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Glutamate Synthase Levels in *Neurospora crassa* Mutants Altered with Respect to Nitrogen Metabolism

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Glutamate synthase catalyzes glutamate formation from 2-oxoglutarate plus glutamine and plays an essential role when glutamate biosynthesis by glutamate dehydrogenase is not possible. Glutamate synthase activity has been determined in a number of *Neurospora crassa* mutant strains with various defects in nitrogen metabolism. Of particular interest were two mutants phenotypically mute except in an *am* (biosynthetic nicotinamide adenine dinucleotide phosphate-glutamate dehydrogenase deficient, glutamate requiring) background. These mutants, *i* and *en-am*, are so-called enhancers of *am*; they have been redesignated herein as *en(am)-1* and *en(am)-2*, respectively. Although glutamate synthase levels in *en(am)-1* were essentially wild type, the *en(am)-2* strain was devoid of glutamate synthase activity under all conditions examined, suggesting that *en(am)-2* may be the structural locus for glutamate synthase. Regulation of glutamate synthase occurred to some extent, presumably in response to glutamate requirements. Glutamate starvation, as in *am* mutants, led to enhanced activity. In contrast, glutamine limitation, as in *gln-1* mutants, depressed glutamate synthase levels.

In the fungus *Neurospora crassa*, ammonium incorporation into organic compounds for the ultimate biosynthesis of cellular nitrogenous constituents is achieved primarily via the sequential action of nicotinamide adenine dinucleotide phosphate (NADP)-glutamate dehydrogenase (GDH) (EC 1.4.1.4) and glutamine synthetase (GS) (EC 6.3.1.2):

$$2\text{-Oxoglutarate} + NH_4^+ + NADPH + H^+ \xrightarrow{\text{NADP-GDH}} \text{glutamate} + NADP^+ \quad (1)$$

$$\text{Glutamate} + NH_4^+ + ATP \xrightarrow{GS} \text{glutamine} + ADP + P_i \quad (2)$$

where ATP is adenosine 5′-triphosphate and ADP is adenosine 5′-diphosphate.

In a secondary reaction, the enzyme glutamate synthase (EC 1.4.1.14, as discussed herein) (glutamine [amide]:2-oxoglutarate aminotransferase, or GOGAT) can catalyze the formation of two equivalents of glutamate from glutamine plus 2-oxoglutarate (α-ketoglutarate):

$$\text{Glutamine} + \text{2-oxoglutarate} + NAD(P)H \xrightarrow{\text{GOGAT}} 2\ \text{glutamate} + NAD(P)^+ \quad (3)$$

Apparently NADPH serves as the electron donor in procaryotes (25), whereas NADH is utilized in yeast (2, 23) and *N. crassa* (14) and ferredoxin is employed in green plants (19). Coupled with GS, GOGAT can function to achieve ammonium assimilation under conditions where this nutrient is limiting or NADP-GDH is absent or ineffective:

$$\text{Glutamine} + \text{2-oxoglutarate} + NADH + H^+ \xrightarrow{\text{GOGAT}} 2\ \text{glutamate} + NAD^+$$

$$\text{Glutamate} + NH_4^+ + ATP \xrightarrow{GS} \text{glutamine} + ADP + P_i$$

$$\text{Net: 2-Oxoglutarate} + NH_4^+ + NADH + H^+ + ATP \rightarrow \text{glutamate} + ADP + P_i + NAD^+$$

Note that the reaction responsible for ammonium fixation in this scheme is that catalyzed by GS. Hummelt and Mora (14) recently reported the presence of GOGAT in *N. crassa*. This activity was detectable in a mutant strain lacking NADP-GDH activity, an *am* strain. Typically, *am* mutants require glutamate for optimal growth but are leaky, i.e., they grow, albeit poorly, on ammonium or nitrate as sole nitrogen source (see Table 1).

