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Control of the Ornithine Cycle in *Neurospora crassa* by the Mitochondrial Membrane

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In *Neurospora crassa*, the mitochondrial membrane separates ornithine used in arginine biosynthesis from ornithine used in the arginine degradative pathway in the cytosol. Ornithine easily exchanges across the mitochondrial membrane under conditions appropriate for synthesis of the immediate biosynthetic product, citrulline. Neither of the two mitochondrial enzymes required for the ornithine-to-citrulline conversion is feedback inhibitable in vitro. Nevertheless, when arginine is added to cells and cytosolic ornithine increases as arginine degradation begins, the rate of citrulline synthesis drops immediately to about 20% of normal (B. J. Bowman and R. H. Davis, *Bacteriol.* 130:285–291, 1977). We have studied this phenomenon in citrulline-accumulating strains carrying the *arg-1* mutation. Citrulline accumulation is blocked when arginine is added to an *arg-1* strain but not to an *arg-1* strain carrying a mutation conferring insensitivity of intramitochondrial ornithine synthesis to arginine. Thus, ornithine is evidently unable to enter mitochondria in normal (feedback-sensitive) cells. Other experiments show that cytosolic ornithine enters mitochondria readily except when arginine or other basic amino acids are present at high levels in the cells. We conclude that in *N. crassa*, the mitochondrial membrane has evolved as a secondary site of feedback inhibition in arginine synthesis and that this prevents a wasteful cycling of catalobolic ornithine back through the anabolic pathway. This is compared to the quite different mechanism by which the yeast *Saccharomyces cerevisiae* prevents a futile ornithine cycle.

The organization of arginine metabolism in the fungus *Neurospora crassa* (Fig. 1) resembles that in most eucaryotes, especially algae, fungi, and plants (R. H. Davis, in K. Hermann and R. Somerville, ed., *Amino Acid Synthesis and Genetic Regulation*, in press). Citrulline is made in the mitochondria (or, in plants, the plastids) from carbamyl phosphate (carbamyl-P) and ornithine (36; Davis, in press). This reaction is catalyzed by ornithine transcarbamylase, and the citrulline formed passes to the cytosol and is transformed to arginine in a terminal, two-step sequence. Ornithine transcarbamylase is neither repressible nor feedback sensitive to arginine in vitro (10). The synthesis of carbamyl-P is catalyzed by carbamyl-P synthetase A, an enzyme specific to the arginine pathway (12, 19). (A second, pyrimidine-specific enzyme of carbamyl-P synthesis exists in the nucleolus [3, 39].) This enzyme is the only repressible enzyme of the pathway, but it is not feedback sensitive to arginine in vitro (19, 39). Many hours of growth are required to dilute carbamyl-P synthetase A to its repressed level. Therefore, no fast control mechanism for this enzyme seems to exist, despite its energy-consuming and, at times, pace-setting role in the pathway. Finally, the synthesis of ornithine begins with acetylglutamate and proceeds cyclically in a path which conserves the acetyl group. No enzyme of ornithine synthesis is repressible (18), but ornithine synthesis is efficiently controlled by feedback inhibition of an early enzyme, acetylglutamate kinase (9, 41). In cells grown in synthetic minimal medium, the pathway as a whole provides arginine for protein synthesis and a large storage pool in the vacuole (22, 24). Despite substantial arginase activity in such cells, no arginine is catabolized, because the cytosolic arginine concentration is too low (11, 34). In cells grown in minimal medium, moreover, enough ornithine escapes the mitochondrion to provide the putrescine moiety of polyamines, a storage pool of ornithine in the vacuole, and a slight amount of proline via the ornithine-catabolic enzyme ornithine aminotransferase (Fig. 1) (20, 24).

When arginine is given to cells grown in minimal medium, the cellular uptake system floods the cytosol with arginine (4, 35). The synthesis of ornithine within the mitochondrion ceases (by feedback inhibition) (41), and further increases in carbamyl-P synthetase A are pre-
ornithine catabolism begins immediately after entry of arginine. The charged amine ion floods the cytosol as arginine floods the vacuole (5). The result is the immediate onset of ornithine catabolism at a high rate (5, 14).

