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Arginine-specific Carbamoyl Phosphate Metabolism in Mitochondria of *Neurospora crassa*

CHANNELING AND CONTROL BY ARGININE*

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Citrulline is synthesized in mitochondria of *Neurospora crassa* from ornithine and carbamoyl phosphate. In mycelia grown in minimal medium, carbamoyl phosphate limits citrulline (and arginine) synthesis. Addition of arginine to such cultures reduces the availability of intramitochondrial ornithine, and ornithine then limits citrulline synthesis. We have found that for some time after addition of excess arginine, carbamoyl phosphate synthesis continued. Very little of this carbamoyl phosphate escaped the mitochondrion to be used in the pyrimidine pathway in the nucleus. Instead, mitochondrial carbamoyl phosphate accumulated over 40-fold and turned over rapidly. This was true in ornithine- or ornithine carbamoyltransferase-deficient mutants and in normal mycelia during feedback inhibition of ornithine synthesis. The data suggest that the rate of carbamoyl phosphate synthesis is dependent to a large extent upon the specific activity of the slowly and incompletely repressible synthetic enzyme, carbamoyl-phosphate synthetase A. In keeping with this conclusion, we found that when carbamoyl-phosphate synthetase A was repressed 2-10-fold by growth of mycelia in arginine, carbamoyl phosphate was still synthesized in excess of that used for residual citrulline synthesis. Again, only a small fraction of the excess carbamoyl phosphate could be accounted for by diversion to the pyrimidine pathway. The continued synthesis and turnover of carbamoyl phosphate in mitochondria of arginine-grown cells may allow rapid resumption of citrulline formation after external arginine disappears and no longer exerts negative control on ornithine biosynthesis.

Two tributaries contribute to the arginine pathway of *Neurospora crassa*, as they do in most lower organisms and plants (1, 2). One is the synthesis of carbamoyl phosphate, a single step catalyzed by the arginine-specific enzyme, carbamoyl-phosphate synthetase A (3). The other comprises the activities of five enzymes that transform glutamate, via acetylated intermediates, to ornithine (Fig. 1). Both tributaries are confined to mitochondria, together with ornithine carbamoyltransferase, which uses carbamoyl phosphate and ornithine to make citrulline (4-7). Citrulline is transformed into arginine in the cytosol. In *N. crassa*, the arginine pathway is independent of pyrimidine synthesis (7). The latter depends upon a second carbamoyl phosphate-synthesizing enzyme, carbamoyl-phosphate synthetase P (Fig. 1). Carbamoyl-phosphate synthetase P is part of a multienzyme complex with aspartate carbamoyltransferase in the nucleolus (8, 9). The product of each carbamoyl-phosphate synthetase is normally directed wholly to its own pathway in wild-type cells growing in minimal medium (7, 10).

The synthesis of ornithine is not coupled to that of carbamoyl phosphate. Ornithine is usually made in excess and is stored in the vacuole or catabolized (11). Mutations of carbamoyl phosphate metabolism do not affect the synthesis of ornithine (12).[1] There is no evidence that the synthesis of carbamoyl phosphate is dependent upon the integrity of ornithine synthetic enzymes (13),[1] and carbamoyl-phosphate synthetase A activity is not stimulated by ornithine *in vitro* (3). Despite these observations, ornithine has not been definitively tested as a factor in carbamoyl phosphate synthesis *in vivo*.

Arginine efficiently feedback-inhibits intramitrochondrial ornithine synthesis, mainly at the acetylglutamate kinase reaction, one of the early steps in the acetylated pathway (Fig. 1) (14-16). This is not sufficient for control, however, because when arginine is added to *N. crassa* cultures, ornithine appears as a catabolic product of arginase, a cytosolic enzyme. Recently, we found that arginine, in addition to feedback-inhibiting ornithine synthesis, also severely inhibited the entry of ornithine into the mitochondrion (Fig. 1) (16). The two actions of arginine led to immediate reduction of citrulline synthesis by 75-90% in normal cultures (17, 18). In the same work, we inferred that neither carbamoyl-phosphate synthetase A nor ornithine carbamoyltransferase were inhibited by arginine *in vivo* in the short term (16). This was shown by continued synthesis of citrulline after arginine was added to a mutant in which the synthesis of intramitochondrial ornithine was feedback-resistant (16).

The question posed by these data is what effect arginine has on carbamoyl phosphate metabolism. One would expect reasonably strict control of carbamoyl-phosphate synthetase A activity, which uses 2 mol of ATP/mol of carbamoyl phosphate synthesized. However, as noted above, no feedback control of the enzyme activity has been detected. The only known control of carbamoyl-phosphate synthetase A by arginine is an incomplete repression of the synthesis of the small subunit of this enzyme (5). We would therefore expect continued synthesis and possibly some diversion of carbamoyl phosphate to the pyrimidine pathway when arginine is present, even in the long term.

Here we report studies on carbamoyl phosphate synthesis, escape from the mitochondrion, and turnover during long- and short-term growth of *N. crassa* in the presence of arginine.

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[1] J. J. DiGangi and R. H. Davis, unpublished observations.

