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N-Acetyl-L-glutamate Synthase of *Neurospora crassa*

CHARACTERISTICS, LOCALIZATION, REGULATION, AND GENETIC CONTROL*

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***N*-Acetylglutamate synthase, an early enzyme of the arginine pathway, provides acetylglutamate for ornithine synthesis in the so-called "acetylglutamate cycle." Because acetylglutamate is regenerated as ornithine is formed, the enzyme has only a catalytic or anaplerotic role in the pathway, maintaining "bound" acetyl groups during growth. We have detected this enzyme in crude extracts of *Neurospora crassa* and have localized it to the mitochondria along with other ornithine biosynthetic enzymes. The enzyme is bound to the mitochondrial membrane. The enzyme has a pH optimum of 9.0 and K_m values for glutamate and CoASAc of 6.3 and 1.6 mM, respectively. It is feedback-inhibited by L-arginine ($I_{0.5}$ = 0.16 mM), and its specific activity is augmented 2–3-fold by arginine starvation of the mycelium. Mutants of the newly recognized *arg-14* locus lack activity for the enzyme. Because these mutants are complete auxotrophs, we conclude that *N*-acetylglutamate synthase is an indispensible enzyme of arginine biosynthesis in *N. crassa*. This work completes the assignment of enzymes of the arginine pathway of *N. crassa* to corresponding genetic loci. The membrane localization of the enzyme suggests a novel mechanism by which feedback inhibition might occur across a semipermeable membrane.**

N-Acetylglutamate synthase is an early enzyme of the arginine pathway which provides acetylglutamate for *de novo* ornithine synthesis in the "acetylglutamate cycle" of many eukaryotes. Acetylglutamate is reduced and aminated to become acetylornithine, and the acetyl group, originating in CoASAc in the original reaction, is conserved by transfer to glutamate as ornithine is formed. Because acetylglutamate is regenerated in this cycle, *N*-acetylglutamate synthase has only a catalytic or anaplerotic role in the pathway, maintaining "bound" acetyl groups during growth (1). The yeast enzyme has been studied in a preliminary way (2–4), but no study of the *Neurospora* enzyme has been published.

The *Neurospora crassa* enzyme is of interest for several reasons. First, it is the only enzyme of arginine biosynthesis that remains to be detected in this organism, and the general regulatory properties of the enzyme must be known in order to complete a coherent view of the pathway. Second, two genetic loci, *arg-13* and *arg-14*, remain to be assigned functions in the pathway; one of them is presumed to be the structural gene for *N*-acetylglutamate synthase inasmuch as all other enzymes have already been assigned to other loci (5). The enzyme is feedback-inhibited by arginine in other organisms. In *N. crassa*, the early enzymes of ornithine synthesis are found associated with the mitochondrial matrix. This poses an interesting biological problem since a similar localization for acetylglutamate synthase would result in separation of the end product and its feedback-sensitive enzyme.

In this paper, we describe the assay, some characteristics, the localization, and the genetic control of *N*-acetylglutamate synthase of *N. crassa*. We propose a novel method for transmembrane control of enzyme activity.

EXPERIMENTAL PROCEDURES

Materials—Radioactive glutamate was purchased from New England Nuclear and purified before use on Dowex 50 columns in the H^+ form to remove radioactivity which passed through this ion exchanger; radioactive aspartate was purchased from ICN. All other biochemicals were purchased from Sigma.

Strains of N. crassa and Growth Conditions—The strains of *N. crassa* used in these experiments, all from the collection of R. H. D., are described in Table I. Besides the wild-type, strain 74A, mutants of the following genetic loci were used: *arg-1, arg-2, arg-3, arg-5, arg-6, arg-7* (synonym: *arg-4*), *arg-10, arg-12, arg-13*, and *arg-14*. All mutants are available from the Fungal Genetics Stock Center, Humboldt State University Foundation, Arcata, CA. Strain IC26 carries the *arg-12*^{s} mutation in the structural gene for ornithine carbamoyltransferase; this mutation leads to a profound decrease in enzyme activity, but no arginine requirement (6); the arginine enzymes of this strain are derepressed. Starved mycelia of strain IC25 (*arg-7*), which lack *N*-acetylornithine:glutamate acetyltransferase activity (7), were used as the standard source of enzyme.