The question we wish to explore is whether, and how, ornithine arising from arginine catabolism is prevented from reentering the anabolic pathway. Our previous work, based on tracer kinetics in vivo, showed that in cells grown in minimal medium, the influx and efflux of ornithine across the mitochondrial membrane are faster than the normal rate of ornithine synthesis (4, 20, 24). Moreover, when arginine is added, carbamyl-P probably continues to be made (5, 40), and no later enzyme is wholly inhibited by arginine. Paradoxically, however, when arginine is added to cells grown in minimal medium, the rate of citrulline synthesis drops by 80% (5). The basis of this phenomenon is shown, in this work, to be inhibition by arginine of the entry of ornithine into the mitochondrion. This mechanism is tantamount to adopting the mitochondrial membrane as a secondary, feedback-regulated step in arginine metabolism. We shall later compare this system to the quite different systems of yeasts and ureotelic mammals.

(Some of the data in this article were presented at the XIII International Congress of Microbiology, Boston, Mass., 8 to 13 August 1982, and will appear in Microbiology—1983.)

**MATERIALS AND METHODS**

**Strains and media.** Strains of *N. crassa* used are given in Table 1 and will be referred to with their component mutations. All mutations except sup-3 were isolated by R.H.D., and multiple mutant strains were derived by standard genetic crosses (15). Strains carrying arg-1 and arg-6 mutations lack argininosuccinate synthetase, *ota* and *aga* mutants lack ornithine transaminase. "Open" arrows indicate biosynthetic steps, solid arrows indicate catabolic steps, and broken arrows indicate membrane passage. OTC, Ornithine transcarbamylase.

FIG. 1. Diagram of arginine metabolism in *N. crassa*, showing the locations of enzymatic steps and metabolic positions of mutations used in this work. Carbamyl-P synthetase A (CPS-A) is a repressible enzyme, as indicated by the circled R. Acetylglutamate kinase, indicated as the first enzyme of ornithine synthesis, is feedback sensitive, as indicated by the circled F. It is lacking in *arg-6* mutants and has greatly reduced feedback sensitivity in strains carrying the *sup-3* mutation (closely linked or allelic to *arg-6* mutations) *arg-1* mutants lack argininosuccinate synthetase, *aga* mutants lack arginine, and *ota* mutants lack ornithine transaminase. "Open" arrows indicate membrane passage.
Citrulline synthesis by arginine-starved arg-1 cells. The arg-1 mutant strain lacks arginosuccinate synthetase (29) (Fig. 1), and therefore it accumulates citrulline when deprived of arginine. We wished to know some of the factors important in this process. Cells grown initially on high levels of arginine were washed and transferred to unsupplemented minimal medium. The cells were monitored thereafter for their ornithine, citrulline, and arginine contents and for the specific activity of carbamyl-P synthetase A. As arginine was exhausted, ornithine and citrulline (especially the latter) accumulated at an accelerating rate. When cycloheximide was added at 3 h, the rate of citrulline synthesis was stabilized and remained linear for over 2 h (Fig. 2A). In keeping with these results, the carbamyl-P synthetase activity (glutamine dependent) began to appear as arginine was exhausted and also increased at an accelerating rate. Addition of cycloheximide usually blocked further enzyme synthesis, but it was not followed by decay of activity present at the time of addition (Fig. 3). (In some experiments, cycloheximide and arginine had little immediate effect upon carbamyl-P synthetase A accumulation.