TABLE I

Enzyme activity, carbamoyl phosphate content, and synthetic rates of citrulline and ureidosuccinate during steady state and starvation and after addition of arginine

Strain (genotype)	Condition[a]	Carbamoyl-P synthetase A activity	Carbamoyl phosphate content	Synthetic rate	
				Cit[b]	US[c]
		units/mg mitochondrial protein	*nmol/mg cell protein*	*nmol/min/mg cell protein*	
A. Steady states					
pyr-3M pyr-1	Urd	4	0.01	2.6	0
	Urd + Arg	0.3–1.0			
pyr-3M pyr-1 arg-1	Urd + Arg	0.5–1.2	0.01	0.03	0
pyr-3M pyr-1 arg-1 sup-3	Urd + Arg	0.3–1.3	0.02	0.25	0
pyr-3M pyr-1 arg-12	Urd + Arg	0.6–2.2	0.06	0	0.04
pyr-3M pyr-1 arg-12[s]	Urd	35.0	0.9	1.28	0.8
B. Starvations and transitions					
pyr-3M pyr-1	U → UA	4	0.45 (pk)		0.34
pyr-3M pyr-1 arg-1	U (starved)	3–7	0.05	4.5	0
	U → UA	Falls	0.4 (pk)	0	0.27
pyr-3M pyr-1 arg-12	U (starved)	2–6	0.9	0	0.25
	U → UA	Falls	Falls	0	0.25
pyr-3M pyr-1 arg-6	U (starved)	ND	0.9		0.5

[a] Where noted, starved refers to 3 h of arginine starvation after transfer to arginine-free medium (U). Addition of 1 mM arginine is designated by U → UA, and values are taken from those found in the first hour after arginine addition. The values are taken from the figures and transformed to a milligram of protein basis.

[b] Cit, citrulline; pk, peak value; ND, not determined.

[c] US refers to the sum of the amounts of ureidosuccinate + dihydroorotate. See "Experimental Procedures."

In this study, we use mutations of arginine enzymes to manipulate the levels of arginine intermediates *in vivo* and mutations of pyrimidine enzymes to trap carbamoyl phosphate overflowing from mitochondria *in vivo* (Fig. 1).

EXPERIMENTAL PROCEDURES[2]

RESULTS

Negative Control of Citrulline Formation at Steady State—The *pyr-3M pyr-1* strain, with a normal arginine pathway, was grown in uridine-supplemented minimal medium. By measurement of the growth rate and of arginine formed during growth, the normal steady-state rate of carbamoyl phosphate utilization for citrulline and arginine synthesis was calculated (Ref. 11; see "Experimental Procedures"). Citrulline was made at a rate of 2.6 nmol/min/mg of protein (Table I). A small pool of carbamoyl phosphate (0.01 nmol/mg of cell protein), approximately enough for 0.25 s of citrulline synthesis, was found (Table I, part A). Carbamoyl phosphate did not escape to the pyrimidine enzymes because no ureidosuccinate + dihydroorotate, referred to hereafter as ureidosuccinate, was trapped by the active segment of the pyrimidine path (aspartate carbamoyltransferase and dihydroorotase in the *pyr-3M pyr-1* mutant strain; see Fig. 1).

Carbamoyl-phosphate synthetase A activity was reduced (Table I, part A) when the *pyr-3M pyr-1* strain was grown in the presence of arginine. (Other arginine enzymes are not repressed by arginine (5).) Unlike all other parameters, including the activity of the ammonia-dependent activity of carbamoyl-phosphate synthetase A, the glutamine-dependent activity of carbamoyl-phosphate synthetase A increased faster than growth (*i.e.* in specific activity) from about 10% to as much as 50% that seen in minimal medium during the interval of growth studied (Table I, part A).

To study the long-term effect of arginine on citrulline synthesis, a *pyr-3M pyr-1 arg-1* strain, which is unable to convert citrulline to argininosuccinate, was used. When the strain was grown in medium supplemented with uridine and arginine, citrulline accumulated at a rate of 0.03 nmol/min/mg of protein, or about 1% the rate calculated in *pyr-3M pyr-1* mycelia from minimal medium (Table I, part A). Carbamoyl phosphate was detected at about the same level in the two cultures.

Arginine efficiently feedback-inhibits mitochondrial ornithine synthesis (15, 19) and, at the same time, severely inhibits its entry of catabolic ornithine (Fig. 1) into mitochondria from the cytosol (16). We wished to know whether arginine inhibited citrulline synthesis wholly by these mechanisms or whether arginine impaired the carbamoyl-phosphate synthetase A reaction as well. By rendering mitochondrial ornithine synthesis feedback-insensitive with the *sup-3* mutation, we could answer this question. The *pyr-3M pyr-1 arg-1 sup-3* strain was grown in arginine. The synthesis of citrulline was about 10-fold the rate seen in the feedback-sensitive strain in the presence of arginine (Table I, part A) and thus about 10% of the rate seen in the *pyr-3M pyr-1* strain grown in minimal medium.

The data suggest that in the presence of arginine, citrulline synthesis in the feedback-sensitive strain is limited by ornithine, whereas citrulline synthesis in the feedback-resistant strain is limited by carbamoyl phosphate. Carbamoyl phosphate synthesis itself is limited mainly by repression; previous work showed that addition of arginine to a feedback-resistant strain growing in minimal medium did not immediately affect citrulline synthesis (16).

Carbamoyl Phosphate Accumulation in Strains Deficient in Ornithine Carbamoyltransferase—To demonstrate further carbamoyl phosphate synthesis during long-term growth in

[2] Portions of this paper (including "Experimental Procedures," part of "Results," part of "Discussion," Figs. 2, 3, and 5–7, and Footnote 4) are presented in miniprint at the end of this paper. The abbreviation used is: TES, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}ethanesulfonic acid. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 86M-3648, cite the authors, and include a check or money order for $4.40 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Wavery Press.

