Intracellular Localization of Enzymes of Arginine Metabolism in Neurospora*

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SUMMARY

The physical basis for the confinement of carbamyl phosphate, ornithine, and arginine to specific anabolic fates has been investigated. Cell extracts of Neurospora grown in minimal medium were fractionated by differential centrifugation to determine the subcellular localization of the relevant enzymes. Carbamyl-P synthase A (arginine-specific, EC 2.7.2.5), ornithine acetyltransferase, and ornithine carbamyltransferase (EC 2.1.2.2) were found to be associated with a particulate (organellar) fraction. All three enzymes co-sediment with mitochondria on linear sorbitol gradients, and appear to be components of the mitochondrial matrix. The mitochondrial membrane limits the ability of exogenous substrates to be metabolized by these enzymes in vitro. Arginine synthesis from citrulline, putrescine synthesis from ornithine, and the catabolism of ornithine and arginine are carried out by cytoplasmic enzymes. The catabolic enzymes are found at significant levels even during growth in minimal medium, but little catabolism occurs in vivo. The results indicate that the anabolic and catabolic pathways of ornithine metabolism are, in part, separated by the mitochondrial membrane. However, the mechanisms responsible for confining carbamyl P and ornithine to arginine synthesis and arginine to protein synthesis are more complex than the simple physical separation of the anabolic and catabolic enzymes.

The ability to catabolize exogenous amino acids is a widespread feature of metabolism. Where exogenous amino acids are lacking, however, cellular economy requires mechanisms to prevent catabolism of endogenous amino acids. In most prokaryotes, the pool sizes of many endogenous amino acids are often maintained at low levels, and catabolic enzymes remain uninduced. In eucaryotic cells, however, endogenous amino acids frequently accumulate despite the presence of significant levels of catabolic enzymes. These large pools are not catabolized (1).

* This research was supported in part by National Institutes of Health Postdoctoral Fellowship 1-F02-GM-51,028-01 from the National Institute of General Medical Sciences to R. L. Weiss and by Research Grant GB-12939 from the National Science Foundation to R. H. Davis. A preliminary report of this work was given at the 72nd Annual Meeting of the American Society for Microbiology, Philadelphia, April 23-28, 1972.

(Received for publication, February 1, 1973)
were extracted and fractionated as outlined in Fig. 2. Urea (0.6 M sorbitol, and 0.14 M 2-mercaptoethanol). The washed cells was then centrifuged at 500 X g for 5 min. The washed pellet was suspended in 50 ml of buffered sorbitol. After the addition of 1 ml of glusulase, the suspension was incubated with gentle agitation for 30 min at 30° in a shaking water bath. The material indicated. The wet mycelial pad was rinsed with cold distilled water and compacted by centrifugation at 500 X g for 5 min. The mycelia were then washed three times with buffered sorbitol (0.1 M citrate, brought to pH 5.8 with K2HPO4 1 mM EDTA, 0.6 M sorbitol, and 0.14 M 2-mercaptoethanol). The washed cells were extracted and fractionated as outlined in Fig. 2. The washed pad, representing approximately 1 g, dry wt, was suspended in 50 ml of buffered sorbitol. After the addition of 1 ml of glusulase, the suspension was incubated with gentle agitation for 30 min at 30° in a shaking water bath. The material was then centrifuged at 500 X g for 5 min. The pellet was washed twice with 1 M sorbitol by gentle resuspension and centrifugation. The pooled supernatants (“glusulase washes”) were saved.

The washed pellet was suspended in 30 ml of fractionation buffer (10 mM potassium phosphate, pH 7.5, 1 mM EDTA, 1 mM sorbitol) and the cells were lysed by homogenization in a Teflon-glass homogenizer. The Teflon pestle was attached to a motor and rotated at 1600 rpm. Initial breakage was accomplished by 6 strokes in the homogenizer. Unbroken cells and cell debris were removed by centrifugation at 600 X g for 10 min. The resulting pellet was re-extracted in 15 ml of fractionation buffer by 3 strokes in the homogenizer. After centrifugation, the combined supernatants were re-centrifuged at 600 X g for 10 min. The resulting crude extract was then fractionated as shown in Fig. 2. Material which was not released into the crude extract was solubilized by treating the 600 X g pellet with 5% Triton X-100.

After fractionation of the crude extract, the glusulase washes, cell fractions, and 600 X g pellet were dialyzed overnight against 6.02 M potassium phosphate, pH 7.5. This treatment effectively solubilized particle-bound enzyme activities (except succinate dehydrogenase, EC 1.3.99.1). Because of the instability of carbamyl-P synthase A, its activity was determined using material desalted by passage through columns of Sephadex G-25.

