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Carbamoyl Phosphate Compartmentation in *Neurospora:* Histochemical Localization of Aspartate and Ornithine Transcarbamoylases

(channelling/arginine/pyrimidines/electron microscope/eukaryotes)

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ABSTRACT **Carbamoyl phosphate is required for arginine and pyrimidine synthesis. In the arginine pathway, it is used in the ornithine transcarbamoylase (EC 2.1.2.1) reaction; in the pyrimidine pathway, it is used in the aspartate transcarbamoylase (EC 2.1.3.2) reaction. In *Neurospora crassa*, two pathway-specific enzymes catalyze the synthesis of carbamoyl phosphate, and two path-specific pools of carbamoyl phosphate are maintained. Histochemical studies show that ornithine transcarbamoylase is located in mitochondria, and, with less certainty, that aspartate transcarbamoylase is confined largely to nuclei. The enzymes that form carbamoyl phosphate are associated with the respective transcarbamoylases in the cell. Therefore, the segregation of carbamoyl phosphate pools could be accounted for by one or both organellar membranes, which demarcate two separate sites of carbamoyl phosphate metabolism in *Neurospora*. The alternative possibility that the enzyme complex that produces and consumes carbamoyl phosphate in the pyrimidine pathway could explain the channelling of carbamoyl phosphate, wholly or in part, is discussed.**

Nonrandom distribution of metabolites within cells as a factor in their metabolic fates is known as channelling. Several compounds involved in oxidative phosphorylation are confined to mitochondria, and certain enzyme aggregates appear to retain intermediates in the course of sequential reactions. The metabolism of carbamoyl phosphate (carbamoyl-P) in eukaryotes has recently been studied in relation to channelling (1–3). Carbamoyl-P is an intermediate of the arginine and pyrimidine pathways, used, respectively, by ornithine transcarbamoylase (OTCase: carbamoylphosphate: ornithine carbamoyltransferase, EC 2.1.3.1) and aspartate transcarbamoylase (ATCase: carbamoylphosphate: aspartate carbamoyltransferase, EC 2.1.3.2). Eukaryotes, in contrast to bacteria, have two enzymes for carbamoyl-P synthesis. In *Neurospora crassa*, both are glutamine-dependent carbamoyl-P synthetases (EC 2.7.2.5). Studies with mutants lacking one or the other of the synthetases show that one (carbamoyl-P synthetase *A*) has a specific role in the arginine pathway, while the other (carbamoyl-P synthetase *P*) functions solely in the pyrimidine pathway (1, 4, 5). These studies suggest that two discrete pools of carbamoyl-P are maintained, since mutants lacking one of the synthetases have an absolute and specific requirement for the corresponding end-product, either uridylic acid or arginine. More direct evidence that supports this view has been published (2).

In mammals, it was established that citrulline synthesis (OTCase and carbamoyl-P synthetase I, which uses ammonia as an N donor) was largely a mitochondrial function (6, 7). Carbamoyl-P synthetase II and ATCase were apparently localized in the high-speed supernatant or light membrane fraction of mammalian tissues according to differential centrifugation studies (8). Recent histochemical localization of OTC in mitochondria (and possibly in the cytosol) of rat hepatocytes has been demonstrated hy Mizutani (9) and by Merker and Spors (10). These authors incubated tissue slices with ornithine and carbamoyl-P in the presence of Pb^{++}. The orthophosphate released in the OTCase reaction was trapped as a lead precipitate *in situ*, and was visualized by electron microscopy. A similar test of ATCase localization by Spors and Merker (11) showed it to be mainly in the rough endoplasmic reticulum in the same cell type.

We have applied these electron microscopic histochemical techniques to *Neurospora*. Our results are discussed in relation to metabolic channelling and to the localization of other arginine and pyrimidine biosynthetic enzymes in *Neurospora.*

MATERIALS AND METHODS

The strains used were wild-type 74A; *arg-12* (UM-107 R2A), lacking OTCase; *pyr-3d* (63902 R1a), lacking ATCase; and the double mutant, *arg-12, pyr-3d* (UM-3, 44502 R2). Mycelia were grown at 25° in Vogel's medium N (12), with 1.5% sucrose, supplemented where appropriate with 200 μg of arginine per ml and 100 μg of uridine per ml.