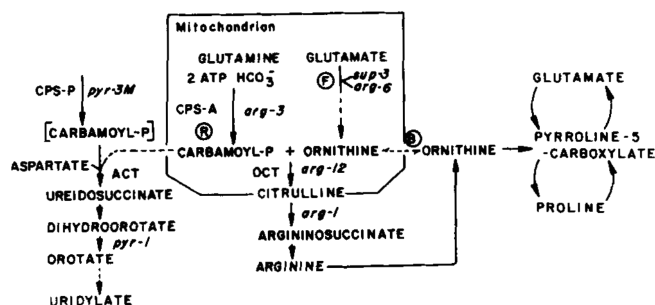

FIG. 1. **Scheme of arginine, pyrimidine, and proline biosynthesis in *N. crassa*.** The *circled symbols* designate arginine-mediated control mechanisms: *R*, repression of carbamoyl-phosphate synthetase A (EC 6.3.5.5); *F*, feedback inhibition of acetylglutamate kinase (EC 2.7.2.8); and *B*, blockage of entry of ornithine into mitochondria. The mutations (*italics*) affecting various steps in the arginine and pyrimidine pathways are as follows: *sup-3*, an allele of the *arg-6* gene, confers feedback resistance on acetylglutamate kinase; *arg-3* blocks carbamoyl-phosphate synthetase A; *arg-12* (and its allele, *arg-12ˢ*) blocks ornithine carbamoyltransferase (*OCT*; EC 2.1.3.3); *arg-1* blocks argininosuccinate synthetase (EC 6.3.4.5); *pyr-3M* blocks carbamoyl-phosphate synthetase P (*CPS-P*); and *pyr-1* blocks dihydroorotate oxidase (EC 1.3.3.1). Carbamoyl phosphate of the pyrimidine pathway is *bracketed* to emphasize that it is not made in strains used in this work. *ACT*, aspartate-carbamoyl transferase (EC 2.1.3.2).

the presence of arginine, a strain wholly lacking ornithine carbamoyltransferase activity, *pyr-3M pyr-1 arg-12*, was grown in arginine-supplemented medium. This strain accumulated carbamoyl phosphate (0.06 nmol/mg of protein), and a small amount of it was diverted to the pyrimidine pathway (Table I, part A). The amount of ureidosuccinate found was only one-sixth the amount of citrulline accumulated by the *pyr-3M pyr-1 arg-1 sup-3* strain under the same conditions. The data suggest that carbamoyl phosphate escapes the mitochondrion with difficulty or that it is trapped inefficiently in the pyrimidine pathway. (We assume that in the *pyr-3M pyr-1 arg-1 sup-3* strain, carbamoyl phosphate synthesis is fully realized in citrulline accumulation.)

The *arg-12ˢ* mutation reduces ornithine carbamoyltransferase activity by about 95% (12, 20). Residual enzyme activity allows strains carrying this mutation to grow in arginine-free medium, and this depends in addition upon the derepression of all arginine biosynthetic enzymes (21). This mutation allowed us to measure diversion of carbamoyl phosphate to the pyrimidine pathway when carbamoyl-phosphate synthetase A activity is maximally derepressed and when its product cannot be used rapidly in the mitochondrion.

The triple mutant *pyr-3M pyr-1 arg-12ˢ* strain was grown in arginine-free medium. Its calculated rate of citrulline synthesis was about one-half normal (Table I, part A). This was manifest in a very low arginine pool, a slightly lower growth rate, and a smaller amount of protein/mg, dry weight (data not shown). Its carbamoyl-phosphate synthetase A activity was 10-fold normal, and its carbamoyl phosphate pool was elevated 50–100-fold. Diversion of carbamoyl phosphate to the pyrimidine pathway was sufficient for considerable ureidosuccinate synthesis (0.8 nmol/min/mg of protein) (Table I, part A).

The amount of ureidosuccinate formed in the *pyr-3M pyr-1 arg-12ˢ* strain was 20-fold that seen in *pyr-3M pyr-1 arg-12* in arginine-supplemented medium. The factor is roughly proportional to the difference in carbamoyl-phosphate synthetase A activities of the two cultures (Table I, part A). However, it was not equal to the citrulline synthesis expected if all of the carbamoyl phosphate synthetic potential was used to make citrulline. This can be seen by comparing the carbamoyl-phosphate synthetase A activity (4.0 units/mg of mitochondrial protein) and citrulline synthesis (2.6 nmol/min/mg of cellular protein) in *pyr-3M pyr-1* with the carbamoyl-phosphate synthetase A activity and the sum of citrulline and ureidosuccinate synthesis in *pyr-3M pyr-1 arg-12ˢ* (35.0 units/mg and 2.16 nmol/min/mg, respectively) (Table I, part A).

To summarize the outcome of the steady-state experiments above, (i) arginine has a 100-fold negative effect on citrulline synthesis, which is due mainly to diminished mitochondrial ornithine because carbamoyl phosphate synthesis continues at 10% of the rate seen in Arg$^+$ cultures grown in minimal medium; and (ii) the mitochondria appear to minimize escape of excess carbamoyl phosphate to the pyrimidine pathway, possibly leading to carbamoyl phosphate turnover within the organelle. The second idea is tested, with alternative hypotheses, in the following short-term experiments.

Onset of Feedback Inhibition—When arginine was added to a *pyr-3M pyr-1* culture growing in minimal medium, the carbamoyl phosphate, per ml culture volume, rose over 40-fold in 90 min and returned to normal in another 2 h (Fig. 2). During the initial increase, ureidosuccinate was formed, and this stopped as carbamoyl phosphate returned to normal. The data are also reported in Table I, part B, in which metabolite determinations are given per mg of cellular protein. The rate of ureidosuccinate synthesis at its greatest was only about 0.34 nmol/min/mg of cell protein, compared to the prior rate of citrulline synthesis (before addition of arginine) of 2.6 nmol/min/mg (Table I, part A). The carbamoyl phosphate pool at its peak, 0.45 nmol/mg of protein, is only a tiny fraction (1.3 min worth) of the ureidosuccinate accumulation (Table I, part A).