Mycelia were grown in sparged cultures (11) of Vogel's minimal medium (medium N) as described previously (8). Auxotrophs were supplemented when necessary with 40 mg/liter L-arginine (for starvation conditions) or 200 mg/liter L-arginine (for fully supplemented conditions). Growth was initiated with an inoculum of approximately 10^6 conidia/ml of culture and continued overnight. Mycelia were harvested at various times from 10 to 18 h after inoculation. Prototrophic and fully supplemented auxotrophic mycelia were in the exponential phase of growth during this time; starved cultures were

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TABLE I
Strains used in this study

Strain	Loci, alleles, mating type	Enzyme deficiencies[a]
IC1	74A	None (wild-type)
LA10	*arg-1* (B369) A	Argininosuccinate synthetase (EC 6.3.4.5)
LA121	*arg-2* (33442) A	Carbamoyl-phosphate synthetase A (small subunit) (EC 6.3.5.5)
IC23	*arg-3, pyr-3* (CD152, DFC3)	Carbamoyl-phosphate synthetase A (large subunit), carbamoyl-phosphate synthetase P, and aspartate carbamoyltransferase (EC 2.1.3.2)
IC24	*arg-5* (CD6) a	Acetylglutamatic semialdehyde:glutamate transaminase (EC 2.6.1.11)
LA322	*arg-6A* (CD136)	Acetylglutamate kinase (EC 2.7.2.8)
LA323	*arg-6A* (CD174)	Same as LA322
LA59	*arg-6B* (CD25)	Acetylglutamyl-P reductase (EC 1.2.1.38)
LA304	*arg-6B* (CD44)	Same as LA59
LA319	*arg-6B* (CD116)	Same as LA59
LA55	*arg-6NC* (CD63)	Acetylglutamate kinase and acetylglutamyl-P reductase
LA320	*arg-6NC* (CD121)	Same as LA55
LA56	*arg-6NC* (CD68)	Same as LA55
IC25	*arg-7* (CD51) a	Acetylornithine:glutamate transacetylase (EC 2.3.1.35)
LA13	*arg-10* (B317) A	Argininosuccinate lyase (EC 4.3.2.1)
IC26	*arg-12* (*arg-12*[a])A	Ornithine carbamoyltransferase (EC 2.1.3.3)
IC27	*arg-13* (CD7) a	Unknown
IC28	*arg-14* (CD197) A	Acetylglutamate synthase (EC 2.3.1.1)
IC29	*arg-14* (CD197) a	Same as IC28

[a] Ref. 5 and this paper.

in a state of starvation at about 15 h, when they were harvested as a source of enzyme.

Preparation of Mitochondria and Enzyme—Mitochondria were prepared from fresh mycelia by breakage with glass beads, differential centrifugation, and discontinuous sucrose gradient centrifugation, as described previously (9). The band of mitochondria was removed from the gradient with a pipette, diluted with an equal volume of a solution which was 25 mM Tris, 5 mM TES,[1] 0.1 mM EDTA, pH 7.5. The suspension was centrifuged at 15,000 × *g* for 20 min. The mitochondria were washed once in the final storage buffer (5 mM Tris-HCl, pH 7.5, 0.33 M sucrose) and stored in this buffer at −70 °C. Preparations were stable for at least 4 months in this condition. Upon thawing (or with fresh mitochondria), mitochondria were used directly or broken in the presence of 1 mM phenylmethylsulfonyl fluoride with glass beads in 1-ml lots as described previously (10). Disrupted mitochondria were centrifuged in an Eppendorf microfuge for 30 min at 4 °C to separate the membrane and soluble materials; the membrane preparation was used for characterization of the enzyme. The membrane preparations were stable for at least 1 week in the storage buffer; enzyme of the supernatant was less stable.