Gradient Centrifugation—Linear sorbitol gradients (4.8 ml) were prepared, in 10 mM potassium phosphate, pH 7.5, 1 mM EDTA, using a Buchler gradient maker. Density gradients of 30 to 60% (w/w) sorbitol were used. Centrifugation was done in a Spinco SW 39L rotor at 4° at 35,000 rpm for 60 min. Fractions were collected in drops after piercing the bottom of the centrifuge tube.

Enzyme Assays—Unless otherwise noted, all enzyme activities were determined at 37°. All assays were done in duplicate and correction was made for nonenzymatic activity. All assays included zero time controls and were checked for substrate and cofactor dependence, linearity with time, and dependence on enzyme concentration. One unit of activity is defined as the formation of 1 μmole of product or the utilization of 1 μmole of substrate in 1 min. Protein was determined by the method of Lowry et al. (14).

Ornithine acetyltransferase was assayed by the method of Dénico (15).

Carbamyl-P synthase A (arginine-specific) was determined by inhibiting the pyrimidine-specific enzyme with UTP. The reaction mixture (0.5 ml) contained 100 mM Tris-acetate, pH 8, 6 mM L-glutamine, 7.5 mM potassium [14C]bicarbonate (specific activity of 0.267 μCi per μmole), 12 mM MgCl2, 12 mM ATP, 1.0 mM UTP, and 0.1 μl of enzyme fractions treated with Sephadex. After incubation for 30 min at 25°, the reaction was stopped and the carbamyl-P was converted to urea by boiling the reaction mixtures for 10 min after adding 0.2 ml of 1.5 M NH4Cl. The mixtures were then cooled and acidified by the addition of 0.1 ml of 1 N HCl. After treatment at 100° for 5 min, the tubes were cooled, covered with filter paper wetted with saturated KOH, and placed in a closed container overnight to remove...
volatile radioactivity. The 0.8-ml mixtures were then brought to approximately pH 5.6 by the addition of 0.2 ml of 1 N (sodium)2 citrate, pH 5.6, which contained 5 μmoles of urea as carrier. Urea (0.5 mg) was then added, and evolved radioactive CO2 (from carbamyl-P converted to urea) was trapped and counted as described by Morris et al. (16).

Ornithine carbamyltransferase (EC 2.1.3.3) was assayed by the method of Davis (17).

Ornithine decarboxylase (EC 4.1.1.17) was assayed in a 0.3-ml reaction mixture containing 100 mM potassium phosphate, pH 6.5, 15 mM pyridoxal phosphate, 14 mM 2 mercaptoethanol, 1 mM L-ornithine, 0.25 μCi dl-[1-14C]ornithine (specific activity 11.9 μCi per μmole), and 0.1 ml of the dialyzed cell fractions. After incubation for 30 min, evolved CO2 was trapped and counted as described by Morris et al. (16).

Ornithine aminotransferase (EC 2.6.1.13) was assayed by the method of Davis and Mora (13).

Argininosuccinate synthetase (EC 6.3.4.5) was assayed as the disappearance of the substrate, citrulline, in a reaction mixture similar to that described by Wampler and Fairley (18). The modifications included the use of a 1-ml reaction volume containing 1 mM citrulline, 2 mM ATP, and the addition of an ATP-regenerating system (1.5 mM P-enolpyruvate and 30 μg of pyruvate kinase).

Argininosuccinate lyase (EC 4.3.2.1) was assayed in a coupled reaction involving activated bovine liver arginase. Activation of arginase was accomplished by incubating it at a concentration of 5 mg per ml for 5 min at 55° in a buffer consisting of 10 mM Tris-HCl, pH 7.5, and 1 mM MnCl2. The 1-ml reaction mixtures contained 50 mM potassium phosphate, pH 7.5, 1 mM potassium argininosuccinate, 0.5 mM MnCl2, 0.5 mg of the activated bovine liver arginase, and appropriate amounts of the dialyzed cell fractions. After incubation for 30 min, the reaction was terminated with 1 ml of 20% trichloroacetic acid. After centrifugation, ornithine in the supernatant was determined by the method of Chinard (19).

Arginase was assayed by the method of Davis and Mora (13).

Succinate dehydrogenase (EC 1.3.99.1) was assayed by the method of King (20).