The fall in the carbamoyl phosphate content and the diminishing rate of ureidosuccinate formation are probably due to the onset of entry of cytosolic ornithine, arising in the cytosol from arginine catabolism, into mitochondria, where it allows carbamoyl phosphate consumption for citrulline synthesis (18). The entry of ornithine into mitochondria is not fully inhibited by arginine in Arg$^+$ cells (18) or, to a more variable extent, in *arg-1* cells (16) for some hours. The diminished carbamoyl phosphate and ureidosuccinate accumulation were not correlated with repression or turnover of carbamoyl-phosphate synthetase A (Fig. 3). Addition of arginine largely prevented the increase of carbamoyl-phosphate synthetase A-specific activity (normally seen in young cultures), but there was no net loss by dilution of enzyme during further growth.

To compare synthesis of the derivatives of carbamoyl phosphate before and after arginine addition, the *pyr-3M pyr-1 arg-1* strain was used. Measurement of citrulline, ureidosuccinate, and carbamoyl phosphate during arginine starvation and after arginine repletion in the *pyr-3M pyr-1 arg-1* strain will detect all known fates of carbamoyl phosphate except turnover. The strain, grown initially in medium supplemented with arginine plus uridine, was transferred to arginine-free medium. After 3 h, arginine starvation assured the relief of feedback inhibition of ornithine synthesis and mild derepression of carbamoyl-phosphate synthetase A. Citrulline accumulated at a constant rate of 4.5 nmol/min/mg of protein thereafter (Fig. 4 and Table I, part B). This rate is higher than in the *pyr-3M pyr-1* strain grown in arginine-free medium owing to a somewhat higher carbamoyl-phosphate synthetase A activity (Table I, part B). The carbamoyl phosphate pool remained low in *pyr-3M pyr-1 arg-1*, and ureidosuccinate did not increase (Fig. 4) during starvation.

The addition of arginine caused an immediate cessation of citrulline synthesis (Fig. 4). (Because starved Arg$^-$ cells do not take up and catabolize arginine rapidly (16), there is even

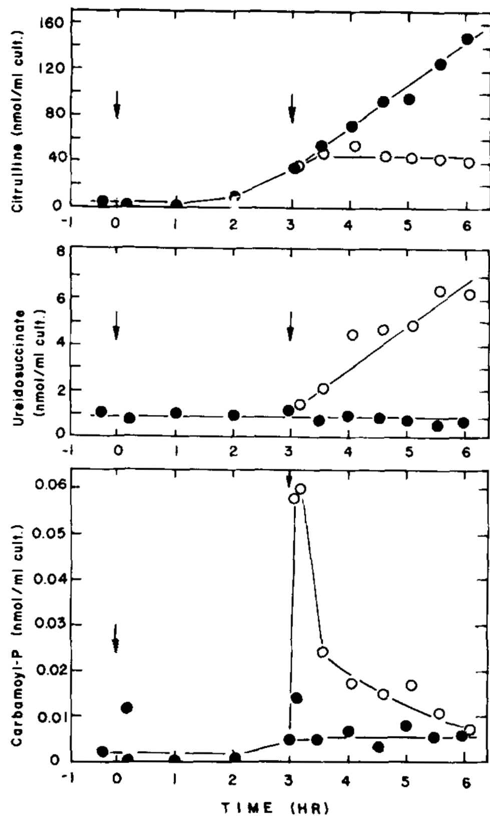

FIG. 4. **Citrulline synthesis, ureidosuccinate synthesis, and carbamoyl phosphate content of strain *pyr-3M pyr-1 arg-1*.** A culture of the strain was grown in arginine- and uridine-supplemented medium until time 0. It was harvested, washed, and resuspended in uridine-supplemented, arginine-free medium (●) at time 0 (*left arrow*). At 3 h (*right arrow*), arginine was added to a portion of the culture (○) to a final concentration of 1 mM. Citrulline (*top*), ureidosuccinate (*middle*), and carbamoyl phosphate (*bottom*) were measured in samples of culture and expressed as nanomoles/milliliter culture volume. Ureidosuccinate represents the accumulation of ureidosuccinate + dihydroorotate in this and other figures. The dry weight of the culture at 3 h was 0.5 mg/ml, and protein was 0.15 mg/ml culture volume. Increase of protein and dry weight stopped at 3.2 h in the arginine-free culture and continued in the arginine-supplemented culture. Data from this culture in terms of protein are reported in Table I.

less recycling of ornithine than in Arg$^+$ strains.) Carbamoyl phosphate abruptly increased by 8-fold, followed by a reduction, first rapidly and then more slowly, over the next 3 h (Fig. 4). An accumulation of ureidosuccinate at an initial rate of 0.27 nmol/min/mg of protein (Table I) was correlated with these events. The rate of ureidosuccinate synthesis was 6% the rate of citrulline synthesis in the control. (Adding the rate of carbamoyl phosphate synthesis required to maintain its pool size is quantitatively insignificant.) The behavior of the strain is similar to the experiment with the *pyr-3M pyr-1* strain: a peak of carbamoyl phosphate and its diversion of this compound at a low rate to the pyrimidine pathway for hours afterward (Fig. 2). The continuation of ureidosuccinate synthesis in the *pyr-3M pyr-1 arg-1* strain may reflect a continued ornithine limitation, as indicated above.