Enzyme Assay—*N*-Acetylglutamate synthase was assayed in a 100-μl reaction mixture containing 100 mM Tris-HCl, pH 9.0, 50 mM [^{14}C]glutamate (0.013 mCi/mM) adjusted to pH 9.0, 3.5 mM CoASAc, and 25–100 μg of protein in storage buffer. Assays were performed in 1.5-ml plastic microfuge tubes at 30 °C, standardized with respect to the sucrose carried over from the storage buffer. Reactions were stopped after 10 min with 50 μl of 1 M formic acid. The reaction tubes were centrifuged in the microfuge for 5–10 min. A volume of 120 μl of the reaction mixture was applied to a Dowex 50-X8 column (200–400 mesh, 0.7 × 3 cm) in the H^+ form equilibrated with 0.1 M formic acid; this was followed by 0.33 ml of 1 M formic acid. Collection of the eluate began when an additional 2 ml of formic acid was added to the column. Of the 2-ml eluate, a 1-ml sample was removed for determination of radioactivity in a liquid scintillation counter. Time-0 and no-enzyme blanks were inescapably high, given the high substrate requirement of the enzyme; they were quite consistent, however, and were subtracted in reporting data. Except where noted, a complete reaction mixture to which 1 mM L-arginine was added was used as the blank. One unit of enzyme is defined as 1 nmol of product/min at 30 °C.

Determination of Submitochondrial Localization—Mycelia of wild-type (74A) strain were grown in 1,200 ml of Vogel's minimal medium at 30 °C for 20 h. The mycelia were harvested in early stationary phase, and mitochondria were purified as previously described (11). The mitochondria were then subjected to osmotic-sonic shock as described by Cassady and Wagner (12) with the following modifications. The purified mitochondria were resuspended in 3 ml of swelling buffer (50 mM phosphate, pH 7.5) and were vigorously homogenized for 2 min in a Teflon-glass homogenizer. After a 30-min period at 0 °C, 1 ml of shrinking buffer was added (50 mM phosphate, pH 7.5, 0.4 M sucrose, 8 mM ATP, 8 mM $MgSO_4$), and the solution was briefly homogenized to blend the two buffers. The mitochondria were sonicated at 0 °C with three 5-s pulses, with 1 min between pulses, to detach the broken outer mitochondrial membranes (12). The 4-ml sonicated suspension was layered over a discontinuous sucrose density gradient containing 2.5 ml of 1.9 M sucrose and 4 ml of 1.0 M sucrose. (Both sucrose gradient solutions also contained 50 mM phosphate, pH 7.5, 1 mM phenylmethylsulfonyl fluoride, and 1 mM EDTA). Centrifugation was performed in an SW 41Ti rotor at 37,000 rpm (167,000 × *g*) for 1 h at 4 °C. Seven-tenths-ml fractions were pumped from the top of the gradient. These fractions were assayed for acetylglutamate synthase, malate dehydrogenase (13), cytochrome oxidase (14), and protein (15).

RESULTS

Optimization of the Assay—At the optimal pH and substrate concentrations described under "Experimental Procedures," the enzymatic reaction was linear with up to 0.7 mg of mitochondrial protein/reaction mixture. Linearity with time, however, was compromised by the instability of the enzyme at pH 9.0. A 10-min incubation was chosen as standard, although linearity was good for 15–20 min in most cases tested. The poor linearity did not reflect the instability of CoASAc nor the accumulation of product (data not shown).

Maximum enzymatic activity was observed between pH 8.8 and 9.0, falling to 38% of maximal activity at pH 8.0 and 25% of maximal activity at pH 9.5. In this experiment, bis-Tris-propane was used. Activity with Tris, CHES, and other buffers having more limited pH ranges was consistent with the profile using bis-Tris-propane; small, regular departures from the bis-Tris-propane curve were seen with other buffers. The buffer chosen for all subsequent work was Tris-HCl. Tris gave

[1] The abbreviations used are: TES, *N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonate; CHES, cyclohexylaminoethanesulfonate; bis-Tris-propane, 1,3-bis[tris(hydroxymethyl)methylamino]propane.

