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Immunochemical Characterization of Glutamine Synthetase from *Neurospora crassa* Glutamine Auxotrophs

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Glutamine synthetase derived from two *Neurospora crassa* glutamine auxotrophs was characterized. Previous genetic studies indicated that the mutations responsible for the glutamine auxotrophy are allelic and map in chromosome V. When measured in crude extracts, both mutant strains had lower glutamine synthetase specific activity than that found in the wild-type strain. The enzyme from both auxotrophs and the wild-type strain was partially purified from cultures grown on glutamine as the sole nitrogen source, and immunochemical studies were performed in crude extracts and purified fractions. Quantitative rocket immunoelectrophoresis indicated that the activity per enzyme molecule is lower in the mutants than in the wild-type strain; immunoelectrophoresis and immunochemical titration of enzyme activity demonstrated structural differences between the enzymes from both auxotrophs. On the other hand, the monomer of glutamine synthetase of both mutants was found to be of a molecular weight similar to that of the wild-type strain. These data indicate that the mutations are located in the structural gene of *N. crassa* glutamine synthetase.

Mutants altered in the structure or the regulation of glutamine synthetase (EC 6.3.1.2) have been of great value towards understanding the mechanisms involved in the control of nitrogen metabolism in procaryotes (5, 14). No such mutants have been characterized in eucaryotes.

In *Neurospora crassa* a regulatory link has been established between the catabolism of nitrogen compounds, the synthesis of glutamine, and the distribution of the amide nitrogen of this amino acid (6, 15, 22). The regulation of glutamine synthetase has been studied in *N. crassa* under different metabolic conditions. During exponential growth glutamine synthetase concentration (23), synthesis (18), and specific mRNA levels (19) depend on the nitrogen source present in the medium. Using fed-batch cultures of *N. crassa* it was found that nitrogen limitation results in an increase in glutamine synthetase specific activity measured in crude extracts, whereas carbon limitation impairs such increase (12). *N. crassa* glutamine synthetase is also regulated in nongrowing conidia where the absence of a nitrogen source results in the induction of the enzyme. This induction is the result of de novo enzyme synthesis (G. Espín, R. Palacios, and J. Mora, J. Gen. Microbiol., in press) due to an increase in specific mRNA levels (W. Hansberg, G. Espín, R. Palacios, and F. Sánchez, Devel. Biol., in press).

To establish the function of glutamine synthetase in the different metabolic conditions studied, the isolation and characterization of glutamine auxotrophic strains is of primary importance. One *N. crassa* glutamine auxotroph, *gln-1a*, has been previously obtained and mapped in chromosome VR by Reich and Silagi (Abstr. Proc. Int. Congr. Genet., 11th, Abstr. no. 1, p. 49-50, 1963). The genetics and physiology of this mutant as well as of another allelic glutamine auxotroph obtained by mutagenesis in our laboratory have been recently reported (4).

The present paper deals with the immunochemical characterization of glutamine synthetase from these auxotrophs. The results indicate that the enzymes from both mutant strains are different from each other and from that of the wild-type strain. These data are useful to identify the structural gene for glutamine synthetase in *N. crassa* and to correlate the previously reported genetic and physiological studies on *N. crassa* glutamine auxotrophs (4) with specific biochemical alterations.

MATERIALS AND METHODS

Strains. *N. crassa* strains used were: 74 A wild type; *gln-1a*, a glutamine auxotroph previously reported (Reich and Silagi, Abstr. Proc. Int. Congr. Genet., 11th, Abstr. no. 1, p. 49-50, 1963), obtained from the Fungal Genetics Stock Center at Humboldt State University Foundation (FGSC no. 1449), and *gln-1b*, a glutamine auxotroph obtained by mutagenesis by Dávila et al. (4). Genetic studies indicated that

the mutations responsible for auxotrophy in *gln-1a* and *gln-1b* are allelic and map in chromatosome VR (4).

Growth conditions. All strains were grown for 12 h at 37°C on Vogel's minimal medium N (24) supplemented with 1.5% sucrose and containing 5 mM glutamine as the sole nitrogen source. Mycelium was grown from a conidia inoculum (20) in liquid medium sparged with hydrated air.