The experiments show that after arginine is added to cells, carbamoyl phosphate was not diverted to the pyrimidine pathway at the rate it was used in citrulline synthesis. Ultimately, the carbamoyl phosphate pool fell. The experiments thus pose the question of whether the synthesis of carbamoyl phosphate is controlled by arginine in some way under these conditions. This might seen unlikely because arginine has no direct effect on carbamoyl-phosphate synthetase A *in vitro* (3) and does not cause short-term inhibition of citrulline synthesis in *sup-3*-bearing strains (16). However, no clear test of carbamoyl phosphate synthesis *in vivo* in the absence of ornithine and the conversion of both to citrulline has been offered. We must therefore consider more subtle possibilities: (i) carbamoyl-phosphate synthetase A may be activated by ornithine, with or without the participation of ornithine carbamoyltransferase (22); (ii) carbamoyl phosphate may inhibit carbamoyl-phosphate synthetase A, with or without the participation of arginine (23); and (iii) carbamoyl phosphate may continue to be made at a high rate, but, trapped in the mitochondrion, it may turn over rapidly (24) or recycle via the carbamoyl phosphokinase activity of the synthetase (25).

Accumulation of Carbomyl Phosphate in Strains Lacking Ornithine or Ornithine Carbamoyltransferase Activity—Strains with the *arg-6* and *arg-12* mutations were used to determine the effect of ornithine or ornithine carbamoyltransferase deficiencies individually upon carbamoyl phosphate accumulation and overflow. The *arg-6* mutation blocks the acetylglutamate kinase reaction (Fig. 1) (19). The *arg-12* allele used (21) is associated with a complete deficiency for ornithine carbamoyltransferase protein.[3] When starved, *arg-12*-bearing strains synthesize large amounts of ornithine in mitochondria (26). The *pyr-3M pyr-1 arg-6* and *pyr-3M pyr-1 arg-12* strains were grown on uridine plus arginine, transferred to arginine-free medium, and sampled over the next 6 h for carbamoyl phosphate and ureidosuccinate. Both strains accumulated carbamoyl phosphate and ureidosuccinate to similar extents (Figs. 5 and 6 and Table I, part B). The effect of arginine upon these cultures will be discussed below.

The data show that neither ornithine nor ornithine carbamoyltransferase is individually required for synthesis and accumulation of carbamoyl phosphate and its admittedly small overflow to the pyrimidine pathway. The amount of ureidosuccinate accumulated (~0.25–0.5 nmol/min/mg of protein) is much less than the carbamoyl phosphate-forming potential. In previous work, addition of ornithine to an arginine- and ornithine-deprived *arg-6 arg-1* strain led to the instant onset of citrulline synthesis at 2.5 nmol/min/mg of protein (16), similar to that of the *pyr-3M pyr-1* strain in minimal medium (Table I, part A).

Inhibition of Carbamoyl-phosphate Synthetase A by Carbamoyl Phosphate and Arginine—The strains carrying the *arg-6* and *arg-12* mutations both accumulate carbamoyl phosphate to a characteristic level of about 0.9–1.0 nmol/mg of cell protein in the absence of arginine. Only about 25% more accumulates if both ornithine and aspartate transcarbamylases are blocked by mutation (data not shown). It is therefore possible that carbamoyl phosphate inhibits its own synthesis.

Mitochondria occupy about 13% of the cell volume in *N. crassa* (27). If matrix volume is 10% of cell volume and one-half the matrix water is free, 5% of the cell water is available as solvent for carbamoyl phosphate. There is about 2.5 ml of cell water/g of mycelial dry weight (28), and one-quarter of the dry weight is protein.[3] Thus, cell water is 10 μl/mg of cell protein. One nmol of carbamoyl phosphate/mg of protein would represent 1 nmol of carbamoyl phosphate/0.5 μl of mitochondrial water, or 2 mM. These figures give only an order of magnitude approximation with which to appreciate the following *in vitro* experiments.

Carbamoyl-phosphate synthetase A was tested for its response to carbamoyl phosphate in the presence and absence of arginine (Fig. 7). Carbamoyl phosphate inhibited only 50% at a concentration of 10 mM. Arginine alone does not inhibit

[3] R. H. Davis and J. L. Ristow, unpublished observation.

the enzyme nor does it intensify the effect of carbamoyl phosphate (data not shown). Thus, we have no evidence that carbamoyl phosphate contributes substantially to inhibition of its own synthesis *in situ*. (The enzymatic test does not measure the recycling of the product through the carbamoyl phosphokinase activity of the enzyme. This is expected to be a minor fate of carbamoyl phosphate *in vivo* (23).)

The insensitivity of carbamoyl-phosphate synthetase A to carbamoyl phosphate *in vitro* and the immediate resumption of citrulline synthesis when ornithine is added to a starved, ornithine-deficient strain (16) suggest that when arginine is not present, carbamoyl phosphate continues to be made and turned over within the mitochondrion. A small amount is diverted to the pyrimidine pathway; but again, our data do not identify the factor that limits this fate: a restricted escape from the mitochondrion or poor trapping of carbamoyl phosphate in the aspartate carbamoyltransferase reaction.

The *pyr-3M pyr-1 arg-12* strain was used to determine the effect of arginine on carbamoyl phosphate accumulation because the strain cannot use the latter compound for citrulline synthesis. Arginine was added to a *pyr-3M pyr-1 arg-12* culture after the carbamoyl phosphate content had reached its maximum. The carbamoyl phosphate level, per ml culture volume, declined (Figs. 5 and 8). The decline was even greater on the basis of milligrams protein, owing to the resumption of growth. Beyond a slight initial excess, the rate of ureidosuccinate formation/ml culture volume remained the same (Fig. 5) or increased slightly (Fig. 8). The data indicate again that arginine does not inhibit the rate of carbamoyl phosphate formation significantly.