RESULTS

Cell Fractionation—Fig. 2 indicates the procedure employed to isolate the cell fractions. The glucose washes and the 600 × g pellet were analyzed for enzyme content. This was done to determine how well the crude extract represented the total cell content (21). The washes contained only minor amounts of protein and of the enzymes examined. Because the 600 × g pellet was enriched in mitochondrial enzyme activities, a typical crude extract contained 80% of the total activity of soluble enzymes, but only 50% of the succinate dehydrogenase. The non-representative nature of the crude extract does not affect the conclusions about enzyme localization, however, and therefore this problem has been ignored in the presentation of the results. No attempt has been made to isolate a nuclear fraction, because most of the nuclei break during cell lysis and the remainder are lost in the 600 × g pellet.

The relatively high speed used to sediment the particulate fraction was designed to separate all intact organelles from the soluble components. Much slower speeds (e.g. 8000 × g) have been found to be adequate for sedimenting mitochondrial activities.

Enzyme Localization—The distribution of the enzymes examined among the cell fractions is shown in Table I. The only enzymes found associated with the particulate fraction are ornithine aminotransferase, carbamyl-P synthase A, and ornithine carbamyltransferase. The small amount (3%) of the argininosuccinate lyase activity in the particulate fraction has not been observed in subsequent experiments. The measurement of succinate dehydrogenase activity was used to monitor mitochondrial distribution. The particulate carbamyl-P synthase activity was insensitive to inhibition by UTP, whereas the soluble activity was inhibited more than 95%. This observation indicates that the particulate activity is that of the arginine-specific enzyme.

Enzymes found largely in the soluble fraction are ornithine decarboxylase, argininosuccinate synthetase, argininosuccinate lyase, arginase, and ornithine aminotransferase. No physical distinction could be observed between the intracellular site of arginine synthesis (argininosuccinate lyase) and catabolism (arginase). However, enzymes of ornithine and carbamyl-P metabolism are found both in the cytosol and in the particulate fractions, and would thus appear to be in different compartments of the cell.

Sedimentation Behavior of Particulate Enzymes—The presence of ornithine aminotransferase and carbamyl-P synthase A in the particulate fraction suggested these enzymes might be mitochondrial. In fact, they are recovered in similar percentages to known mitochondrial enzymes, ornithine carbamyltransferase (9) and succinate dehydrogenase. However, electron microscopic examination of the particulate fraction revealed several types of membrane-enclosed structures, thus requiring further fractionation to determine the exact localization of the particulate enzymes.

The distribution of each of the particulate enzymes in linear density gradients was compared with that of succinate dehydrogenase, the mitochondrial marker. Fig. 3 indicates that all three arginine biosynthetic enzymes band at the density of succinate dehydrogenase after 1 hour of centrifugation. No change in their

### Table I

| Enzyme                                | Total units | Enzyme units recovered |
|---------------------------------------|-------------|-------------------------|
|                                       | Cytoplasm   | Particulate late wash   | Particulate | Microsome |
|                                       | µMoles/mg    | %                       |             |           |
| Protein                               | 254         | 66                      | 3           | 17        | 8         |
| Ornithine decarboxylase                | 0.010       | 104                     | <1          | <1        | <1        |
| Carbamyl-P synthase A                  | 0.0018      | 10                      | n.d.        | 85        | n.d.      |
| Ornithine decarboxylase transferase    | 2.7         | 10                      | 2           | 3         | 2         |
| Argininosuccinate lyase                | 2.02        | 90                      | <1          | <1        | <1        |
| Ornithine aminotransferase             | 4.6         | 100                     | <1          | <1        | <1        |
| Succinate dehydrogenase                | 16.3        | <1                      | <1          | 98        | 1         |

* Measured in milligrams.

**Not determined.
positions in the gradients was observed if centrifugation was continued for 18 hours. This density (approximately 1.19 g per cm$^3$) is the same as that determined by Luck (22) for Neurospora mitochondria.

Other organelles of Neurospora which might contain these enzyme activities are glyoxysomes and vacuoles. Attempts to detect these organelles in the gradients shown in Fig. 3 were unsuccessful. The enzymes characteristic of these organelles were undetectable in the vigorously aerated cultures used here, and are evidently repressed. However, the reported densities of these organelles (1.22 g per cm$^3$ for glyoxysomes and greater than 1.35 g per cm$^3$ for vacuoles) are sufficiently greater than mitochondria that they would have been distinguishable from the latter in the density gradients (23, 24).