Cells were obtained by filtration of 10–15 ml volumes of exponential cultures (0.5–0.7 mg of dry weight per ml) on 2.5-cm circles of Whatman no. 540 filter paper. The mycelial pads were minced and placed in 3% glutaraldehyde–0.1 M sodium cacodylate buffer (pH 7.8) for 2 hr at 4°. The glutaraldehyde solution also contained either 5 mM L-ornithine or 5 mM of L-aspartate, or both, to protect the transcarbamoylases. The tissue was then rinsed successively in 0.1 M sodium cacodylate buffer and 0.05 M Tris–maleate buffer (pH 8.9).

The fixed mycelia were placed in a centrifuge tube in the appropriate enzyme reaction mixture. The standard mixture (10 ml) contained 5 mM Li_2carbamoyl-P, 3 mM $Pb(NO_3)_2$, 5 mM sodium citrate, and 50 mM Tris–maleate (pH 8.9), plus one or both amino-acid substrates, 10 mM L-ornithine and 10 mM L-aspartate. Incubations were at 37° for 15–60

Abbreviations: carbamoyl phosphate, carbamoyl-P; ornithine transcarbamoylase, OTCase; aspartate transcarbamoylase, ATCase.

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min. Because of spontaneous carbamoyl-P decomposition and consequent turbidity, the reaction mixture for longer incubations was replaced with fresh solution every 15–20 min.

Mycelia were postfixed in 1% osmium tetroxide for at least 2 hr, rinsed with distilled water, dehydrated in a graded series of alcohol solutions and propylene oxide, and embedded in Epon 812 (Ernest Fullham, Inc.). Blocks were sectioned on a Sorvall Porter–Blum ultramicrotome, stained with uranyl acetate and lead hydroxide, and examined with a Zeiss model 9A electron microscope.

RESULTS

The fundamental observations made with wild-type mycelia were as follows. After incubation with ornithine and carbamoyl-P, the cells contained precipitated lead only in the mitochondria. After incubation with aspartate and carbamoyl-P, the cells contained precipitated lead in their nuclei, with an occasional, slight cytoplasmic precipitate, and no precipitated lead in their mitochondria. Cells incubated with carbamoyl-P and both amino-acid substrates had precipitate in all the above locations, as though the two transcarbamoylase activities were additive. Upon omission of both amino-acid substrates, mycelia had no intracellular precipitate. In all reaction mixtures, precipitated lead appeared on the outside of the cell wall. The simplest interpretation of these results is that OTCase is mitochondrial, ATCase is mainly nuclear, and extracellular precipitate is nonenzymically generated. It should be noted that the reaction mixtures containing only one of the amino-acid substrates are mutual controls for substrate specificity.

Several controls were performed, the first of which provides the best illustrative material. Fig. 1 demonstrates the requirement for the enzymes by comparison of wild-type and mutant strains in 15- to 60-min incubations. All strains were prepared similarly and were then incubated with carbamoyl-P and both ornithine and aspartate. The wild-type cells show nuclear and mitochondrial precipitate (Fig. 1*A*). Cells of *pyr-3d*, which lack ATCase, lack nuclear precipitate and have the mitochondrial precipitate (Fig. 1*B*). Cells of *arg-12*, which lack OTCase, lack mitochondrial precipitate and have only nuclear precipitate (Fig. 1*C*). Cells of the double mutant, *pyr-3d arg-12*, are devoid of intracellular precipitate (Fig. 1*D*). All types of cells displayed extracellular precipitate (e.g., Fig. 1*A* and *B*) when grids were surveyed.

In a further test of substrate specificity, wild-type cells were incubated in reaction mixtures containing carbamoyl-P and either lysine (an ornithine analogue to which OTCase is indifferent) or glutamate (an aspartate analogue to which ATCase is indifferent). No intracellular precipitate was seen in either case.

We are reasonably sure that the organellar locations of precipitate are not the result of selective affinity of the organelles for phosphate or its lead salt (13). This lack of affinity is indicated by the different locations of precipitate in single mutant preparations, even though both generate intracellular orthophosphate in identical conditions. It is less certain that there is little or no activity in the cytosol. Three artifacts could obscure enzyme activity in the cytosol: (*a*) selective inactivation of enzyme during fixation; (*b*) an unsatisfied ultrastructural requirement for visible lead precipitate; and (*c*) failure to reach a threshold concentration of phosphate or its lead salt in the precipitation step. While these possibilities were not explored directly, they should be kept in mind in the discussion below.