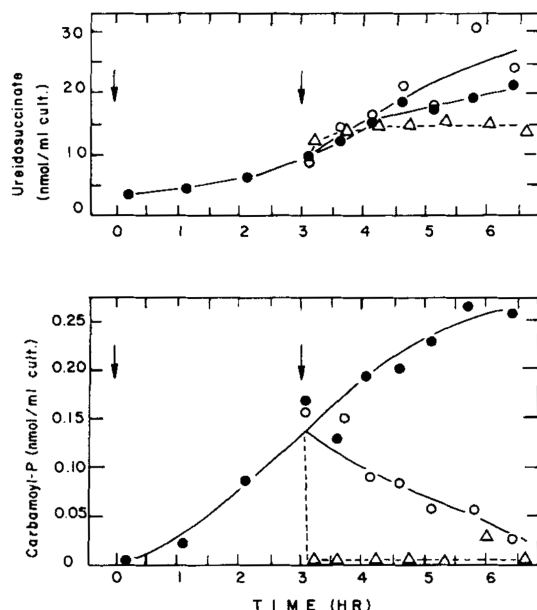

FIG. 8. **Ureidosuccinate synthesis and carbamoyl phosphate content of strain *pyr-3M pyr-1 arg-12*.** The conditions of growth, transfer, and supplementation were the same as described for Figs. 4 and 5, in which ureidosuccinate (*top*) and carbamoyl phosphate (*bottom*) are determined in cultures with (○) and without (●) arginine. In addition, a third portion of the arginine-free culture was taken at 3 h, and sodium azide (1 mM) was added (△). The dry weight of the culture at 3 h was 0.57 mg/ml, and protein was 0.16 mg/ml culture volume. Increases of protein and dry weight stopped at 3 h in the arginine-free culture and continued in the arginine-supplemented culture. A similar experiment (not shown) demonstrated that the carbamoyl phosphate pool disappeared (i) when sodium azide was added to the arginine-supplemented portion of the culture at 4 h and (ii) when oligomycin (1 μl/ml) was added to the arginine-free culture at 3 h.

Addition of lysine, which did not lead to resumption of growth, led to an increase, rather than a decrease, of the carbamoyl phosphate pool and led to a slight stimulation of ureidosuccinate formation (data not shown). The different effect of lysine and arginine on the carbamoyl phosphate pool size cannot be interpreted clearly, but may be a trivial correlate of the resumption of growth in the latter case. The carbamoyl phosphate pool is so small in relation to the rate of ureidosuccinate synthesis that a very small alteration in the balance of synthesis, consumption, and degradation would have a significant effect.

Turnover of Carbamoyl Phosphate—The synthesis and the turnover of carbamoyl phosphate cannot be measured separately with our methods. However, evidence of rapid turnover of carbamoyl phosphate during its accumulation was obtained with the mitochondrial energy poisons sodium azide and oligomycin. These compounds deprive mitochondria of ATP by different mechanisms and are therefore expected to block carbamoyl phosphate synthesis *in vivo*. When sodium azide was added to a culture of *pyr-1 pyr-3 arg-12* that had accumulated 0.9 nmol of carbamoyl phosphate/mg of protein, the carbamoyl phosphate pool was wholly lost by the first time point (12 min), and the synthesis of ureidosuccinate stopped quickly thereafter (Fig. 8). A repetition of the experiment demonstrated that this also happened 1 h after arginine addition and upon the addition of oligomycin (data not shown). Sodium azide had no effect on the slow decay of carbamoyl phosphate in solution at pH 8.5 or on the trapping system used to assay it in extracts.

The data show that carbamoyl phosphate turned over very rapidly, at least when it accumulated. Because the pool diminished only slowly after arginine was added, it follows that rapid carbamoyl phosphate synthesis continued in the presence of arginine. This is borne out by continued ureidosuccinate synthesis even while the carbamoyl phosphate pool declined (Figs. 5 and 8). Unfortunately, the rate of turnover cannot be measured and thus related to the rate of consumption in ureidosuccinate synthesis before the inhibitors were added.

DISCUSSION

Summary of Results—Our results can be summarized as follows: (i) During long-term growth in arginine, endogenous synthesis of this amino acid is limited mainly by ornithine availability in the mitochondrion. (ii) Under these conditions, carbamoyl phosphate continued to be made at a rate roughly proportional to the partially repressed specific activity of carbamoyl-phosphate synthetase A. This was shown in a strain in which the synthesis of ornithine was feedback-insensitive. (iii) Under all conditions in which carbamoyl phosphate consumption in the mitochondrion was limited, excess carbamoyl phosphate was captured inefficiently by the extramitochondrial pyrimidine enzyme, aspartate carbamoyltransferase. This included conditions under which carbamoyl-phosphate synthetase A was maximally derepressed. (iv) At the onset of feedback inhibition (when arginine was added to cells grown in its absence), cellular carbamoyl phosphate levels rose greatly, but the intermediate was not diverted efficiently to the pyrimidine pathway. (v) In ornithine- or ornithine carbamoyltransferase-deficient mycelia in which carbamoyl phosphate had established a maximal rate of escape to the pyrimidine pathway and a maximal pool size, arginine did not affect the rate of escape and only slowly diminished the pool size. (vi) Under the same conditions, addition of respiratory inhibitors that blocked ATP formation (and thus carbamoyl phosphate synthesis) led to an immedi-

ate loss of the carbamoyl phosphate pool. (vii) Arginine did not inhibit carbamoyl-phosphate synthetase A *in vitro*, and carbamoyl phosphate inhibited carbamoyl-phosphate synthetase A significantly only at concentrations above 2 mM. Arginine did not intensify the latter effect.