Mutation at either of two separate loci in *N. crassa*, namely, the *i* locus (on linkage group VR) or the *en-am* locus (on linkage group IIR), results in no easily discernible phenotype in either case, except that in an *am* mutant background, these mutants enhance the *am* phenotype. That is, the poor growth of *am* (NADP-

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GDH defective) mutants on ammonium becomes even poorer in *am,i* or *am;en-am* double mutants. This property is precisely what would be expected of a strain lacking both NADP-GDH and GOGAT. This paper reports the results of studies on nitrogen metabolism in these *Neurospora* strains which demonstrate that the *en-am* mutant is devoid of GOGAT activity, suggesting that the *en-am* locus may be the structural gene for GOGAT. For clarity and consistency with current *Neurospora* nomenclature, we propose the new symbols *en(am)-1* for *i* and *en(am)-2* for *en-am*, and we use these designations below.

MATERIALS AND METHODS

Strains and growth conditions. *N. crassa* 74-OR23-1A was used as wild type. Mutant strains variously affected in nitrogen metabolism included: *am* strains am_1 (allele no. 32213), am_2 (allele no. 47305), or am_{17} (allele no. am_{17}) as indicated; *gln-1* strains *gln-1a* (allele no. R1015) or *gln-1b*, kindly provided by J. Mora; *nit-2* (allele no. nr37); *i* (allele no. 1444), designated as *en(am)-1* in this paper; *mea-1*, a methylammonium-resistant strain isolated in this laboratory; and the double mutants *en-am;am* (alleles C24 and 32213, respectively) [referred to as *en(am)-2;am* herein], *i,am*, isolated in this laboratory from crosses of $am_{17} \times i$ (allele no. 1444) and designated as *en(am)-1;am* herein, and *nit-2;mea-1*, isolated from *nit-2* (nr37) × *mea-1* crosses carried out in this laboratory. The *en(am)-2* single mutant used in this study was obtained from crosses of *en(am)-2;am* × 74-OR8-1a wild type by selecting among the progeny for isolates lacking the *am* defect which grew more poorly than wild type. Although such growth differences are not strikingly obvious, they are discernible, and two such isolates were independently obtained.

Mycelia for enzyme extraction were grown for 40 h from conidial inocula in minimal salts medium containing 3% (wt/vol) cane sugar, 80 mM NH_4Cl, 5 mM glutamate and 2.5 mM glutamine, harvested by filtration, washed, and transferred to indicated media for 6 h, and then harvested, washed, and frozen at −70°C until used.

Enzyme extraction. Typically, frozen mycelia were homogenized in an ice-cold Ten Broeck glass homogenizer with 0.1 M potassium phosphate buffer (pH 7.3) containing 5 mM cysteine, 5 mM ethylenediaminetetraacetic acid, and 10% glycerol. Crude homogenates were then centrifuged at 20,000 × *g* for 20 min, and the supernatant was taken as the crude extract.

Enzyme assays. NADPH-nitrate reductase was assayed as directed by Garrett and Nason (10) as modified from Nason and Evans (21); NADPH-nitrite reductase was assayed by nitrite-dependent NADPH oxidation as described by Greenbaum et al. (12); GS was determined by the transferase assay described by Ferguson and Sims (8); and NADP-GDH and NAD-GDH were assayed as given in Dantzig et al. (5) and Dantzig et al. (4), respectively. GOGAT activity was measured using NADH as electron donor and 1% β-mercaptoethanol in the buffer according to the protocol of Boland and Benny (1). All activities are expressed as nanomoles of substrate transformed per minute per milligram of protein.

Protein determinations. The procedure of Lowry et al. (16) was followed. Bovine serum albumin served as the standard.

Growth tests. Growth tests were performed on solid medium in petri plates. The medium contained Fries minimal salts, 0.05% glucose, 0.05% fructose, 2% sorbose, 2% agar, and nitrogen sources as indicated. Conidial suspensions of the various mutant strains were independently spotted onto the surface of this medium, and the plates were incubated for 2 to 4 days at 25°C. Growth was scored as: −, none; +, weak; ++, subnormal; or +++, vigorous.

