa* was grown on glutamate and 169 when grown on glutamine. (b) Indirect immunological titration (see Methods) of glutamine synthetase grown on NH_4NO_3 ($\circ$) or glutamate ($\bullet$). The titre was obtained by subtracting the enzyme activity remaining at high concentrations of anti-rabbit γ -globulin (200 μl) from the initial activity and was 0.26 when *N. crassa* was grown on glutamate and 0.25 when grown on NH_4NO_3 .

Fig. 3. Sedimentation of glutamine synthetase from *N. crassa* cultures grown on different nitrogen sources. (a) An extract of culture grown on glutamate as nitrogen source was mixed with purified *N. crassa* glutamine synthetase labelled 'in vivo' with [^3H]methionine, used as a marker for the octameric structure of the enzyme (Palacios, 1976). Of the total glutamine synthetase activity of the mixture, more than 95 % corresponded to that present in the extract. The preparation was layered on to a 5 to 20 % (w/v) continuous sucrose gradient prepared in extraction buffer (see Methods) and centrifuged for 12 h at 40000 rev. min^{-1} in a Beckman SW40 rotor. The gradient was fractionated and enzyme activity ($\circ$) and radioactivity ($\bullet$) were determined in each fraction. Globular proteins were run in separate gradients to calibrate for molecular weight (Palacios, 1976). (b) Extracts from *N. crassa* grown on NH_4NO_3 ($\circ$), glutamine ($\bullet$) or glutamate ($\square$) were run in separate gradients and processed as described above. Data were normalized to the fraction with highest activity or radioactivity.

excess (data not shown). Thus, NH_4^+ is limiting when glutamate is the nitrogen source and both substrates are in excess when NH_4^+ is present as the nitrogen source.

Glutamine synthetase activity, as measured by both the transferase and synthetase assays, was very low in spores and increased during outgrowth in the presence of either substrate, reaching levels about fivefold higher when grown in glutamate, i.e. limited NH_4^+ , than when excess NH_4^+ was present (see Discussion). On the other hand, enzyme activity decreased slightly when glutamine was present as nitrogen source. The ratio of synthetase to transferase activities of the enzyme remained constant with glutamate and glutamine as nitrogen sources for growth, whereas with NH_4^+ the ratio was higher at 6 h (Fig. 1b). The intracellular concentrations of glutamine and glutamate were two to threefold higher when these compounds were used as sole nitrogen source (Fig. 1c, d).

Specific antibodies against glutamine synthetase gave similar titration curves for glutamine synthetase activity when either glutamate or glutamine was used as nitrogen source (Fig. 2a), indicating that the activity per enzyme molecule was the same under both conditions. In addition, indirect immunological titrations with cell-free extracts from cultures grown on NH_4^+ or glutamate (Fig. 2b) gave similar antibody titres. Therefore, the differences in the specific activity of glutamine synthetase found in mycelium grown with different nitrogen sources corresponded to differences in enzyme concentration.

The oligomeric structure of the enzyme from cells grown with different nitrogen sources corresponded mainly to the octameric form (Fig. 3).

Fig. 4. Shift of *N. crassa* from glutamate to glutamine as nitrogen source. *Neurospora crassa* was grown for 12 h with glutamate as the sole nitrogen source. The mycelium was then washed with fresh medium and transferred to medium containing glutamine as the sole nitrogen source. At intervals after the shift, samples were taken to determine: (a) dry weight per ml culture; (b) glutamine synthetase specific activity measured by the transferase (○) or synthetase (●) assays, and expressed as a percentage of the activity at zero time (0.58 units of transferase, 0.19 units of synthetase); ---, theoretical dilution of activity expected by growth; (c) direct antibody titre.

Shift from substrate to product of glutamine synthetase as nitrogen source

To determine the kinetics of adjustment from glutamate to glutamine as nitrogen source, *N. crassa* was grown for 12 h on glutamate and then transferred to medium containing glutamine as the sole nitrogen source. After a lag of about 40 min, growth resumed and the organism grew exponentially with a doubling time of 3 h (Fig. 4a). The activity of the enzyme (Fig. 4b) decreased at a faster rate than that expected from dilution during the first minutes; afterwards the decrease in activity corresponded to the theoretical dilution expected by growth. There was no change in the ratio of synthetase to transferase activities of glutamine synthetase. As shown by direct immunoprecipitation of enzyme activity (Fig. 4c), the changes in enzyme specific activity corresponded to changes in enzyme concentration. The enzyme was in the octameric form at different times after the shift (data not shown).