Preparation of extracts and determination of glutamine synthetase activity. Mycelium was filtered through Whatman no. 41 paper and washed with distilled water. Acetone powders were prepared from the mycelium, ground with Dry Ice, and homogenized in the cold with buffer A (5 mM phosphate, 50 mM K_2SO_4, 0.5 mM EDTA, pH 7.2). The homogenates were centrifuged for 15 min at 12,500 × *g*, and the supernatants were used as the source of enzyme. Glutamine synthetase was measured as transferase or synthetase activity (see figure legends) as described by Ferguson and Sims (7). Protein was determined by the method of Lowry et al. (13).

Preparation and characterization of anti-glutamine synthetase antibodies. *N. crassa* glutamine synthetase was purified to homogeneity by a procedure based on affinity chromatography on anthranilate-bound Sepharose (16). Rabbits were immunized as previously described (17) and serum was fractionated with ammonium sulfate to obtain the total gamma globulin fraction. The specific antibody was further purified from this fraction by affinity chromatography on a matrix of glutamine synthetase bound to Sepharose. This antibody has been characterized and found to be monospecific for *N. crassa* glutamine synthetase (17). Goat anti-rabbit gamma globulin, used to form indirect immunoprecipitates, was prepared as described (17).

Purification of glutamine synthetase from glutamine auxotrophs. Glutamine synthetase from both auxotrophs and the wild-type strain grown on glutamine as the sole nitrogen source was purified by a previously reported procedure (16) with some modifications. All steps were performed at 4°C. Extracts were prepared on buffer B (5 mM imidazole, 5 mM glutamate, 8 mM $MgSO_4$, 50 μM EDTA, 500 μM β-mercaptoethanol, and 2.5 mM sodium bisulfite, pH 7.2). The extracts were chromatographed on DEAE-cellulose equilibrated with buffer B, and elution was performed with buffer C (50 mM imidazole, 50 mM glutamate, 80 mM $MgSO_4$, 500 μM EDTA, pH 7.2). Fractions with glutamine synthetase activity were pooled, made 2.25 mM $MnCl_2$, and chromatographed on a matrix of anthranilate bound to Sepharose equilibrated with buffer C containing 2.25 mM $MnCl_2$. Elution was made with buffer C containing 2.25 mM $MnCl_2$ and 30 mM AMP. Fractions with activity were pooled, precipitated with 70% saturation $(NH_4)_2SO_4$, resuspended in a small volume, and dialyzed overnight against buffer B; purified fractions were divided in small aliquots and frozen at −70°C. Enzymes from wild-type, *gln-1a* and *gln-1b* strains were purified 150-, 80-, and 65-fold, respectively.

Immunochemical titration of glutamine synthetase activity. Crude or purified fractions containing a constant amount of enzyme activity were incubated with different concentrations of the anti-glutamine synthetase total gamma globulin fraction. Incubation was for 90 min at 4°C in the presence of buffer C containing 2 mg of bovine serum albumin per ml. The final volume of the reaction was 0.5 ml. After incubation, glutamine synthetase was measured in the total reaction or in aliquots of the supernatant or the pellet after centrifugation at 7,000 × *g* for 5 min.

Electrophoresis, immunodiffusion, and immunoelectrophoresis studies. Slab gel electrophoresis in the presence of sodium dodecyl sulfate was performed as reported by Laemmli (10); fluorography was according to Bonner and Laskey (1). Double immunodiffusion and immunoelectrophoresis were performed as described by Campbell et al. (3) using 10 mM sodium phosphate buffer. Rocket immunoelectrophoresis was carried out as described by Weeke (25) using a 1% agarose gel containing 1% of anti-glutamine synthetase gamma globulin fraction. The buffer used was 25 mM sodium barbital (pH 8.6) containing 0.025% sodium azide. The gel was run for 18 h at 5 V/cm. After electrophoresis the gel was washed extensively, dried, and stained with Coomassie brilliant blue R-250.

Immunoprecipitation of in vivo-labeled glutamine synthetase. Exponentially growing cultures received a 30-min pulse with 2 μCi of [^{35}S]methionine per ml (New England Nuclear Corp.; specific radioactivity 100 to 400 Ci/mmol). Extracts were prepared and incubated for 90 min in the presence of purified anti-glutamine synthetase antibody. After incubation, anti-rabbit gamma globulin was added and the reaction mixture was centrifugated through 20% sucrose at 20,000 rpm for 60 min in a Beckman SW50.1 rotor. Details for this technique are given elsewhere (18). Immunoprecipitates were processed for slab gel electrophoresis in the presence of sodium dodecyl sulfate, followed by fluorography.

