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Neurospora crassa Mutants Deficient in Asparagine Synthetase

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Neurospora crassa mutants deficient in asparagine synthetase were selected by using the procedure of inositol-less death. Complementation tests among the 100 mutants isolated suggested that their alterations were genetically allelic. Recombination analysis with strain S1007t, an asparagine auxotroph, indicated that the mutations were located near or within the *asn* gene on linkage group V. In vitro assays with a heterokaryon indicated that the mutation was dominant. Thermal instability of cell extracts from temperature-sensitive strains in an in vitro asparagine synthetase assay determined that the mutations were in the structural gene(s) for asparagine synthetase.

Asparagine biosynthesis is catalyzed by asparagine synthetase:

$$\text{L-Asp} + \text{L-Gln} + \text{ATP} \xrightarrow{Mg^{2+}} \text{L-Asn} + \text{L-Glu} + \text{AMP} + PP_i$$

The asparagine synthetase enzyme from procaryotes and mutations which affect this activity have been described (3, 4, 19). The role of amino donor is filled by NH_4^+ (2, 4, 19). Eucaryotic organisms, however, utilize glutamine as the preferred nitrogen donor (10, 14). Genetic analyses of *Saccharomyces cerevisiae*, *Klebsiella aerogenes*, and *Escherichia coli* indicate that complete auxotrophy is obtained only as the result of two unlinked mutations. In *E. coli*, this situation is not thought to be the result of gene duplication; however, in *S. cerevisiae*, the two enzymes required for complete auxotrophy are remarkably similar (8, 11, 12, 18, 20). In the case of the two different genes in *K. aerogenes*, one codes for ammonia-dependent asparagine synthetase, whereas the other codes for glutamine-dependent asparagine synthetase (20). A mutant of *Neurospora crassa* which requires asparagine for growth was first reported by Tanenbaum et al. (22). The inability to detect asparagine synthetase activity prevented the identification of the biochemical defect. The development of a new assay for asparagine synthetase has alleviated this problem (15).

A total of 111 mutants requiring asparagine were isolated from ca. 15,000 survivors of UV mutagenesis by inositol-less death selection (13). Conidia from an inositol (*inl*) auxotroph, suspended in water at a concentration of 10^6 conidia per ml, were irradiated for 7 min with UV light (254 nm) and then incubated for 6 h at 37°C in a shaker bath. The growth medium contained minimal Fries salts, 1.5% sucrose, and 150 μg of inositol per ml. After germination, the conidia were washed twice, suspended in molten medium (43°C) containing 2% sorbose, 0.5% fructose, 0.5% glucose, Fries minimal salts, and 1% agar, and then poured into petri plates. After 24, 48, or 72 h at 37°C, the plates were overlaid with 10 ml of a 0.8% molten agar medium containing 3 mM asparagine, 0.6% sodium acetate, and 450 μg of inositol per ml. Complementation and dominance testing were done on sorbose minimal medium petri plates by spotting loopfuls of conidial suspensions of different auxotrophs on top of each other. The asparagine auxotrophs were crossed with the fluffy (*fl*) strain (FGSC 1838). Among the mutants, 12 were temperature sensitive: *asn-100*(Ts) through *asn-111*(Ts). Of these, none showed any growth at 37°C without added asparagine. All of the temperature-sensitive mutants grew as well as wild type at 22°C; most of them were leaky at 33°C. None of the asparagine auxotrophs grew on aspartate-, glycine-, or glutamine-supplemented media without asparagine, and all were tight, clean mutants.

Complementation testing was done by pairing all mutants with the randomly picked strains *asn-13* and *asn-37* on minimal medium plus inositol and incubating them at 25, 33, and 37°C. Only combinations *asn* (*13-35*), (*13-48*), (*13-89*), (*13-90*), (*13-91*), (*13-93*), (*13-97*), and (*37-35*) demonstrated any detectable growth, and that growth was approximately 10 to 30% that of wild type. Growth patterns were the same at all

temperatures with the exception of the temperature-sensitive strains which, of course, grew at 25°C (data not shown). This extremely slow and inconsistent growth suggested partial complementation.

Dominance testing was done in nutritionally forced heterokaryons of *asn inl ad*$^+$, *asn*$^+$ *inl*$^+$ *ad*, *asn inl nic*$^+$, and *asn*$^+$ *inl*$^+$ *nic* (ad [FGSC 674] and *nic* [FGSC 763]). These strains were spotted jointly on solid medium both with and without asparagine. All *asn* auxotrophs except *asn-22* appeared to be dominant to the wild type when adenine was used as the forcing marker. The 1 mutant (*asn-22*) out of 111 which appeared to be recessive to the wild type was probably the result of either a reversion or a contamination. However, most of the *asn* auxotrophs were recessive to the wild type in the nicotinamide heterokaryons (data not shown). Because of this conflict, the dominance of one auxotroph was analyzed by using in vitro enzyme analysis of a temperature-sensitive *asn* strain.