RESULTS

Growth tests. The growth of various *Neurospora* mutant strains impaired in glutamate or glutamine metabolism was determined on different nitrogen sources and compared to that of the wild type (Table 1). Wild-type *N. crassa* grew well on all nitrogen sources tested here and was resistant to chlorate on ammonium, glutamate, or glutamine because these reduced nitrogen forms effectively repress nitrate reductase synthesis and thereby prevent the conversion of chlorate to the toxic chlorite, as catalyzed by this enzyme. Although the growth of wild-type mycelia on nitrate, ammonium, or glutamate has been reported to be inhibited extensively by the glutamate analog L-methionine-DL-sulfoximine in liquid culture (22), in plate tests on solid medium the wild type was relatively unaffected by this analog. Wild type also grew satisfactorily in the absence of sucrose when using 2-oxoglutarate and glutamine as its sole carbon and nitrogen sources, a test reflecting the ability of *N. crassa* to carry out the GOGAT reaction in order to meet its requirements for glutamate. The *gln-1* mutant alleles, *gln-1a* and *gln-1b*, as well as the *am* mutant, displayed growth responses consistent with their enzymatic capabilities. That is, the *gln-1* mutants were leaky on NO_3^-, NH_4^+, and glutamate since they retain some GS activity. Although the *am* mutant was devoid of detectable NADP-GDH activity, it was nevertheless also leaky on NO_3^- and NH_4^+, suggesting that an alternative pathway for glutamate formation exists. Growth of *am* on 2-oxoglutarate plus glutamine supported this notion. The mutants *en(am)-1* and *en(am)-2* in *am* backgrounds resulted in an enhancement of the *am* phenotype. As such, *en(am)-2* appeared to have the greater effect, and the *en(am)-2;am* double mutant only grew normally on glutamate. As single mutants, *en(am)-1* and *en(am)-2* displayed wild-type growth patterns except that *en(am)-2* seemed to grow slightly more

TABLE 1. *Results of growth tests on various Neurospora mutants*

Genotype	Nitrogen source utilization[a]				Analog sensitivity[b]				Growth on αKG + Gln[c]
					Chlorate plus			MSX + NH_4^+	
	NO_3^-	NH_4^+	Glu	Gln	NH_4^+	Glu	Gln		
Wild type	+++	+++	+++	+++	R	R	R	R	+++
gln-1A,B	+	+	+	+++	−	−	R	−	+++
am	+	+	+++	+++	−	R	R	−	+++
en(am)-2	++	++	+++	++	R	R	R	S	++
en(am)-2;am	−	−	+++	++	−	R	SR	−	+/−
en(am)-1	+++	+++	+++	+++	R	R	R	R	+++
en(am)-1;am	+	+/−	+	+++	−	S	SR	−	+++

[a] N sources: NO_3^- and NH_4^+, 10 mM; glutamate (Glu) and glutamine (Gln), 5 mM.

[b] Analogs: Chlorate, 100 mM; L-methionine DL-sulfoximine (MSX), 5 mM. R, Resistant; SR, slightly resistant; S, sensitive; −, extremely sensitive (no growth).

[c] This medium contained Vogel salts, 2% agar, and 5 mM 2-oxoglutaric acid (αKG) plus 5 mM glutamine as the sole carbon and nitrogen sources.

poorly on N-sources other than glutamate and showed sensitivity to L-methionine-DL-sulfoximine, probably because its glutamine levels are limiting. It is significant that *en(am)-2;am* could not grow on 2-oxoglutarate plus glutamine as sole carbon and nitrogen sources.

Levels of glutamate-metabolizing enzymes in various *N. crassa* strains. The absence of GOGAT activity due to mutation might be anticipated to have minimal consequence under most conditions but to be effectively lethal when other routes of glutamate production are lost (as in *am* mutants). The levels of GOGAT, NADP-GDH, and NAD-GDH were surveyed in selected *N. crassa* strains to examine this hypothesis (Table 2). Mycelia were incubated in media containing the indicated nitrogen source for 6 h, a period which was found to give optimal GOGAT expression, and crude extracts were prepared as described in Materials and Methods. The striking result was that strains carrying the *en(am)-2* mutation were devoid of GOGAT activity on any nitrogen source. Strains carrying the am_1 mutation were totally lacking NADP-GDH as anticipated. It is interesting that the NAD-GDH levels in the *en(am)-2;*am_1 double mutant strain were severely depressed compared to the levels in wild type or either single mutant strain.