RESULTS

The mutations *gln-1a* and *gln-1b* impair the conversion of glutamate to glutamine and result in a low glutamine synthetase specific activity (4). To search for structural differences between glutamine synthetase from the glutamine auxotrophs and the wild-type strain, immunochemical studies were performed. That the monomer of glutamine synthetase from both mutant strains has a molecular weight similar to that of the wild-type strain was evidenced by the experiment presented in Fig. 1. Cultures from the three strains grown on glutamine as the sole nitrogen source were pulse-labeled with [^{35}S]methionine; extracts were prepared, and glutamine synthetase was precipitated with specific antibodies. The immunoprecipitates were subjected to acrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, followed by fluorography. The main radioactive band of both mutant strains moved similarly to that of the wild-type strain.

In the experiments presented in Fig. 2 and 3,

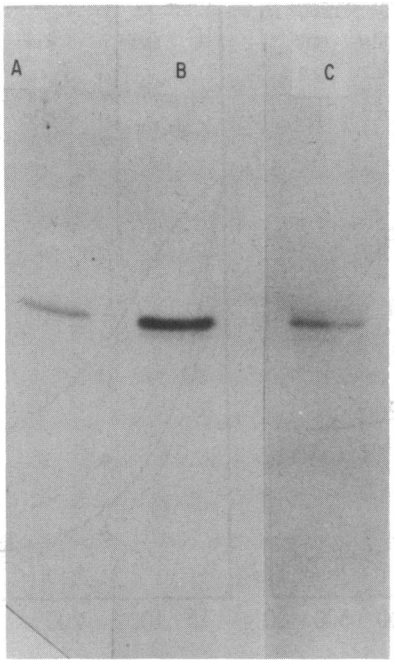

FIG. 1. *Electrophoresis in the presence of sodium dodecyl sulfate of immunoprecipitated glutamine synthetase from wild-type and mutant strains. Wild-type and mutant strains were labeled in vivo with [^{35}S]-methionine, and glutamine synthetase was immunoprecipitated as described in the text. Immunoprecipitates were subjected to electrophoresis in the presence of sodium dodecyl sulfate followed by fluorography. (A) Wild type; (B) gln-1b; (C) gln-1a.*

immunological titrations of glutamine synthetase activity were performed. Figure 2 shows the specificity of the immunoprecipitation titration assay. Wild-type enzyme was incubated in the presence of different concentrations of the anti-glutamine synthetase gamma globulin fraction. After incubation, the reaction mixture was centrifuged, and transferase and synthetase activities were measured in both the supernatant and the pellet. At large concentrations of antibody, the supernatant was completely devoid of enzyme activity and less than 30% was recovered in the pellet. It is also important that the ratio of synthetase to transferase activity of the supernatant decreased as the antibody concentration was increased. Since both activities are present in the same molecule (16), this could be explained by assuming that the enzyme remaining in the supernatant is partially combined and inactivated by antibody molecules, the synthetase being more sensitive than the transferase activity. In this experiment a control of the specificity of the immunological titration was included and showed that when the extract was incubated with increasing amounts of an antibody directed against a protein not synthesized by *N. crassa*, glutamine synthetase activity remained unchanged.

In the experiment presented in Fig. 3A, constant amounts of enzyme activity from extracts derived from either the wild type or the mutant strains were incubated with increasing concentrations of anti-glutamine synthetase gamma globulin fraction. After incubation, the reaction mixture was centrifuged, and enzyme transferase activity was quantified in the supernatant. The amount of antibody required to eliminate 50% of enzyme activity from the supernatant was taken as the antibody titer. Strain *gln-1b* had a higher antibody titer than the wild type (from three- to fivefold using different antibody preparations); on the other hand, *gln-1a* presented antibody titers similar to or lower than those found with the wild-type strain (0.6 to 1.1 using different antibody preparations). As suggested by the data presented in Fig. 2, the titration curves obtained are the result of both immunoprecipitation and inactivation of enzyme activity by the