![Graphs showing citrulline accumulation](image)

**FIG. 2.** Effect of arginine on the accumulation of citrulline in arg-1 (A) and arg-1 sup-3 (B) strains. Strains were grown in arginine-supplemented medium to 0.4 mg (dry weight) per ml (ca. 10 h). At time zero, mycelia were transferred to arginine-free medium. Exhaustion of intracellular arginine (not shown) took place at about 2.5 h, and net protein accumulation (not shown) stopped at about 2.75 to 3 h. Cycloheximide (10 μg/ml) was added at 3 h, and arginine was added to a final concentration of 1 mM to half the cultures. Accumulation of ornithine (triangles) and citrulline (circles) was determined thereafter. Open symbols: No arginine; closed symbols: arginine added. CULT., Culture; CIT, citrulline; ARG, arginine; ORN, ornithine; CHX, cycloheximide.
This was probably due to continued entry of a pool of enzyme precursor, already synthesized, into the mitochondrion from the cytosol. In no case did carbamyl-P synthetase A activity decline after cycloheximide addition.) We presume that the limiting factor in citrulline synthesis under these conditions is carbamyl-P, the availability of which is controlled by carbamyl-P synthetase A activity.

Having a system capable of synthesizing citrulline within the mitochondrion, we wished to know how it would respond to arginine. When arginine was added to cycloheximide-treated, starving cells, the rate of citrulline synthesis immediately dropped to 7% of the control rate (Fig. 2A; Table 2). Cellular ornithine stopped rising at this point, but it remained present in the cells. It is not known what proportion of the ornithine was in the cytosol, as opposed to the vacuole; this matter will be taken up below.

The effect of arginine in blocking citrulline synthesis in this system was specific. Addition of L-lysine or L-ornithine, two other basic amino acids, had no major effect on citrulline synthesis (Table 2). Thus, the effect of arginine is not strictly chemical. It can best be understood as the action of the end product of the pathway having a negative effect on its own synthesis.

The mechanisms by which arginine might inhibit citrulline synthesis in vivo are: (i) inhibition of carbamyl-P synthetase A or ornithine carbamyltransferase; (ii) inhibition of intramitochondrial ornithine synthesis, with the proviso that the remaining ornithine in the cell cannot easily enter the mitochondrion. The latter hypothesis may be subdivided into several possibilities, which will be described in a later section.

Citrulline synthesis in a feedback-resistant strain. The sup-3 mutation imparts feedback resistance to the intramitochondrial enzyme acetamidoglutamate kinase (37, 41), an initial enzyme of ornithine synthesis (Fig. 1). Arginine, therefore, cannot block intramitochondrial ornithine synthesis in the arg-1 sup-3 double mutant (41). It is therefore useful in testing both hypotheses outlined above. The addition of arginine (with cycloheximide) to the arg-1 sup-3 strain as it synthesized citrulline had almost no effect (Fig. 2B; Table 2). These data also demonstrate that arginine has no noticeable effect in vivo upon carbamyl-P synthetase A activity (once derepressed) or upon ornithine transcarbamylase.

The data strongly indicate that the lack of a mitochondrial ornithine pool is the factor which limits citrulline synthesis after arginine is added to feedback-sensitive cells, a factor which does not prevail in the feedback-resistant mutant.

Efficiency of feedback inhibition. A point which requires further analysis is the efficiency of feedback inhibition in the sup-3 and sup+ strains. Several mutations allow analysis of this phenomenon. The aga mutation blocks arginase activity (16) and, thereby, the formation of cytosolic ornithine from arginine. If one also uses the ota mutation to block ornithine catabolism (17), one can test (by measurement of ornithine accumulation) the efficiency with which de novo ornithine synthesis is controlled. When the arg-1 ota aga strain and its feedback-resistant counterpart arg-1 sup-3 ota aga were starved for arginine, both accumulated citrulline as usual. The addition of arginine completely inhibited further increase in the sum of ornithine and citrulline in the arg-1 ota aga strain. In the hour after arginine was added, all citrulline synthesis took place slowly at the expense of the large ornithine pool (Table 3). Thus, feedback inhibition was complete. (The additional loss of ornithine, 8 nmol/mg of protein, was minor and can be accounted for by polyamine synthesis [20, 30].) A similar analysis of the feedback-resistant counterpart strain, arg-1 sup-3 ota aga revealed that the sum of ornithine plus citrulline increased after arginine addition at 59% of its previous rate and that, in fact, new citrulline was made at 80% of the control rate. That the added arginine has an effect on the rate of de novo synthesis of ornithine in this strain is consistent with the known, residual sensitivity of the ace-
tylglutamate kinase of sup-3 strains to arginine ($K_i = 10 \text{ mM}$) (37).