25% higher activity at pH 9.0 than did bis-Tris-propane.

The radioactive product of the reaction was identified as acetylglutamate by co-chromatography with the authentic compound by the method of Wolf and Weiss (16). The product was stable in the reaction mixture when authentic [^{14}C]acetylglutamate was tested at 1 mM with 0.16 mg of mitochondrial protein. Similarly, broken mitochondria, mitochondrial supernatants (matrix fraction), and mitochondrial membranes had no effect on the product.

The standard blank chosen for routine experiments was a complete reaction mixture to which 1 mM L-arginine was added. In such reaction mixtures, arginine-insensitive, CoASAc-dependent, time-dependent radioactivity was seen (Table II). All but the arginine-sensitive reaction were found in mitochondrial preparations of the *arg-14* mutant strain IC28 (Table II), and thus only the arginine-sensitive counts were taken to be products of the *N*-acetylglutamate synthase reaction (see below).

Substrates and Inhibitors—The apparent K_m values of the enzyme were 6.3 mM for glutamate and 1.6 mM for CoASAc. The concentrations in the standard assay, 50 mM glutamate and 3.5 mM CoASAc, gave maximal activity and a low radioactive blank.

The reaction was not significantly inhibited by 1 mM concentrations of acetylglutamate, α-*N*-acetylarginine, *N*-acetylaspartate, α-*N*-acetylornithine, *N*-acetylalanine, histidine, lysine, ornithine, proline, citrulline, 2-oxoglutarate, or pyruvate. CoASH (1 mM) inhibited the reaction approximately 30%, and 1 mM oxalacetate inhibited the reaction 68% in the same experiments. A more detailed study of inhibition by arginine showed that half-maximal inhibition was achieved at 0.16 mM (Fig. 1), if the inhibition at 1 mM is taken as maximal (see above). At a final concentration of 3.5 mM, propionyl-CoA yielded 18% the amount of product (presumably *N*-propionyl-L-glutamate) as did 3.5 mM CoASAc. The activity with propionyl-coenzyme A was inhibited by 1 mM arginine. L-[^{14}C]Aspartate did not serve as a substrate in place of [^{14}C]glutamate.

Results using the *Saccharomyces cerevisiae* enzyme show that acetylglutamate and L-arginine interact, such that the effect of arginine is much greater in the presence of 1 mM acetylglutamate. The latter has little effect of its own (3). Tests with the *N. crassa* enzyme failed to reveal the interaction, even at lower glutamate concentrations (*e.g.* 1 mM). In addition, the *N. crassa* enzyme appears to be less sensitive than the *S. cerevisiae* enzyme to L-arginine: the yeast enzyme was one-half inhibited at 0.02 mM in the standard yeast reaction mixture (similar to the one described here except for a lower glutamate concentration of 20 mM); that of *N. crassa* required 0.16 mM, as noted above.

Wild-type mitochondrial preparations yielded large amounts of radioactive product when acetylglutamate was added; again, this was CoASAc-independent and insensitive to L-arginine (data not shown). We tested the hypothesis that the arginine enzyme, α-*N*-acetylornithine:glutamate acetyltransferase (EC 2.3.1.35), was responsible. This mitochondrial enzyme normally functions to regenerate *N*-acetylglutamate from acetylornithine and glutamate in the cyclic pathway. It would be expected that the enzyme would catalyze an exchange of [^{14}C]glutamate and acetylglutamate, such that radioactivity from the former would enter the pool of the latter and thereby be able to pass through Dowex 50. Mitochondria from strain IC25, which lack the acetyltransferase activity, were tested. They failed to produce radioactive product from [^{14}C]glutamate and acetylglutamate (data not shown). Substitution of α-*N*-acetylornithine for acetylglutamate in reaction mixtures containing the wild-type enzyme yielded even greater amounts of product. Moreover, the activity was largely confined to the supernatant of centrifuged, disrupted mitochondria, as previously found for the acetyltransferase (17). *N*-Acetylglutamate synthase, by contrast, was found principally in the residue (see below). These experiments led us to use strain IC25 for most of the work on *N*-acetylglutamate synthase.