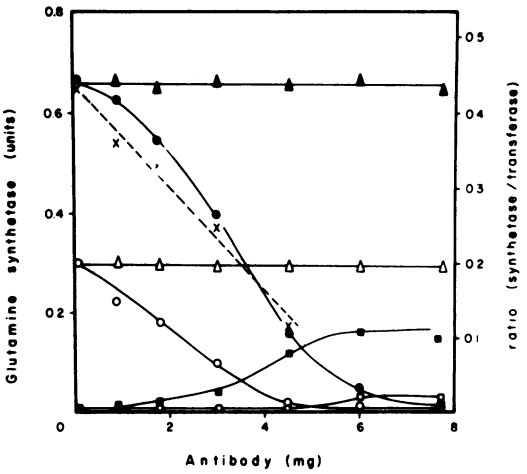

FIG. 2. *Specificity of immunological titration of glutamine synthetase activity. A constant amount of purified N. crassa glutamine synthetase from the wild-type strain was incubated for 90 min in the presence of different concentrations of anti-glutamine synthetase or anti-ovalbumin gamma globulin fractions. Enzyme activity was measured in both the supernatant and the pellet after centrifugation. Incubation with anti-glutamine synthetase:* (●——●) *transferase activity in the supernatant;* (■——■) *transferase activity in the pellet;* (○——○) *synthetase activity in the supernatant;* (□——□) *synthetase activity in the pellet;* (×- - -×) *ratio of synthetase to transferase activities in the supernatant. Incubation with anti-ovalbumin:* (▲——▲) *transferase activity in the supernatant;* (△——△) *synthetase activity in the supernatant.*