Levels of GOGAT and other enzymes of nitrogen metabolism in *N. crassa* strains altered in glutamate metabolism. The absence of GOGAT in *en(am)-2* strains permitted an assessment of the influence of this enzyme on the expression of other nitrogen-metabolizing activities in *N. crassa*. The results of such studies are presented in Table 3. In addition to GOGAT and GS, the enzymes of nitrate assimilation, nitrate reductase and nitrite reductase, were assayed. The absence of GOGAT in *en(am)-2* single-mutant strains did not appreciably alter the regulation of nitrate assimilation as evidenced by nitrate reductase and nitrite reductase levels, nor did it affect GS activity. Activity levels observed in wild type provide the basis for these comparisons. The am_1 single mutant manifested little change in GS activity as well, compared to wild- type. Note, however, that nitrate assimilation in am_1 escaped ammonium repression but was still subject to glutamate and glutamine repression, as reported previously (4). The *en(am)-2;*am_1 double mutant displayed very high levels of the nitrate assimilation enzymes and GS under all conditions of nitrogen nutrition tested here. Apparently nitrogen metabolite repression is relatively ineffective in this strain, even in the presence of glutamine.

GOGAT levels in other *N. crassa* mutant strains. Table 4 presents the results of studies on the response of GOGAT activity to different conditions of nitrogen nutrition in other *Neurospora* mutants altered in nitrogen metabolism. Several conclusions can be drawn, in addition to the fact that GOGAT levels in the wild type showed little variation with nitrogen source. Where NADP-GDH activity was missing, as in the am_2 mutant, GOGAT levels achieved significantly higher levels than those in the wild type. This response may help to ameliorate the glutamate-starved condition that is imposed by *am* mutations. The defect in *en(am)-1* which leads to enhancement of the *am* phenotype is apparently unrelated to GOGAT since this activity resembles wild type in its expression in *en(am)-1*. The *nit-2* mutant, which affects a wide spectrum of nitrogen utilization in *Neurospora*, has no effect on GOGAT. The mutant *mea-1* is a recent isolate in our laboratory, selected on the basis of its resistance to the toxic ammonium analog methylamine. It is presumably impaired

TABLE 2. *GOGAT, NADP-GDH, and NAD-GDH specific activities in various N. crassa mutants*

N source	Genotype	GOGAT	NADP-GDH	NAD-GDH
20 mM $NaNO_3$	Wild type	6.5	591	128
	en(am)-2	0	472	57
	en(am)-2;am$_1$	0	0	25
	am$_1$	6.0	0	102
10 mM $NaNO_3$ + 25 mM NH_4Cl	Wild type	5.0	365	86
	en(am)-2	0	356	133
	en(am)-2;am$_1$	0	0	24
	am$_1$	6.0	0	104
10 mM $NaNO_3$ + 20 mM glutamate	Wild type	2.0	320	111
	en(am)-2	0	380	134
	en(am)-2;am$_1$	0	0	21
	am$_1$	4.2	0	106
10 mM $NaNO_3$ + 20 mM glutamine	Wild type	6.3	181	77
	en(am)-2	0	306	137
	en(am)-2;am$_1$	0	0	32
	am$_1$	8.9	0	122
10 mM $NaNO_3$ + 5 mM uric acid	Wild type	7.1	450	67
	en(am)-2	0	540	112
	en(am)-2;am$_1$	0	0	22
	am$_1$	5.4	0	76

TABLE 3. *GOGAT, nitrate reductase, nitrite reductase, and GS specific activities in various N. crassa mutants*