**Ornithine pool.** At this point, we know that the blockage of citrulline synthesis by arginine in arg-1 sup$^+$ strains is due to the inability of the remaining cellular ornithine to enter the mitochondrion. This may be because cytosolic ornithine is too low in concentration (the majority being in the vacuole), because ornithine may be intrinsically impermeant to mitochondria (unlikely in view of previous data), or because arginine competitively inhibits the normal passage of ornithine between the cytosol and mitochondrion.

The best information on this matter comes from isotopic tracer studies of wild-type mycelia, for which it was shown that upon addition of arginine, catabolic and vacuolar ornithine flood the cytosol (5). A similar isotopic tracer analysis of the arg-1 strain, however, showed that addition of radioactive arginine was not followed by ornithine flooding the cytosol. During the hour after arginine addition, the small amount of new citrulline made was much more radioactive than the resident cellular ornithine during the same interval (data not shown). This suggests that the nonradioactive, resident ornithine was sequestered in the vacuole (20, 24). Thus, the starved

### Table 2. Ornithine pools and rates of citrulline synthesis in various strains after amino acid additions

<table>
<thead>
<tr>
<th>Strain</th>
<th>Addition (1 mM)</th>
<th>Ornithine pool (nmol/mg of protein)</th>
<th>Citrulline synthesis (nmol/mg of protein per h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3 h</td>
<td>3.5 h</td>
<td></td>
</tr>
<tr>
<td>arg-1</td>
<td>None</td>
<td>76</td>
<td>171 (100)</td>
</tr>
<tr>
<td></td>
<td>Ornithine</td>
<td>94</td>
<td>153</td>
</tr>
<tr>
<td></td>
<td>Lysine</td>
<td>135</td>
<td>153</td>
</tr>
<tr>
<td></td>
<td>Arginine</td>
<td>64</td>
<td>12</td>
</tr>
<tr>
<td>arg-1 sup-3</td>
<td>None</td>
<td>81</td>
<td>206 (100)</td>
</tr>
<tr>
<td></td>
<td>Arginine</td>
<td>73</td>
<td>183</td>
</tr>
<tr>
<td>arg-1 ota</td>
<td>None</td>
<td>200</td>
<td>183 (100)</td>
</tr>
<tr>
<td></td>
<td>Arginine</td>
<td>183</td>
<td>75</td>
</tr>
<tr>
<td>arg-1 arg-6</td>
<td>None</td>
<td>&lt;3</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Ornithine</td>
<td>167</td>
<td>147 (100)</td>
</tr>
<tr>
<td></td>
<td>Arginine</td>
<td>167</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>Lysine</td>
<td>167</td>
<td>73</td>
</tr>
<tr>
<td></td>
<td>Histidine</td>
<td>167</td>
<td>107</td>
</tr>
<tr>
<td>arg-1 arg-6 ota</td>
<td>Ornithine</td>
<td>261</td>
<td>133 (100)</td>
</tr>
<tr>
<td></td>
<td>Arginine</td>
<td>261</td>
<td>78</td>
</tr>
<tr>
<td></td>
<td>Lysine</td>
<td>261</td>
<td>111</td>
</tr>
<tr>
<td></td>
<td>Histidine</td>
<td>261</td>
<td>106</td>
</tr>
<tr>
<td>arg-1 arg-6</td>
<td>Ornithine</td>
<td>187</td>
<td>106 (100)</td>
</tr>
<tr>
<td></td>
<td>Arginine</td>
<td>187</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td>Alamine</td>
<td>187</td>
<td>106</td>
</tr>
<tr>
<td></td>
<td>Phenylalanine</td>
<td>187</td>
<td>112</td>
</tr>
<tr>
<td></td>
<td>Glutamine</td>
<td>187</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Leucine</td>
<td>187</td>
<td>106</td>
</tr>
</tbody>
</table>

* Cultures were analyzed, as described in the legend to Fig. 2, after onset of arginine starvation at time zero. Ornithine pools were measured at 3 h (first four strains) or at 3.5 h (last three cultures), and rates of citrulline synthesis were for the hour after the last addition.

b Parentheses indicate control values.