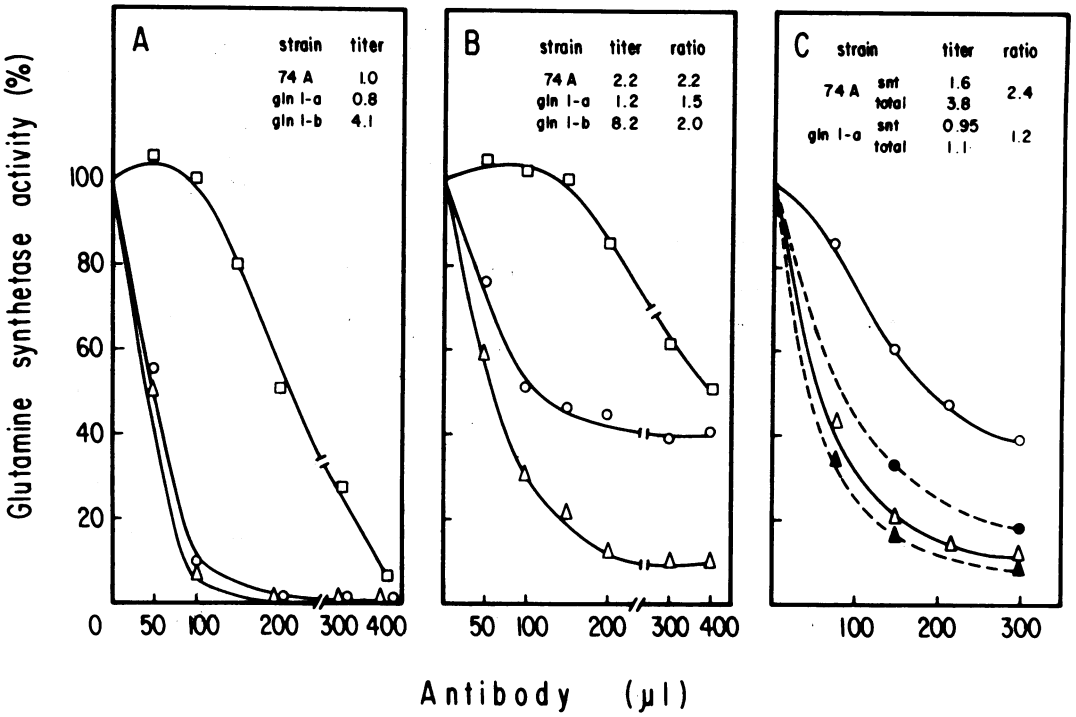

FIG. 3. *Immunological titration of glutamine synthetase activity from wild type and glutamine auxotrophs. A constant amount of activity from crude extracts was incubated in the presence of different concentrations of antibody (see the text), and transferase activity was measured in the supernatant after centrifugation (A) or in the total reaction mixture without centrifugation (B).* (○) *Wild type;* (△) *gln-1a;* (□) *gln-1b. (C) A constant amount of enzyme activity from wild-type and gln-1a partially purified enzymes was incubated with different concentrations of antibody, and transferase activity was measured in the supernatant after centrifugation and in the total reaction mixture without centrifugation. Wild type:* (●- - -●) *supernatant;* (○——○) *total reaction. gln-1a:* (▲- - -▲) *supernatant;* (△——△) *total reaction. The titer was obtained by dividing the amount of antibody by the units of enzyme activity at the point of 50% decrease in enzyme activity. The values were normalized by taking as 1.0 the titer obtained for the wild-type strain in (A). The ratio was obtained by dividing the titer of the supernatant by that of the total reaction mixture. Starting enzyme activity was 0.6 unit in the experiments presented in (A) and (B) and 0.4 unit in the experiment presented in (C). Experiment in (C) was performed with a different antibody preparation from that used for the experiments in (A) and (B).*

antibody. To obtain an indication of the sensitivity of the different enzymes to inactivation by the antibody, enzyme activity was measured in the total reaction mixture (without centrifugation) after the extracts were incubated in the presence of different concentrations of the specific antibody (Fig. 3B). The ratio of titers obtained by measuring glutamine synthetase activity in the total reaction (Fig. 3B) and in the supernatant after centrifugation (Fig. 3A), compared with the ratio obtained in the wild-type strain, should give an indication of the relative sensitivity to inactivation of the different enzymes. The ratios obtained were similar for wild-type and *gln-1b* enzymes and lower for that of *gln-1a*, thus suggesting that the enzyme from *gln-1a* strain is more sensitive to inactivation by the antibody. The higher sensitivity of the enzyme from *gln-1a* strain could also be demonstrated in the partially purified preparations (Fig. 3C). Immunological titrations similar to those shown in Fig. 3 were performed starting with different concentrations of enzyme activity (data not shown). The results obtained were similar to those presented. Furthermore, when a constant amount of anti-glutamine synthetase was incubated with increasing concentrations of extracts derived from the different strains, conclusions similar to those derived from the experiment depicted in Fig. 3A were obtained, i.e., *gln-1b* required more antibody than the wild type to eliminate to a given amount of enzyme activity, whereas *gln-1a* required the same or, with some preparations, a smaller amount of antibody (data not shown).

An experimental approach to determine activ-

ity per enzyme molecule of the different enzymes was based on rocket quantitative immunoelectrophoresis. In this method the antigen is run electrophoretically in an antibody containing agarose gel. The area under the immunoprecipitate is proportional to antigen concentration (25). In the experiment presented in Fig. 4, samples of the partially purified fractions from wild-type and mutant strains containing the same amount of enzyme activity were subjected to rocket immunoelectrophoresis. Both mutant strains gave larger immunoprecipitate areas than the wild-type strain. The activity per enzyme molecule, determined as a percentage of that of wild type, was about 40 and 60% for *gln-1a* and *gln-1b*, respectively, when the experiment was based on the transferase assay, whereas it was 30 and 6% for *gln-1a* and *gln-1b*, respectively, when based on the synthetase assay. When this experiment was performed using crude extracts as the source of enzyme (not shown), the activity per enzyme molecule obtained was 50% for both mutants when based on the transferase assay, but 30 and 15% for *gln-1a* and *gln-1b*, respectively, when based on the synthetase assay.

Strain	Activity per enzyme molecule (%)	
	Transferase	Synthetase
74A	100	100
gln1-a	40	30
gln1-b	60	6