Low-resolution recombination analysis was done by crossing 12 of the mutants to strain FGSC S1007a, an asparagine-requiring mutant which has been mapped to the right arm of linkage group V. None of the crosses displayed the vigorous growth characteristic of wild-type progeny. Spores spread on plates with added asparagine were able to grow vigorously.

Cells were assayed for asparagine synthetase activity by the method of Luehr and Schuster (15). Cell extracts from a temperature-conditional mutant, *asn-108*(Ts), and wild-type cells were incubated at 50°C for 0, 15, 30, and 45 min and then were assayed at 25°C for 20 min. The activity of the temperature-conditional mutant decreased rapidly as a function of time at 50°C (Fig. 1).

A heterokaryon was formed between one temperature-sensitive mutant, *asn-108*(Ts), and the nicotinamide-requiring mutant *asn-108*(Ts) *inl nic*$^+$/*asn*$^+$ *inl*$^+$ *nic*. Cultures were grown in sparge flasks containing Fries minimal salts, 1.5% sucrose, and any appropriate supplements for 24 h at 25°C. The mycelia were collected on filter paper, washed with 4°C distilled water, and ground in a 4°C porcelain mortar with sand in 100 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer containing 1 mM dithiothreitol and 7 mM $MgCl_2$ at pH 7.8. This homogenate was sonicated for 45 s at the maximum setting with an Ultrasonic Sonifier Cell Disruptor microtip and was centrifuged at 20,000 rpm in an SS-34 rotor (Sorvall) for 30 min. The supernatant was again sonicated and assayed. Cell extracts were incubated at 50°C for the indicated time periods and then immediately assayed at 25°C for asparagine synthetase activity. The specific activity of wild type was

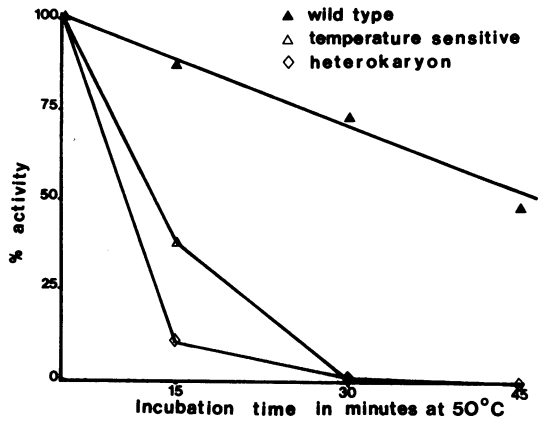

FIG. 1. Relative heat stability at 50°C of asparagine synthetase activity in crude extracts from strains FGSC 497 (wild type), mutant *asn-108*(Ts), and the heterokaryon *asn-108*(Ts)*inl nic*$^+$/*asn*$^+$ *inl*$^+$ *nic*.

3.2 nmol/min per mg, that of the mutant *asn-108*(Ts) was 2.5 nmol/min per mg, and that of the heterokaryon was 2.4 nmol/min per mg. The assay medium contained 5 mM L-glutamine, 4 mM ATP, 8 mM L-[4-^{14}C]aspartate (5.25×10^4 cpm/μmol), 25 mM HEPES, and 0.4 ml of enzyme extract in a 0.6-ml volume (pH 7.8). Assays were incubated at 37°C for 30 min and then terminated by pipetting 0.5 ml of the assay mixture into 1 ml of 1.2 M sodium acetate (pH 5.7). After heating in a boiling-water bath for 5 min, the solution was centrifuged, and the supernatant was tested for [^{14}C]asparagine by preferential decarboxylation of aspartate, using pyridoxal and Al^{3+} ions to β-decarboxylate nonenzymatically the ^{14}C of L-[4-^{14}C]aspartate. Because the β-carboxyl leaving group of aspartate is much more electronegative than the corresponding amidated β-carbon of asparagine, aspartate is selectively decarboxylated at the 4-carbon after Schiff-base formation with pyridoxal (15). Protein concentrations were determined by the Biuret method (12).

Asparagine synthetase activity in the heterokaryon decreased rapidly with increasing time intervals of preincubation at 50°C (Fig. 1). As expected, this decrease was not a simple biphasic curve. The wild-type enzyme was significantly more stable.

Table 1 illustrates the percentage of asparagine synthetase activity found in mutants which were grown in the presence of asparagine. These mutants exhibited 1.3 to 23.8% of the asparagine synthetase relative activity in wild-type cells. The mutants *asn-104*(Ts) and *asn-108*(Ts), when assayed without being incubated at 50°C before assay, had activity ranging from 80 to 130% of that of wild type. When these isolates were incubated at 50°C for 45 min before assay, they

TABLE 1. Asparagine synthetase activity in mutant strains

Strain	Enzyme activity (nmol/min per mg)
Wild type	1.92
Mutants	
asn-1	0.46
asn-5	0.31
asn-14	0.02
asn-16	0.22
asn-26	0.05
asn-40	0.29
asn-104(Ts)[a]	0.27
asn-108(Ts)[a]	0.07

[a] Incubation at 50°C for 30 min before assay. *asn-104*(Ts) and *asn-108*(Ts) were grown at the permissive temperature (25°C); their specific activities were 1.95 and 1.52 nmol/min per mg, respectively, when not incubated at 50°C for 30 min. Strain FGSC 477 *inl* was used as the wild-type strain. All FGSC strains were obtained from the Fungal Genetics Stock Center, Humbolt State University Foundation, Arcata, Calif. Cultures were maintained at 37°C on Fries minimal salts medium (1) plus 1.5% (wt/vol) sucrose and appropriate supplements for auxotrophic strains. Amino acid supplements were provided at 1 mM, and inositol was provided at 50 μg/ml. Standard methods were used for the maintenance and storage of these strains (7). Extracts were prepared and assays were performed as reported in Fig. 1.

had no detectable activity, as compared with the wild-type enzyme activity which decreased to 75% of its original activity after 45 min at 50°C.