N source	Genotype	GOGAT	NaR[a]	NiR[b]	GS
20 mM $NaNO_3$	Wild type	5.6	56.9	63	17.9
	en(am)-2	0	26.7	56	12.2
	en(am)-2;am$_1$	0	64.9	70.3	55.1
	am$_1$	6.0	72.1	66	13.5
10 mM $NaNO_3$ + 25 mM NH_4Cl	Wild type	6.5	1.2	2.9	9.4
	en(am)-2	0	1.8	6.0	17.1
	en(am)-2am$_1$	0	62.7	48.9	68.7
	am$_1$	6.0	62.0	91	12.7
10 mM $NaNO_3$ + 20 mM glutamate	Wild type	4.1	5.9	15.7	23.0
	en(am)-2	0	7.7	16	17.1
	en(am)-2;am$_1$	0	20.5	49.0	29.6
	am$_1$	4.2	1.8	16	17.8
10 mM $NaNO_3$ + 20 mM glutamine	Wild type	6.4	0.1	0	4.7
	en(am)-2	0	0	0	1.0
	en(am)-2;am$_1$	0	23.7	41.6	33.2
	am$_1$	8.9	0.1	0	3.4
10 mM $NaNO_3$ + 5 mM uric acid	Wild type	6.5	25.7	54.2	18.8
	en(am)-2	0	20.8	37	15.9
	en(am)-2;am$_1$	0	54.9	34.8	68.8
	am$_1$	5.4	45.6	22	14.8

[a] NaR, Nitrate reductase.
[b] NiR, Nitrite reductase.

TABLE 4. *Glutamate synthase specific activity in wild type and N. crassa mutants exposed to various conditions of nitrogen nutrition*

N source in medium	Wild type	am_2	*en(am)-1*	*nit-2*	*mea-1*	*nit-2;mea-1*	*gln-1b*
2.5 mM NH_4Cl	5.0	8.8	5.2	8.6	7.5	3.4	2.4
5 mM NH_4Cl	5.1	9.6	4.6	5.8	7.8	8.5	2.4
5 mM glutamate	4.0	7.5	1.6	4.3	6.0	4.4	2.4
5 mM glutamine	4.9	5.8	5.1	4.3	4.8	5.5	1.5
5 mM $NaNO_3$	4.1	14.7	4.7	8.1	8.7	4.5	1.6
5 mM NH_4Cl + 5 mM $NaNO_3$	6.9	8.7	7.7	9.0	6.9	6.8	1.4

in ammonium uptake; GOGAT levels in *mea-1* may be slightly elevated over wild type but the effect does not appear significant. In a *nit-2;mea-1* double mutant, GOGAT remains unaltered. It is interesting that the GOGAT levels in the *gln-1b* GS-impaired strain are markedly depressed.

DISCUSSION

In *N. crassa* there exist two separate loci in which mutations appear phenotypically silent, except in the presence of an *am* mutation. These mutants have been termed "enhancer of *am*" or *en-am* and "inhibitor of *am*" or *i*. Herein *en-am* has been redesignated as *en(am)-2*, and *i* has been designated *en(am)-1*. The *en(am)-1* locus is on linkage group V; *en(am)-2* is on linkage group II. The *am* locus is near *en(am)-1* on linkage group V, showing about 8% recombination with it (3). The *am* locus specifies NADP-GDH, the enzyme responsible for glutamate biosynthesis from 2-oxoglutarate and ammonium (9). This reaction is the major biological route for glutamate formation. Nevertheless, in many organisms including *N. crassa*, mutants totally devoid of detectable NADP-GDH activity are not strict glutamate auxotrophs. That is, these mutants can subsist, albeit somewhat less robustly, in the absence of glutamate on ammonium as the sole nitrogen source. These facts alone suggest an alternative pathway for glutamate synthesis. The existence of GOGAT provides this alternative, mediating the formation of two equivalents of glutamate from 2-oxoglutarate plus glutamine in the presence of reduced pyridine nucleotide. The occurrence of GOGAT in procaryotes in widely reported, and its presence in yeast (2, 23), green plants (17), and, just recently, *N. crassa* (14) has also been established.