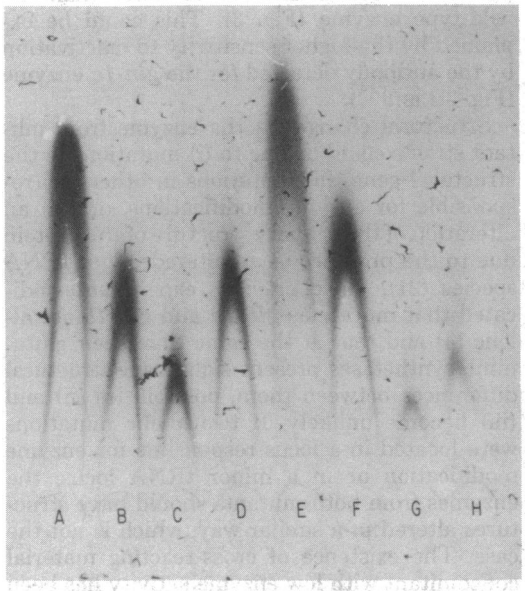

FIG. 4. *Rocket quantitative immunoelectrophoresis of glutamine synthetase from partially purified preparations of wild type and glutamine auxotrophs. Procedures were performed according to Weeke (25) as described in the text. (A-C) Calibration curve of wild-type glutamine synthetase at progressive 1:2.5 dilutions; (D) 3 × 10^{-2} unit of transferase activity from wild-type enzyme; (E) 3 × 10^{-2} unit of transferase activity from gln-1a enzyme; (F) 3 × 10^{-2} unit of transferase activity, equivalent to 10^{-3} unit of synthetase activity from gln-1b enzyme; (G) 10^{-3} unit of synthetase activity from wild-type enzyme; (H) 10^{-3} unit of synthetase activity from gln-1a enzyme. Transferase activity per enzyme molecule was calculated with samples D to F, and synthetase activity was calculated with samples F to H. To obtain activity per enzyme molecule, the amount of enzyme units was divided by the antigen concentration. The results were normalized by taking as 1.0 the activity per enzyme molecule of the wild-type enzyme. Antigen concentration was obtained by measuring the area under the immunoprecipitates using the standard curve (samples A to C) to calculate the relative concentration.*

The above-described experiments suggest structural differences between *gln-1a*, *gln-1b*, and wild-type glutamine synthetase. These differences were confirmed by immunoelectrophoresis studies (Fig. 5). Extracts or partially purified fractions from both mutants and the wild-type strain gave single immunoprecipitation bands. The enzyme from the wild-type strain moved faster towards the anode than that from either auxotroph; on the other hand, the enzyme from mutant *gln-1a* moved faster than that of *gln-1b* (Fig. 5A to D). The enzyme from each strain moved similarly whether the experiment was performed with crude extracts or with purified fractions (Fig. 5I to N). When mixtures of extracts from each mutant strain and purified wild-type enzyme were coelectrophoresed, two fused immunoprecipitation bands, localized in their corresponding positions, were observed (Fig. 5E to H). These data indicate that, under the experimental conditions used, there was no exchange of subunits from the wild-type and mutant strains and that the extract from mutant strains did not significantly alter the wild-type enzyme.

DISCUSSION

The molecular weight of the monomer of glutamine synthetase from both mutant strains studied is similar to that of the wild-type enzyme, as indicated by acrylamide gel electrophoresis in the presence of sodium dodecyl sulfate of the immunoprecipitates from in vivo-labeled protein (Fig. 1). On the other hand, the native enzyme from both mutant strains presented lower activity per enzyme molecule than that from the wild-type strain (Fig. 4), as well as different electrophoretic mobility (Fig. 5). These data indicate structural alterations in the gluta-