### Table 3. Feedback inhibition by arginine of ornithine and citrulline accumulation

<table>
<thead>
<tr>
<th>Strain</th>
<th>Addition (1 mM at 3h)</th>
<th>Ornithine pool (nmol/mg of protein)</th>
<th>Change between 3 and 4 h (nmol/mg of protein per h)</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ornithine</td>
<td>Citrulline</td>
</tr>
<tr>
<td>arg-1 ota aga</td>
<td>None</td>
<td>90</td>
<td>+71</td>
<td>+174</td>
</tr>
<tr>
<td></td>
<td>Arginine</td>
<td>103</td>
<td>-52</td>
<td>+44</td>
</tr>
<tr>
<td>arg-1 sup-3 ota aga</td>
<td>None</td>
<td>156</td>
<td>+81</td>
<td>+178</td>
</tr>
<tr>
<td></td>
<td>Arginine</td>
<td>177</td>
<td>+11</td>
<td>+143</td>
</tr>
</tbody>
</table>

* Cultures were analyzed as described in the legend to Fig. 2. Arginine starvation was initiated at time zero, and arginine was added to half of each culture at 3 h. Ornithine and citrulline determinations were made thereafter to measure amounts made during the hour after arginine additions.
arg-1 culture is not a wholly suitable model for the wild type, and the extreme inhibition of citrulline synthesis by arginine may be explained at least in part by the deprivation of both intramitochondrial and cytosolic ornithine. Thus, it was necessary to determine whether arginine inhibited citrulline synthesis in arg-1 strains with elevated cytosolic ornithine pools.

The effect of elevating the cytosolic ornithine concentration was tested first by using the ota mutation to block ornithine catabolism. We wished to know whether the barrier to ornithine entry into the mitochondrion could be overcome by a high ornithine concentration. The arg-1 ota strain was allowed to begin citrulline synthesis as usual. As expected, an unusually large ornithine pool also accumulated (Table 2). Upon addition of arginine, the culture stopped ornithine accumulation (ornithine severely inhibits arginase activity, whereas arginine feedback-inhibits de novo ornithine synthesis). At the twofold-higher cellular levels of ornithine, the effect of arginine on the arg-1 ota culture was less pronounced than on arg-1 cultures. The rate of citrulline synthesis dropped to 41%, rather than to 7 to 15% of the control rate seen previously (Table 2). This indicates that citrulline synthesis is indeed limited by ornithine and that ornithine entry into mitochondria in the presence of arginine is concentration dependent.

The second type of experiment designed to manipulate ornithine concentration also allowed us to control the presence or absence of arginine. The arg-1 arg-6 double mutant is not only unable to utilize citrulline for arginine synthesis, but is also unable to make ornithine as well: arg-6 blocks acetylglutamate kinase (Fig. 1). If the strain is to make citrulline, it must have exogenous ornithine to do so. The first question asked was whether the arg-1 arg-6 strain could make citrulline when given ornithine. It did so almost as well as the starving arg-1 strain itself (Fig. 4; Table 2). This shows that there is no intrinsic barrier to ornithine entry into the mitochondrion from the cytosol. (It is well known, moreover, that single mutants unable to synthesize ornithine will grow on ornithine as an arginine source.)