OTCase appears to be located almost wholly in mitochondria, even in preparations incubated for 60 min (e.g., Fig. 1*B*). In such reactions, the mitochondria sometimes become so heavily labeled with lead phosphate that they are completely obscured. The small amount of extramitochondrial precipitate that occasionally appears is too variable to judge its dependence upon OTCase. It would not be surprising to find some OTCase in the cytoplasm if the enzyme, encoded by a nuclear gene, is made on cytoplasmic ribosomes. The histochemical localization of OTCase correlates well with differential centrifugation studies in *Neurospora*, in which 85% of the activity can be recovered in the mitochondrial fraction (14). Therefore, questions of selective inactivation of cytoplasmic OTCase and special requirements for lead deposition can be safely disregarded.

The distribution of OTCase within mitochondria is also of interest. The precipitate that forms is associated with cristae, both in intact cells (Fig. 2*A*) and in mitochondria isolated by differential centrifugation (Weiss and Bernhardt, unpublished observations). The precipitated lead is not associated with the outer or inner peripheral mitochondrial membranes. Because the precipitate frequently appears in double lines, presumably separated by cristal membranes and intracristal space, we feel that the enzyme faces, or is within, the inner mitochondrial compartment. It is unknown whether the enzyme itself is on the membrane or whether the lead salt preferentially precipitates there. We have not yet adequately tested the latency of OTCase in isolated mitochondria, but we have found that mitochondria readily release OTCase as a soluble activity (14). Any attachment of OTCase to mitochondrial membranes is, therefore, weak.

In the case of ATCase, there is more evidence for a small amount of activity in the cytosol, particularly in long incubations. Nevertheless, most of the activity visualized is nuclear, and no ATCase activity is detectable in mitochondria by any technique (2). Unfortunately, the distribution of ATCase between nucleus and cytosol is hard to determine by differential centrifugation, because nuclei cannot easily be separated from whole cells and other debris after gentle cell breakage. In such tests in this laboratory, a "heavy" ATCase fraction and a second, soluble fraction of ATCase are usually found. The latter may well be a cytosol fraction *in vivo*, too diffuse to see in electron micrographs.

The nuclear precipitate formed in the ATCase histochemical reaction is not evenly distributed in this organelle. Instead, it is disposed in a diffuse (Fig. 1*A*) or sharply defined (Figs. 1*C* and 2*B*) cluster of particles. The cluster may represent the nucleolus (Fig. 2*B*), and particles of precipitate could be associated with groups of ribosomes or of preribosomal components. An association of ATCase with ribosomes is consistent with *in vitro* studies of complementation of ATCase mutants that showed that ribosomes were the best complementing component (15). Spors and Merker (11) also showed a close relation of ATCase with ribosomes by histochemical tests.

DISCUSSION

Our studies show OTCase to be localized in the mitochondria of *Neurospora* mycelia. This localization is also true for rat hepatocytes, although there is some evidence of OTCase

activity in the endoplasmic reticulum by histochemical tests (10). A mitochondrial localization of OTCase is supported by differential centrifugation studies of both *Neurospora* and rat. Differential centrifugation reveals that certain earlier enzymes of the *Neurospora* arginine pathway, *N*-acetylornithine:2-oxoglutarate amino-transferase (EC 2.6.1.11) and carbamoyl-P synthetase *A*, are also localized in mitochondria (2, 14). The same technique shows that the two enzymes that follow OTCase in the pathway, that transform citrulline to arginine, are *not* associated with mitochondria (14). This result is also found in rats (6, 16).

ATCase appears in histochemical tests to be in the nucleus, although a small amount of activity may appear in the cytoplasm. Because differential centrifugation data are lacking, it is unwise to assume that the histochemical strain accurately reflects the distribution of ATCase *in vivo*. In rat hepatocytes, Spors and Merker (11) demonstrated most of the ATCase in rough endoplasmic reticulum, and this result is consistent with centrifugation data for this tissue. They found a very slight nuclear precipitate in histochemical tests that they felt might be an artifact. In both organisms, ATCase may have a definite relation to ribosomes, but the significance