The possibility that NAD-GDH provides the alternative pathway for glutamate synthesis is discounted by a consideration of the results in Table 2. NAD-GDH, which is generally assigned a catabolic role in glutamate metabolism, apparently cannot fulfill a biosynthetic function. The levels of this enzyme, though substantially lower in the *en(am)-2;* am_1 double mutant than in the other strains, are nevertheless roughly equivalent to typical GS levels (about 20 nmol of substrate metabolized per min per mg of protein in crude extracts; see Tables 2 and 3) and four- to fivefold greater than normal GOGAT levels in wild type (Table 2). Thus it seems more likely that the occurrence of GOGAT, not the presence of NAD-GDH accounts for the characteristic leakiness of *am* mutants. However, the possibility that both of these enzymes contribute cannot be ruled out; the structural gene for NAD-GDH in *N. crassa* is unknown. In *Aspergillus nidulans*, where both the NADP-GDH gene (*gdhA* locus) and the NAD-GDH gene (*gdhB* locus) have been described, the *gdhA1;gdhB1* double mutant still shows leakiness on ammonium (24).

It was anticipated that GOGAT activity in *N. crassa* might somehow be absent or altered in *en(am)-1* or *en(am)-2*, thus accounting for the phenotypic enhancement of *am* seen in these strains. Examination of the expression of GOGAT activity in these and other *N. crassa* mutant strains revealed that this enzyme is affected only in *en(am)-2*. Under no conditions could any GOGAT activity be detected in this strain. Conversely, this activity was essentially unaffected in virtually all other instances. The simplest interpretation is that *en(am)-2* is the structural gene locus for GOGAT.

Deshpande and Kane (7) have reported that mutation at two loci, designated *gltA* and *gltB*, results in loss in GOGAT activity in *Bacillus subtilis*. The enzyme in this procaryote is a heteromultimer composed of two nonidentical subunits, as is the GOGAT from *Escherichia coli* (17, 18, 20), *Klebsiella aerogenes* (11, 26), and *Bacillus megaterium* (23). Although *en(am)-2* strains are devoid of GOGAT activity, the other locus leading to enhancement of the *am* phenotype, *en(am)-1*, has normal GOGAT levels. A second locus in *N. crassa* which results in loss of GOGAT activity has not been reported. No evidence on the subunit composition of *N. crassa* GOGAT is available, and whether this fungal enzyme is hetero- or homomultimeric is unknown.

The nature of *en(am)-1* remains enigmatic. Its effects are minimal except in combination with

an *am* mutant. Since GOGAT is normal in *en(am)-1*, this secondary pathway of glutamate synthesis should salvage the *en(am)-1,am* double mutants unless ammonium uptake is drastically impaired in such strains. This possibility seems unlikely since the effects of the *en(am)-1* single mutation are minimal. Further, the GOGAT/GS route apparently has a greater avidity for ammonium ion than the usual NADP-GDH/GS pathway (14, 27). Resolution of this question must await further experimentation.

The results in Tables 2 and 3 permit the comparison of relative GOGAT, NADP-GDH, and GS levels in *N. crassa* under typical conditions. As an approximation, in terms of nanomoles of substrate transformed per minute per milligram of crude extract protein, the relative values for GOGAT, NADP-GDH, and GS are 5, 300, and 20, respectively. Since the GS was assayed by the transferase assay, which is typically only 0.33 as effective as the synthetase activity of the enzyme (6), the ratio is more appropriately indicated as 5:300:60. The point to be made here is that the GOGAT specific activity is about an order of magnitude less than that of GS and only 2% that of NADP-GDH. Thus, glutamate auxotrophy is readily apparent in *am* mutants, whereas mutations in *en(am)-2* are essentially unrecognizable in standard growth tests. These results also support the assignment of the major role of glutamate biosynthesis in *N. crassa* to NADP-GDH and the designation of GOGAT as a secondary route. The importance of GOGAT in the fixation of ammonium at low concentrations has already been emphasized (14, 26).

The relative GOGAT activity levels in wild type and various mutant strains under different nutritional regimens suggest that some modulation of these levels occurs, but not to a dramatic extent. An *am* mutant (am_2; Table 4), wherein the greatest demand for GOGAT expression might be anticipated due to loss of the biosynthetic NADP-GDH, displayed only twice the normal wild-type levels of this enzyme. An interesting observation was the occurrence of low GOGAT activity in the *gln-1b* mutant under all conditions tested. One interpretation is that this depression in activity would lessen consumption of the limiting glutamine levels in this strain. An alternative speculation, for which no evidence exists, is that glutamine or GS somehow regulates GOGAT in a positive fashion.

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