Some yeast microbodies have been found to band at the same density as mitochondria, but to be distinguishable during rate sedimentation (21). Sedimentation rates of the particulate arginine enzymes were therefore compared with succinate dehydrogenase. Each enzyme appears to co-sediment with sucrose in the gradient. This behavior distinguishes these enzymes from succinate dehydrogenase, an enzyme integrated into the inner membrane of the mitochondrion.

Since only the more drastic treatments fully solubilized the enzyme activities, their mitochondrial association appears strong. The inability of KCl to solubilize the activities suggests that the association is not simply an ionic interaction. Combined with the distinction in behavior described above, the fact that recoveries of the arginine enzymes in the particulate fraction are always less than those for succinate dehydrogenase suggests that these enzymes are associated with the mitochondrial matrix. Whatever the nature of the mitochondrial localization, the similarity in the behavior of ornithine carbamyltransferase and ornithine acetyltransferase suggests that they are in the same mitochondrial compartment.

**Effect of Intact Mitochondrial Structure on Observed Enzyme Activity**—The effect of maintaining intact mitochondrial structure on the apparent activity of ornithine carbamyltransferase and ornithine acetyltransferase is shown in Table III. While decreasing the osmotic strength results in increasing activity, additional activity is observed in the presence of Triton X-100 (the latter has no effect on soluble enzyme activity).

These results indicate that an intact mitochondrial membrane serves as a permeability barrier to at least one of the substrates of each reaction. No reliable method has been found to examine the ability of the individual substrates to penetrate the mitochondrial membrane. Similarly, there is no means of assessing whether the nonlatent activity observed at the highest sorbitol

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**TABLE II**

**Solubilization of mitochondrial enzymes**

Samples of a washed particulate fraction were suspended in 5 ml of a solution containing 10 mM potassium phosphate, pH 7.5, 1 mM EDTA, 1 M sorbitol (except the "No sorbitol" sample) and the indicated additions. Each sample was kept at 0° for 2 hours. During this time they received the indicated treatments. The percentage of the ornithine carbamyltransferase, ornithine acetyltransferase, and succinate dehydrogenase which no longer sedimented at 15,000 X g in 20 min was determined as described under "Materials and Methods."

| Treatment                  | Ornithine carbamyltransferase | Ornithine acetyltransferase | Succinate dehydrogenase |
|----------------------------|-------------------------------|----------------------------|-------------------------|
| Control                    | 8                             | 4                          | 0                       |
| No sorbitol                | 28                            | 31                         | 2                       |
| Plus 0.2 M KCl             | 9                             | 9                          | 0                       |
| Plus 0.5% Triton X-100     | 95                            | 100                        | 90                      |
| Sonication                 | 86                            | 92                         | 48                      |
| Freeze-thaw (3 times at -70°) | 8 6                          | 0                          | 0                       |
and is converted to arginine in the cytoplasm. Similar conclu-
Because argininosuccinate synthetase and argininosuccinate
carbamyl-P synthesis, and their utilization to form citrulline.
brane during isolation of the organ&.
concentration is the result of partial permeability of the mem-
brane to the substrates, or damage to the mitochondrial mem-
fragment at various sorbitol concentrations in the pres-
mlr). The fact, that it is not significant,ly catabolizcrl 1)~ orn-
sis of citrulline. Therefore, although most of the ornithine made
and this pool is evidently not a direct intermediate in the synthe-
system for investigation of the role of subcellular organization
in regulation (7-11). A complete understanding requires identi-
the requirements for growth, but is prototrophic if identical levels
of the endogenous source of ornithine arc available (29, 30).
The mitochondrial membrane in Neurospora may minimize the
amount of catabolic ornithine which re-enters the biosynthetic
pathway.
Because the synthesis and catabolism of arginine and its
utilization for protein synthesis occur in the cytoplasm, we might
expect the arginine pool to be readily available for catabolism.
Despite the fact that intracellular arginine (8 m&r in cell water)
is more than adequate to satisfy arginase (Km = 5 mM), no
detectable catabolism is observed (8, 11). The paradox is re-
solved by the finding that the pool of arginine is confined to the
cellular vesicle in which we find ornithine (10).
Arginine metabolism in Neurospora has proven to be a fruitful
ly for investigation of the role of subcellular organization in
regulation (7-11). A complete understanding requires identifi-
cation and characterization of the membrane systems which
separate intracellular enzymes and intermediates. Localization
of the pools of ornithine and arginine has proven to be possible
and amenable to further analysis (10). These investigations are
continuing in an attempt to define the role and importance of
compartmentation in regulating amino acid metabolism in
Neurospora.

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*J. Biol. Chem.* 1973, 248:5403-5408.

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