Channeling of Carbamoyl Phosphate—Upon arginine addition to mycelia grown in the absence of arginine, the transient, 40-fold increase in the carbamoyl phosphate pool is correlated with a rate of ureidosuccinate formation of about 6–13% the prior rate of citrulline synthesis. The rate of capture of carbamoyl phosphate does not respond to variations in aspartate carbamoyltransferase activity (see "Experimental Procedures").[3] It is therefore likely that the limiting factor in ureidosuccinate synthesis is the escape of carbamoyl phosphate from mitochondria and that the bulk of the carbamoyl phosphate we measure is confined to mitochondria.

Prior evidence for this view comes, first, from earlier experiments in which carbamoyl phosphate, generated by carbamoyl-phosphate synthetase A at rates characteristic of mycelia grown in minimal medium, was not used by aspartate carbamoyltransferase fast enough to sustain growth of a uridine-starved strain lacking carbamoyl-phosphate synthetase P of the pyrimidine pathway (10). This was true despite the presence of substantially more carbamoyl phosphate in the cell (~0.05 nmol/mg of protein) than is normally associated with normal flux of carbamoyl phosphate in the pyrimidine pathway (10). Only when carbamoyl-phosphate synthetase A is highly derepressed does it sustain the demands of the pyrimidine pathway for growth (7).

Second, in earlier work (7, 10), we studied the reciprocal overflow of pyrimidine-specific carbamoyl phosphate, diverted by a mutational block in aspartate carbamoyltransferase, *into* the mitochondrion and its use in the ornithine carbamoyltransferase reaction. Under these circumstances, sufficient carbamoyl phosphate enters mitochondria to sustain growth of a mutant lacking carbamoyl-phosphate synthetase A. The associated cellular carbamoyl phosphate pool was about 10-fold the amount normally found in the pyrimidine path, or about what is associated with a normal arginine pathway (~0.01–0.02 nmol/mg of protein). The two observations suggest that carbamoyl phosphate is not only retained by mitochondria that produce it, but may actually be taken up actively by mitochondria when it is present in the cytosol.

Synthesis and Turnover of Carbamoyl Phosphate in Mitochondrion—As noted above, cells unable to use carbamoyl phosphate in the mitochondrion fail to divert carbamoyl phosphate quantitatively to the pyrimidine pathway. Any means of blocking the use of carbamoyl phosphate in the mitochondrion leads to its accumulation to similar levels and diversion to the pyrimidine pathway at similar, slow rates. These means include feedback inhibition of ornithine synthesis, starvation of a mutant blocked in ornithine synthesis, or starvation of a mutant that lacks ornithine carbamoyltransferase. Significantly, arginine does not limit the amount of carbamoyl phosphate that escapes if it cannot be used in the mitochondrion. The insensitivity of carbamoyl-phosphate synthetase A to arginine or to physiological concentrations of carbamoyl phosphate suggests that these factors do not seriously limit the rate of carbamoyl phosphate synthesis when its utilization is blocked. The data suggest instead that carbamoyl phosphate continues to be made, rises in concentration within the mitochondrion, and turns over rapidly. However, we do not exclude the possibility that carbamoyl phosphate exerts some effect upon its synthesis. Carbamoyl phosphate is a very weak inhibitor of its synthesis, but it may be more concentrated near the enzyme *in situ*; in any case, we are uncertain about the properties of this enzyme in the mitochondrial environment.

Addition of inhibitors of the synthesis of ATP, a substrate of carbamoyl-phosphate synthetase A, led to an immediate disappearance of the carbamoyl phosphate pool. This suggests that carbamoyl phosphate is rapidly turned over when it accumulates. The conclusion is not definitive because the first samples was taken at 12 min, and in that time, residual leakage and ureidosuccinate synthesis (the latter unmeasurably small) might have depleted the entire carbamoyl phosphate pool. However, results with the inhibitors are in significant contrast to the effect of arginine, which leads only to a very slow decline. The contrast suggests again that arginine does not block carbamoyl phosphate synthesis.

Control of Mitochondrial Carbamoyl Phosphate Metabolism—The simplest interpretation of our data is that upon addition of arginine to cells, citrulline synthesis is severely inhibited, owing to the deprivation of ornithine. Carbamoyl phosphate, confined by the mitochondrial membrane, accumulates rapidly and turns over, and its synthetic rate is not greatly inhibited. The accumulation of carbamoyl phosphate in the mitochondrion leads to a small amount of leakage into the cytosol from which it is used (in the nucleus) for pyrimidine synthesis.

In wild-type cells, this state can change in two ways. First, the entry of ornithine (derived by catabolism) into the mitochondrion (17) or the reinitiation of ornithine synthesis within the mitochondrion (15) will lower the pool of carbamoyl phosphate and prevent its further leakage to the cytosol. Second, repression of carbamoyl-phosphate synthetase A during the next several doublings of mass in the presence of arginine (5, 16) will lower the rate of synthesis of carbamoyl phosphate. Combined with the increasing efficiency of excluding ornithine from the mitochondrion (17, 18), citrulline synthesis will fall to 1% of the level characteristic of minimal grown cells. It requires at least 8 h of growth of Arg^+ mycelia to achieve this state (17). At steady state, synthesis and degradation of carbamoyl phosphate still continue within mitochondria. The advantage of incomplete repression and lack of feedback inhibition of carbamoyl-phosphate synthetase A may lie in the ease with which citrulline synthesis can resume once arginine is no longer in excess. This point has been made by Goodman and Weiss (29) in their studies of the control of arginine synthesis in feedback-sensitive and feedback-resistant strains of *N. crassa*. Because *N. crassa* normally grows in a carbon-rich environment, energy conservation may not have been paramount in the evolution of this apparently wasteful metabolic system.