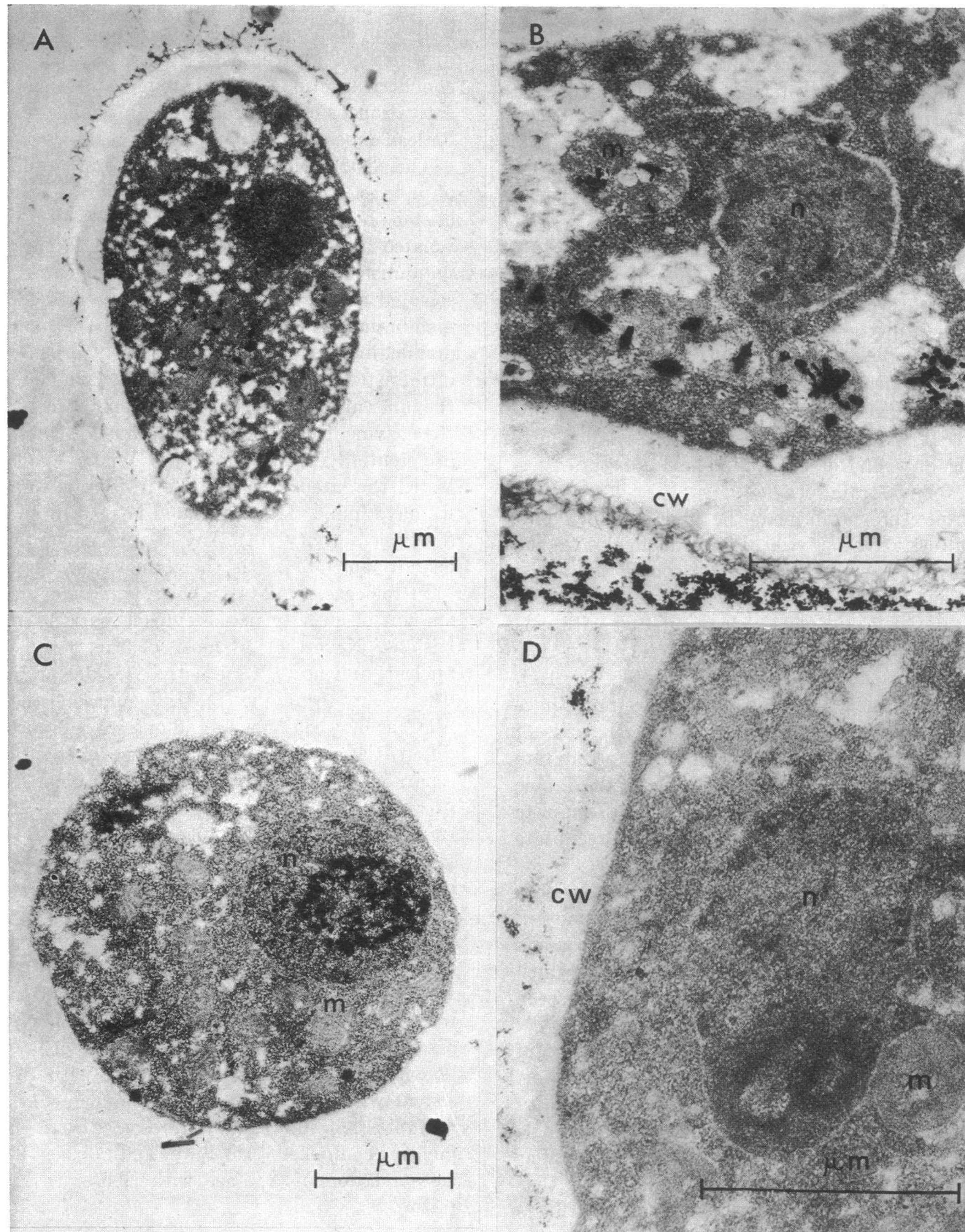

FIG. 1. Histochemical demonstration of OTCase and ATCase in wild-type and mutant *Neurospora* cells. All preparations were incubated in carbamoyl phosphate and both asparate and ornithine. (*A*) Wild type, containing both enzymes. (*B*) *pyr-3d*, containing only OTCase. (*C*) *arg-12*, containing only ATCase. (*D*) *arg-12, pyr-3d*, a double mutant containing neither transcarbamoylase. Symbols: *m*, mitochondrion; *n*, nucleus; *cw*, cell wall; *v*, vacuole. *Bar*, 1 μm.