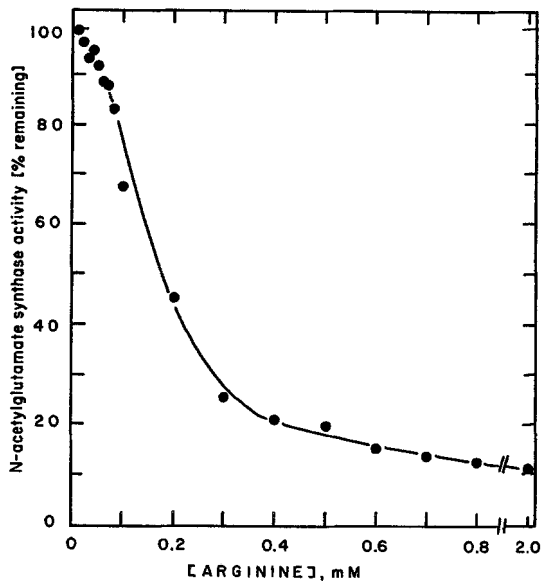

FIG. 1. **Relationship of activity to arginine concentration in the reaction mixture.** Thirty μl of mitochondrial membranes of *arg-7* were used in each reaction mixture. In these assays, the blank used was one in which CoASAc was omitted, in order to reveal the additional, arginine-insensitive reaction at high arginine concentrations (see text).

TABLE II

Dependence of product formation on CoASAc, arginine, and genotype of extracts

Standard reaction mixtures with 40 μl of mitochondrial membranes were used with additions of arginine (1 mM) and other conditions as noted.

Condition	Mitochondrial preparation	
	IC25 (*arg-7*)	IC28 (*arg-14*)
	nmol/mg protein/min	
Complete	16.2	4.9
−CoASAc	1.6	0.7
+Arginine	6.1	4.6
−CoASAc, +arginine	1.2	<0.1

In experiments with keto acids, radioactive products were formed by mitochondrial membrane preparations of strain IC25 (*arg-7*) in the absence of CoASAc and in the presence of L-arginine. The keto acids were probably serving as amino acceptors from [^{14}C]glutamate in a transaminase reaction; the [^{14}C]oxoglutarate thus formed would have passed through the Dowex 50 column after the standard assay. This phenomenon was not affected by the *arg-14* mutation and was not pursued further.

Localization of the Enzyme—Cell fractionation procedures designed to minimize damage to organelles yielded preparations in which 65% of the *N*-acetylglutamate synthase activity of the cell homogenate was recovered in the organellar pellet. In the same experiments, 50% of ornithine carbamoyltransferase, a mitochondrial matrix marker, was found in the organellar pellet. The pellet was resuspended and centrifuged on a 15–45% sucrose gradient. The synthase co-sedimented

at 1.18 g/ml with the marker enzyme (Fig. 2).

An initially surprising observation was the fact that intact mitochondria display two times more activity for the synthase than do disrupted mitochondria. This was not expected because many substrates do not normally permeate mitochondrial membranes. However, loss of activity during breakage cannot be estimated, and the standard reaction mixture was pH 9.0. At this pH, the latency of matrix enzymes may be lost.

When mitochondria purified on a discontinuous gradient (see "Experimental Procedures") were broken by agitation with glass beads and then centrifuged at high speed, ornithine carbamoyltransferase was found wholly in the supernatant; the synthase was distributed between supernatant and pellet in a variable ratio. This was true of both repressed and derepressed activities. This suggested that the enzyme was loosely bound to the membrane fraction. The identity of the synthase activity in both fractions was verified by the observation that the *arg-14* mutation caused the loss of both. The recovery of total activity was relatively low, and the activity in the supernatant fractions was unstable.

The submitochondrial localization of the synthase was determined with mild breakage procedures (see "Experimental Procedures") in which the majority of matrix marker enzymes were solubilized with a high recovery of synthase activity. Combined osmotic shock and brief sonication followed by centrifugation on a discontinuous sucrose gradient demonstrated that, in conditions liberating about half of the matrix marker enzymes, the synthase was not rendered soluble (Table III).