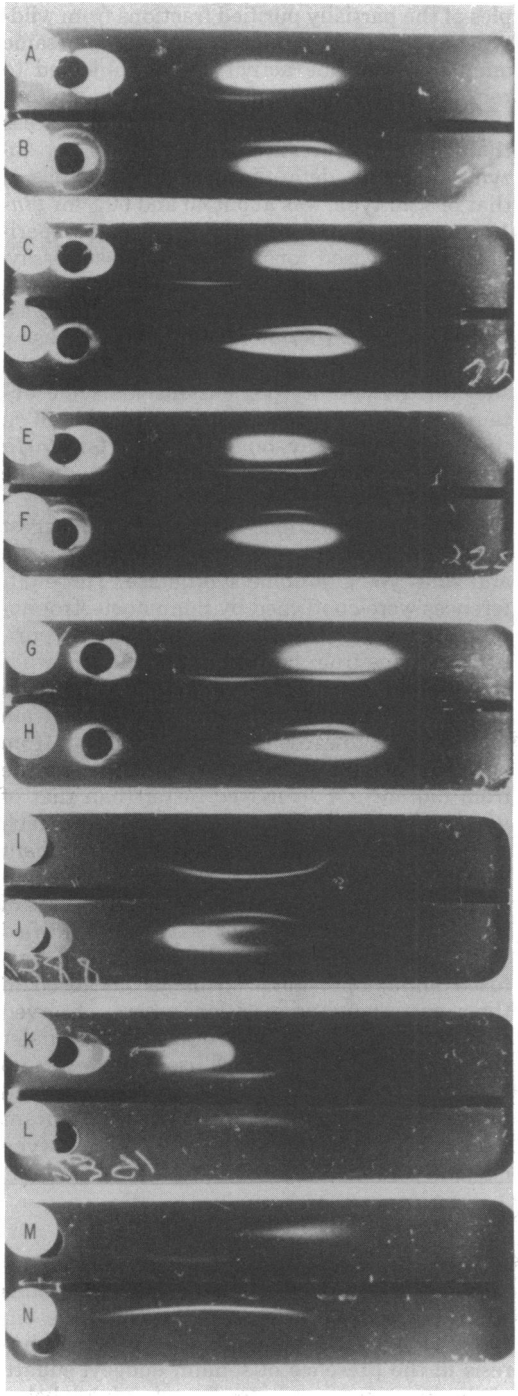

FIG. 5. *Immunoelectrophoresis of glutamine synthetase from wild type and glutamine auxotrophs. (B), (D), (F), (H), Wild-type extract; (A), gln-1a extract; (C) gln-1b extract; (E) gln-1a extract mixed with purified glutamine synthetase from wild-type strain; (G) gln-1b extract mixed with purified glutamine synthetase from wild-type strain; (I) purified wild-type enzyme; (J) wild-type extract; (K) gln-1a extract; (L) purified gln-1a enzyme; (M) gln-1b extract; (N) gln-1b purified enzyme. Samples A to H, I to L, and M and N were run in three different experiments.*

mine synthetase from both auxotrophs. Moreover, significant differences were observed between *gln-1a* and *gln-1b* enzymes. The enzyme from *gln-1a* strain had higher synthetase activity per enzyme molecule (Fig. 4), lower antibody titer (Fig. 3), and faster electrophoretic mobility towards the anode (Fig. 5) than the *gln-1b* enzyme.

Since glutamine synthetase from mutant strains presented lower activity per enzyme molecule than that of the wild-type strain, higher values in the immunological titration of the enzyme activity should be expected. This is the case for *gln-1b* strain; however, *gln-1a* enzyme presented titers similar to or lower than the wild-type enzyme (Fig. 3). This could be explained by the higher sensitivity to inactivation by the antibody detected for the *gln-1a* enzyme (Fig. 3B and C).

Structural changes in the enzyme from mutant strains could be due to (i) mutations in the structural gene, (ii) mutations in other loci responsible for enzyme modifications, or (iii) an alteration of the primary structure of the protein due to the presence of an altered minor tRNA species (21). Since previous experiments indicated that mutations *gln-1a* and *gln-1b* are allelic (4) and that at the same time their glutamine synthetases present significant structural differences between them, possibilities (ii) and (iii) become unlikely. If two allelic mutations were located in a locus responsible for enzyme modification or in a minor tRNA locus, the enzymes from both mutants should have structures altered in a similar way, which is not the case. The existence of cross-reacting material concomitant with low enzyme activity has been used as a criterion of mutations in structural genes (9, 11); accordingly, our results indicate that mutations *gln-1a* and *gln-1b* are located in different regions of the glutamine synthetase structural gene. However, a definite conclusion must wait for the determination of specific amino acid substitutions, something that has been accomplished in *N. crassa* only in the case of biosynthetic glutamate dehydrogenase (2).

The quantitative data obtained in this study in regard to the activity per enzyme molecule and the immunological titration of glutamine synthetase activity depend on the assumption that the mutant enzymes present the same antigenicity as the wild-type enzyme. That this is the case is suggested by the fact that in double

immunodiffusion (data not shown) and immunoelectrophoresis studies (Fig. 5) antigenic identity bands were obtained even when different protein concentrations were used. In any case, if some antigenic differences not detected by our experimental procedures were present in the mutant enzymes, the main conclusion derived from this work would still hold, i.e., that the glutamine auxotrophy of *gln-1a* and *gln-1b* strains is due to alterations in the structure of glutamine synthetase.

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