Fig. 5. Shift of *N. crassa* from limiting glutamate to excess NH_4^+ as nitrogen source. *Neurospora crassa* was grown on limiting glutamate as nitrogen source (see Methods) and (a) total protein per ml culture was measured during this period. After 15 h, NH_4Cl was added at a final concentration of 5 mM and incubation was continued for a further 5 h. At intervals after the shift, samples were taken to determine: (b) glutamine (●) and glutamate (○) pools; (c) glutamine synthetase specific activity measured by the transferase (○) or synthetase (●) assays, and expressed as a percentage of the activity at zero time (0.53 units of transferase, 0.12 units of synthetase); (d) direct antibody titre; (e, f, g) sedimentation of glutamine synthetase (see Fig. 3) at zero time (e), 2 h (f) and 5 h (g) after addition of NH_4^+ .

Shift from limiting glutamate to excess NH_4^+ as nitrogen source

Glutamate was a suboptimal nitrogen source (see Fig. 1*a*), and in an attempt to obtain a more severe nitrogen limitation, glutamic acid was added in limiting amounts at a constant rate in fed-batch cultures (Limón-Lason *et al.*, 1977). This resulted in linear growth (Fig. 5*a*) and a glutamate pool that was about fourfold lower than that found when glutamate was added in excess to the culture medium. Glutamine synthetase activity was similar to that found with excess glutamate; however, the ratio of synthetase to transferase activities was lower and, in addition to the octameric form of the enzyme, a lower oligomer possibly corresponding to a tetramer was evident (Fig. 5*e*).

When, after this nitrogen limitation, excess NH_4^+ was added to the culture medium, the rate of growth increased (Fig. 5*a*) and the glutamine pool rose to a level about 20-fold higher while the glutamate pool remained constant (Fig. 5*b*). Enzyme activity maintained its original level during the first hour after the shift and then decreased slowly (Fig. 5*c*), which again parallels the dilution expected by growth. Immunological titrations indicated that glutamine synthetase activity corresponded to enzyme concentration (Fig. 5*d*). The oligomeric pattern of the enzyme was preserved during this time (Fig. 5*f,g*) and the low ratio of synthetase to transferase activities was maintained.

DISCUSSION

When *Neurospora crassa* is grown with NH_4^+ as nitrogen source, glutamine synthetase is repressed. When grown on glutamate, this compound is in excess but it must also provide NH_4^+ . Since growth on glutamate is suboptimal, we must conclude that the high activity of glutamine synthetase found under this condition only partially compensates for the NH_4^+ deficiency. When glutamine is present as the sole nitrogen source, this compound is present in excess inside the cell, the organism grows optimally and glutamine synthetase activity is very low. These results led us to investigate the effect of limiting amounts of NH_4^+ in the regulation of glutamine synthetase by *N. crassa* and, as expected, we found an optimum induction of the enzyme under these conditions. On the other hand, limitation of sucrose in an excess of NH_4^+ results in a low glutamine synthetase activity (Limón-Lason *et al.*, 1977), but this is due to different biosynthetic rates (Quinto, Mora & Palacios, 1977). Since strain *am-1*, which lacks biosynthetic glutamate dehydrogenase activity, induces its glutamine synthetase even in the presence of NH_4^+ , a direct effect of this compound can be ruled out (Limón-Lason *et al.*, 1977). We propose, therefore, that the carbon source has a positive role on the regulation of glutamine synthetase whereas glutamine has a negative one. The level at which this regulation occurs is now being investigated.

Ferguson & Sims (1974*b*) and Sims *et al.* (1974*a*) reported that in *Candida utilis* glutamine synthetase is present as an octamer when glutamic acid is used as nitrogen source. Addition of NH_4^+ or glutamine to such cells increases the intracellular pool of glutamine and ultimately causes a rapid decrease in enzyme activity concomitant with the appearance of a tetrameric form of the enzyme. This contrasts with our results where the kinetics of the shift from glutamate to glutamine as nitrogen source indicate that the cell adjusts the concentration of glutamine synthetase slowly and no tetrameric form is apparent. Assuming that enzyme synthesis is prevented, the decrease in enzyme concentration is in close correlation with the theoretical dilution expected by growth. A minor contribution to the decrease in enzyme activity is possibly due to degradation or inactivation of the enzyme during the first minutes after the shift. Even in fed-batch cultures where glutamic acid is the limiting nitrogen compound, the addition of NH_4^+ , which results in a large increase in the glutamine pool, causes only a slight decrease in enzyme activity, and for 5 h after the addition of NH_4^+ , the oligomeric pattern of the enzyme is not modified. Under these limiting conditions two oligomeric forms of the enzyme corresponding to an octamer and a tetramer are present.

These experiments indicate that mycelium of *N. crassa* does not adjust its glutamine synthetase activity by inactivation and that the slow adaptation is achieved mainly by growth dilution. The tetrameric form that appears under glutamate limitation could be related to the capacity of the cells to fix NH_4^+ at low concentration since the proportion of this oligomeric form increases under conditions where nitrogen limitation is more severe (Limón-Lason *et al.*, 1977).

Although no explanation can be offered at present, the absence of a steady state in glutamine synthetase concentration with glutamate as nitrogen source is possibly related to the fact that *Neurospora* is a filamentous organism where developmental processes are not excluded.

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