Regulation and Genetics—Using whole mitochondria for comparison, the specific activities of strains grown in various states of arginine sufficiency were measured. A wild-type strain growing in minimal medium had a specific activity of 16.1 units/mg of mitochondrial protein. The specific activity of the enzyme in strain IC25 grown on high levels of arginine was 10.6 (67% of the previous figure), whereas that of an arginine-starved mycelium was 23–33. This behavior is similar to all other arginine enzymes of *N. crassa* except carbamoylphosphate synthetase A, which is almost wholly repressible (18).

N-Acetylglutamate synthase, as noted previously (Tables II and III), is not found in the mutant strain IC28 (*arg-14*). This is true for intact mitochondria and for submitochondrial fractions. Mixtures of extracts from strains IC25 (*arg-7*) and

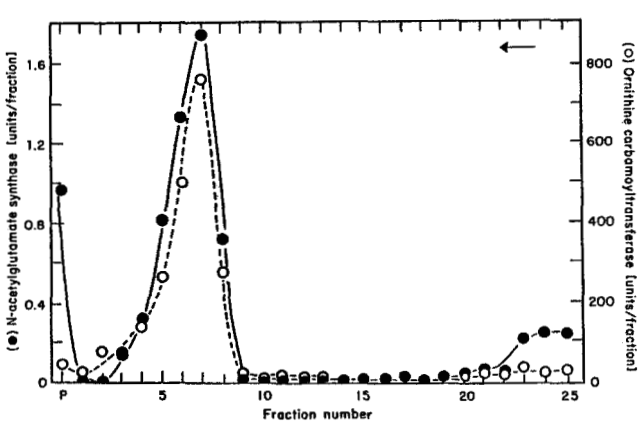

FIG. 2. **Sedimentation of organellar pellet on a continuous 15–50% sucrose gradient.** Mitochondria (approximately 3.8 mg of protein) from the *arg-7* strain were used; the total units of *N*-acetylglutamate synthase and of ornithine carbamoyltransferase were measured in each fraction. The sucrose concentration at fraction 7 is 39% (density = 1.18 g/ml).

TABLE III

Submitochondrial localization of acetylglutamate synthase

Mitochondria were subjected to osmotic shock and brief sonication followed by separation on a discontinuous sucrose gradient as described under "Experimental Procedures." All fractions were assayed for enzyme activity. Numbers represent the enzyme activity not entering the gradient as a fraction of the total recovered enzyme activity.

	Activity solubilized	Activity recovered
	%	
Acetylglutamate synthase	1.7	74[a]
Malate dehydrogenase	58.6	100
Ornithine carbamoyltransferase	47.8	90
Cytochrome oxidase	0.5	125

[a] In other experiments, 100% of the synthase activity was recovered.

TABLE IV

Effect of mutations at the arg-6 locus on acetylglutamate synthase activity

Data for acetylglutamate kinase and acetylglutamyl-P reductase are from Ref. 11. Activities are expressed per milligram of mitochondrial protein.

Strain	Complementation group	Acetylglutamate kinase	Acetylglutamyl-P reductase	Acetylglutamate synthase
		milliunits/mg		
IC1	Wild-type	0.177	0.275	16.2
LA322	A	0.003	0.409	15.8
LA323	A	0.004	0.351	16.1
LA59	B	0.004	0.001	0
LA304	B	0.010	0	11.1
LA319	B	0.036	0.001	14.4
LA55	NC	0	0	0.8
LA56	NC	0.005	0	0
LA320	NC	0	0	0.2

IC28 (*arg-14*) revealed no inhibitory or stimulatory interactions. The auxotrophic phenotype is what is expected of a mutant strain lacking an indispensable enzyme of arginine biosynthesis. Mutants lacking individual enzymes of the pathway (mutations = *arg-1, arg-2, arg-3, arg-5, arg-6A, arg-6B, arg-7, arg-10, arg-12, arg-13*) displayed at least 80% the normal activity. Certain mutations at the *arg-6* locus, which encodes both acetylglutamate kinase and acetylglutamyl-P reductase (19), had unusual effects (Table IV). All three mutants tested which lacked both activities also lacked acetylglutamate synthase activity. One of three mutants tested which lacked the reductase also lacked synthase activity. Both of two kinase-deficient mutants had normal synthase activity.