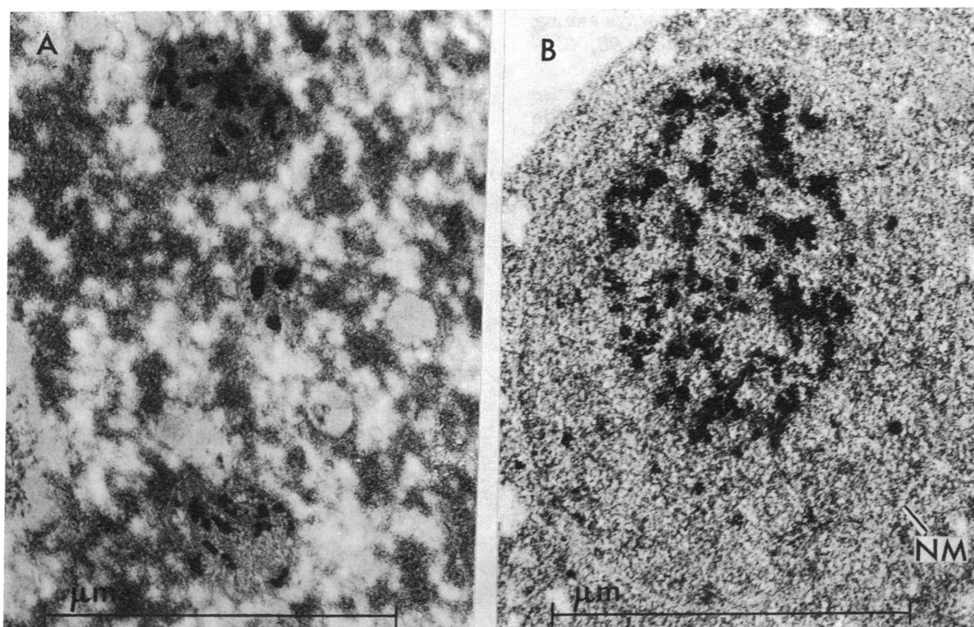

FIG. 2. (*A*) Three mitochondria of a *pyr-3d* cell, showing localization of OTCase reaction product. (*B*) Nucleus of an *arg-12* cell, showing intranuclear distribution of ATCase reaction product. Symbol: *nm*, nuclear membrane. *Bar*, 1 μm.

of this hypothetical relationship is not clear. In other studies with *Neurospora*, certain other pyrimidine enzymes are not in the nuclei; in fact, dihydro-orotate dehydrogenase (EC 1.3.3.1) is in mitochondria (17). Therefore, neither the arginine nor the pyrimidine pathway is completely confined to either organelle.

Our histochemical studies were prompted by evidence for two discrete carbamoyl-P pools in *Neurospora* (2). The localization of the transcarbamoylases is similar to the localization of the corresponding, path-specific, carbamoyl-P synthetases. Carbamoyl-P synthetase *A* is in mitochondria with OTCase (2). Carbamoyl-P synthetase *P* of *Neurospora* (18), yeast (19), and rat (20) is associated with ATCase in an enzyme aggregate, and is therefore in the same locations as ATCase. The metabolism of carbamoyl-P in the arginine pathway, therefore, proceeds at a different site in a cell from carbamoyl-P metabolism in the pyrimidine pathway, and this separation correlates well with the observed channelling of carbamoyl-P pools in *Neurospora*. These pools are very small (0.4 nmol/g of dry weight for the pyrimidine pathway, and about 4 nmol/g of dry weight for the arginine pathway), and their discreteness is in part dependent on their small size (2).

The most significant barrier between the two carbamoyl-P pools is probably the mitochondrial membrane. The nuclear membrane, although it may be quite porous, may also play a role in carbamoyl-P channelling, particularly because it persists during nuclear division. It would be premature to conclude with certainty, however, that channelling is a membrane-related phenomenon. First, recent experiments (3, 21) have cast doubt upon the ability of rat hepatocyte mitochondria to confine carbamoyl-P produced in the mitochondria to the arginine pathway. Second, the enzyme complex carrying carbamoyl-P synthetase *P* and ATCase activities may be able to confine carbamoyl-P as an enzyme-bound intermediate, and conceivably to exclude low concentrations of carbamoyl-P produced elsewhere (22). An enzyme complex with both properties could wholly explain the observed discreteness of both carbamoyl-P pools. A study, now in progress, of *Neurospora* mutants with mislocated arginine enzymes will allow us to judge more clearly the role of the mitochondrial membrane in channelling carbamoyl-P and other intermediates.

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