The effect of adding arginine to arg-1 arg-6 cells already replete with ornithine was then tested. The result was that arginine immediately reduced the rate of citrulline synthesis by 55 to 75% (Fig. 4; Table 2). A similar test of arginine on an ornithine-replete arg-1 arg-6 ota strain showed a lesser effect (40% inhibition), in keeping with the higher ornithine pool of this strain (Table 2). The specificity of the phenomenon was indicated by a test of other amino acids upon citrulline synthesis in ornithine-replete arg-1 arg-6 strains (Table 2). The basic amino acids all had inhibitory effects (Arg > Lys > His), whereas various neutral amino acids had virtually none.

The data indicate that basic amino acids inhibit intracellular ornithine from entering the mitochondrion. However, basic amino acids compete with ornithine for entry into the cell itself. Their effect, therefore, may be to reduce the amount of ornithine in the cytosolic compartment, the bulk of ornithine being in the vacuole. If this is true, it may be that arginine has its effect by merely reducing the availability of ornithine to the mitochondrion. It is extremely difficult, if not impossible, to determine rigorously the concentrations of vacuolar, cytosolic, and mitochondrial ornithine in these non-steady-state conditions. Therefore, the strongest evidence that arginine actually blocks ornithine entry into mitochondria comes from the original experiments of Bowman and Davis (4, 5). Despite a 15- to 25-fold-higher cytosolic ornithine concentration, arginine reduces citrulline synthesis in wild-type cells by 75%. The data drawn from the arg-1 ota, the arg-1 arg-6, and the arg-1 arg-6 ota strains in the present experiments are quite consistent with the postulated arginine blockade of ornithine entry into mitochondrion, even when the cytosolic ornithine concentration is quite high.

**DISCUSSION**

Our data show that arginine blocks the use of extramitochondrial, but not intramitochondrial,
ornithine for citrulline synthesis. The results thereby demonstrate that neither carbamyl-P synthetase A nor ornithine transcarbamylase is sensitive to feedback inhibition by arginine. Our data thus suggest that the mitochondrial membrane of *N. crassa* is a regulatory element in the pathway, which is responsive to arginine and which supplements feedback inhibition by controlling the access of ornithine to ornithine transcarbamylase. In this role, the mitochondrial membrane adaptively minimizes a wasteful ornithine cycle. It is not unlikely that most eucaryotic microbes and plants which have both a de novo glutamate-ornithine-arginine synthetic pathway and an arginase mode of arginine degradation use similar strategies, because enzyme localizations are similar in most of these organisms (Davis, in press). The details of the mechanism by which the mitochondrial membrane performs its adaptive role in ornithine transport is not known. It is reasonable to predict a basic amino acid carrier in the inner mitochondrial membrane. Such a carrier would enable arginine to enter the mitochondrion for mitochondrial protein synthesis, as well as, in an arginine-poor environment, facilitating the demonstrated rapid exchange of ornithine between cytosolic and mitochondrial compartments. Such a basic amino acid transport system would be ideally suited to minimize cycling under conditions of arginine excess. High levels of arginine would competitively (but not completely) block entry of ornithine into mitochondria while allowing arginine to enter and to feedback-inhibit acetylglutamate kinase, as shown by Goodman and Weiss (22).

Although the cytosolic ornithine pool is also high under these conditions, catabolism via ornithine transaminase maintains ornithine at a level which allows it to enter mitochondria at only 13% the rate characteristic of cultures grown without arginine (5). A similar competition of basic amino acids for entry into the cell itself is known and has been proposed for the vacuolar membrane as well (5). If the proposed mechanism is borne out by in vitro studies, it will be a rare example of an organellar membrane having an end-product sensitivity which supplements feedback inhibition of a biosynthetic pathway. Our current work with isolated, resting mitochondria has shown them to be extremely impermeable to basic amino acids; even energized mitochondria so far show no definite evidence of carrier-mediated entry of such molecules (T. J. Paulus and R. H. Davis, unpublished data). The interaction of arginine and ornithine at the mitochondrial membrane, therefore, cannot yet be investigated in detail in vitro.

A comparable example of the mitochondrial membrane having an organizational role in amino acid metabolism is that of ornithine degrada-

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LITERATURE CITED