DISCUSSION

We have shown that *N*-acetylglutamate synthase of *N. crassa* is mitochondrial, that it is arginine-inhibitable, and that it has a 2–3-fold regulatory amplitude in response to excess or insufficient mycelial arginine. In these respects, the enzyme is like all other ornithine biosynthetic enzymes (17). The auxotrophy of the *N*-acetylglutamate-deficient mutant, IC28, demonstrates that the activity is indispensable to arginine synthesis by *N. crassa*. The assignment of the *arg-14* locus to this enzyme completes gene-enzyme assignments. (The present assignment, like many of the others, lacks definitive proof of a structural role of the locus.) The remaining unassigned locus, *arg-13*, is an unusual one. All *arg-13*

mutants isolated so far are "leaky," but they grow well in ornithine-supplemented media (5). The determination of the biochemical defect in *arg-13* strains may reveal involvement with a particular enzymatic step, or a more general or indirect effect upon ornithine synthesis.

A number of possibilities might account for the reduced activity observed in strains of the noncomplementing class of the *arg-6* locus (19). Such mutations might define a closely linked locus encoding a positively acting regulatory protein required for expression of the unlinked *arg-6* and *arg-14* genes. Alternatively, interactions between the various gene products may be necessary for proper processing or functioning of the *arg-14* gene product. These possibilities are being explored, beginning with a more extensive survey of the many available *arg-6* mutants (19).

N-Acetylglutamate synthase of *N. crassa* is similar to that of *S. cerevisiae* in most of its properties and its location in mitochondria. Unlike the case of the yeast enzyme (2, 3), the feedback sensitivity of *N*-acetylglutamate synthase from *N. crassa* is not greater in the presence of acetylglutamate. The significance of this cannot be judged easily; however, the consumption of glutamate and CoASAc may be small enough to make differences in the efficiency of regulation economically negligible.

The *N. crassa* enzyme has a high pH optimum and a high K_m for both substrates. *N*-Acetylglutamate synthases of most prokaryotic and lower eukaryotic organisms share these properties (3, 20–22). Technically, the high K_m of glutamate for the enzyme imposes high no-enzyme blanks; for this reason, continued work with the enzyme will be more practical with reaction mixtures containing 20 mM, rather than 50 mM, L-[^{14}C]glutamate. Again, the significance of these properties cannot be judged, especially because the nature of the intracellular environment of the enzyme is not understood. *N*-Acetylglutamate synthase of mammals, which produces *N*-acetylglutamate as a cofactor for carbamoyl-phosphate synthetase I, has a lower pH optimum and lower substrate requirements than the fungal activities (23). Other properties, such as activation by arginine, equip it to function optimally in the urea cycle (1).

The association of most of the activity with the mitoplast or its sedimentable components suggests that most of the enzyme is bound to the mitochondrial inner membrane (a similar association of the yeast enzyme with a mitochondrial membrane has been reported (4)). The results cannot rule out the possibility that a fraction of the enzyme is targeted to the mitochondrial matrix, but it is not in the intermembrane space. The data do not reveal whether the catalytic site of the enzyme faces the matrix or the intermembrane space, because, possibly for technical reasons (*e.g.* the high pH of enzyme reaction mixtures), we have not detected latency of the activity in intact mitochondria.

The membrane localization of much of the synthase activity suggests an interesting mechanism by which cytosolic arginine concentrations could cause feedback inhibition. This could occur if the arginine-binding site were exposed to the intermembrane space (freely accessible to cytoplasmic arginine) while the active (catalytic) site was exposed on the interior of the mitochondrion: the site of other ornithine-synthesizing enzymes. This possibility is under investigation.

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