In assays with wild-type cells, when glutamine was compared with ammonia as nitrogen donor, the apparent activity of asparagine synthetase was three times greater with 5 mM glutamine (1.62 nmol/min per mg) than with 200 mM ammonia (0.54 nmol/min per mg). These assays were done with saturating substrate concentrations. Since the ammonia-dependent asparagine synthetase activity was so much lower than the glutamine-dependent activity, it was not routinely measured.

Studies in mammalian systems have shown that asparagine synthetase exists as a multimeric enzyme (16). The complementation and dominance data presented here indicate the presence of a multimeric enzyme in *N. crassa* also. Intragenic complementation occurs in proteins whose active form is not a single polypeptide but an aggregate of two or more polypeptides. In some mutants, the enzyme subunit is defective or distorted in such a way that when it is associated with the subunit of another mutant, as in complementation, the abnormal conformation is corrected and at least some activity is achieved. This type of complementation (an interaction between different mutant derivatives of the same subunit) has been documented in *am* mutants of *N. crassa* (5, 6) and the alkaline phosphatase mutants of *E. coli* (21). In asparagine synthetase of *N. crassa*, these subunits appear to be identical. This is unlike the situation recently elucidated in *S. cerevisiae* and in *E. coli*, where total auxotrophy requires a double mutant (11, 17, 18). However, both asparagine synthetases from *K. aerogenes* are polypeptide aggregates. The glutamine-dependent enzyme is a tetramer of four identical subunits, whereas the ammonia-dependent enzyme is only a dimer (20). In *S. cerevisiae*, the two enzymes produced are very similar and are thought to be the result of gene duplication. This observation correlates with the data presented here supporting the concept of a single eucaryotic gene. A recent report of mutational analysis in cultured Chinese hamster ovary cells also identified only a single gene which coded for asparagine synthetase (9). As shown here, total auxotrophy in *N. crassa* was produced by a single gene mutation. The only complementation observed was the slow-inconsistent growth indicative of intragenic complementation. The recombination analysis provides further proof of a single gene; there was less than 0.1% recombination, an amount low enough to escape detection in low-resolution analysis.

The fact that glutamine is the preferred amide donor in *N. crassa* further supports the hypothesis that its asparagine synthetase is similar to that found in higher eucaryotes rather than that shown to be present in procaryotes (10, 13). It must be pointed out that although these studies indicate that asparagine synthetase from *N. crassa* can use both glutamine and ammonia as the nitrogen donor, it is difficult to know which is the in vivo substrate. If the cellular pH were high enough, conceivably the free NH_3 could be used as it is in procaryotes. However, the fact that the *N. crassa* asparagine synthetase utilizes glutamine suggests the presence of a glutaminase activity that releases NH_3 in close to the reactive intermediate form of aspartic acid, as has been suggested for the enzyme from eucaryotes (10).

The association of an altered phenotype with an altered gene product was characterized by thermal stability studies. The mutation in temperature-sensitive strains must be the result of a defect in enzyme stability rather than in enzyme synthesis, as the dramatic decrease of enzyme activity of the temperature-sensitive mutant, compared with the heat stability of the wild-type enzyme shown in Fig. 1, clearly demonstrates. If the defect were in synthesis, temperature would not have any effect on activity in a cell extract. Thus the lesion is in a structural rather than a regulatory gene. Furthermore, when *ad* was used as the forcing marker in vivo, 110 of 111

mutants were determined to be dominant. This mutation has been shown to be dominant in vitro in the heterokaryon when *nic* was used as the forcing marker, at least with *asn-108*(Ts). The fact that the temperature-sensitive heterokaryon data did not conform to a simple biphasic curve suggests that a negative synergism was occurring (Fig. 1). The active enzyme must be multimeric with interacting subunits, since in vitro the heterokaryon displays a progressive loss of enzyme activity which parallels the temperature-sensitive mutant. If the mutant *asn-108*(Ts) in the heterokaryon were not dominant, the activity would have paralleled that observed for the wild-type enzyme; that is, it would have been a biphasic curve characteristic of independent deactivation.

Dominance can occur if a defective mutant protein is a subunit of an oligomeric enzyme. The mutant monomer may modify the conformation of the wild-type monomers in an oligomeric enzyme, making the enzyme inactive. Dominance is a relatively uncommon occurrence in mutant genes, which makes this mutation particularly valuable for genetic analysis.

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