A discussion of related work on *N. crassa*, *Saccharomyces cerevisiae*, and ureotelic vertebrates is included in the Miniprint.

Acknowledgments—We thank Drs. Luisa Raijman, Natalie Cohen, and Dr. Richard L. Weiss for discussion of this work and criticism of the manuscript. We thank Dr. Weiss for antiserum against *N. crassa* ornithine carbamoyltransferase, with which the deficiency of this protein in our *arg-12* mutant was established.

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SUPPLEMENTAL MATERIAL TO

Arginine-Specific Carbamoyl Phosphate Metabolism in Mitochondria of *Neurospora crassa*. Channeling and Control by Arginine

Rowland H. Davis and Janet L. Ristow

This section contains the Experimental Procedures, Figures 2, 3, 5, 6 and 7 of the RESULTS, and a final portion of the DISCUSSION.

EXPERIMENTAL PROCEDURES

Strains. The mutations of *N. crassa* used in this study are placed on the metabolic map in Fig. 1 (see main text), and strains are referred to by their genotypes. The pyrimidine pathway mutations were *pyr-3M*, allele DFC8 (30), lacking carbamoyl phosphate synthetase P activity (but retaining aspartate carbamoyltransferase activity), and *pyr-1*, allele DFC33 (30), lacking dihydroorotic acid oxidase activity. The arginine-pathway mutations, also shown in Fig. 1, were *arg-1*, allele CD145 (21), lacking argininosuccinate synthetase activity; *arg-3*, allele CD186 (21), lacking carbamoyl phosphate synthetase A activity (large subunit); *arg-6*, allele CD29 (21), lacking acetylglutamate kinase activity (31); *arg-12*, allele CD109 (21), lacking ornithine carbamoyltransferase activity and protein; *arg12^{s}*, (no allele number) (20, 32), having a severe (95-98%) deficiency in ornithine carbamoyltransferase activity, but prototrophic; and *sup-3* (no allele number) (14), which confers feedback resistance on acetylglutamate kinase, an early enzyme of ornithine synthesis (6). The *al-2* allele 15300 (albino conidia) was combined with the *sup-3* mutation to follow the latter more easily in crosses. The strain designations (not used in the text) for multiple mutants constructed in the authors' laboratory are as follows: IC-31, *pyr-3M pyr-1*; IC-32, *pyr-3M pyr-1 arg-1*; IC-33, *pyr-3M pyr-1 arg-3*; IC-34, *pyr-3M pyr-1 arg-12*; IC-35, *pyr-3M pyr-1 arg-12^{s}*; IC-49, *pyr-3M pyr-1 sup-3 al-2*; IC-50, *pyr-3M*, *pyr-1*, *arg-6*. They are available to individual investigators on request for research purposes. Strains carrying most of the individual mutations are available from the Fungal Genetics Stock Center, Department of Microbiology, University of Kansas Medical Center, Kansas City, KS 66103.

Growth. Growth and maintenance of stock cultures was done in Vogel's medium N (33), supplemented with 200 µg L-arginine.HCl and/or 100 µg uridine per ml as appropriate for the auxotrophic mutations. Growth of exponential cultures for enzyme and metabolite determinations was done in 1 l boiling flasks, aerated with forced air and maintained at 25°C as described earlier (16, 34). Transfers of cultures to a second medium was accomplished by gentle filtration and washing in sterile water, taking care not to compress the pad on the filter. The mycelia, resuspended to half their original volume in water, were diluted 1:1 with double-strength, fresh medium. Samples (10 to 20 ml) for dry weight were taken with a wide-bore pipette, filtered on 24-mm filter circles (Whatman #540) and dried immediately with acetone.

Carbamoyl phosphate synthetase A assay. Samples (at least 200 ml) for carbamoyl phosphate synthetase A assays were taken and rinsed on a filter circle (Whatman #1) in a Buchner funnel, sand ground in buffer (10 mM TES, 1 mM EDTA, 0.33 M sucrose and 3% bovine serum albumin, pH 7.5) designed to stabilize organelles osmotically. Mitochondria were isolated from the homogenate as described previously (35). Mitochondria were used for carbamoyl phosphate synthetase A assay. Carbamoyl phosphate synthetase A assay was performed on whole mitochondria in reactions containing Triton X-100 as described earlier (3). Protein was determined by the method of Lowry *et al.* (36), using ovalbumin as a standard. Specific activities are expressed as nmole per hour per mg mitochondrial protein at 25°C.

Metabolite determinations. Samples for amino acid and pyrimidine intermediate determination (20 - 50 ml) were filtered and washed with water on 25-mm membrane filters (Metricel, 5 µm pore size, Gelman Co.). Mycelia were transferred with a spatula to 2 ml 5% trichloroacetic acid, extracted for at least 20 min, and centrifuged. The supernatants were used for metabolite determinations. Arginine, ornithine and citrulline were determined after fractionation on multiple, short AG-50W cation exchange columns as described earlier (16). Ureidosuccinate and dihydroorotate, which accumulate in some conditions in the strains used (see below), were determined as ureidosuccinate after converting the dihydroorotate to ureidosuccinate with alkali, as follows. To 1-ml samples of the trichloroacetic acid extract, 100 µl of 9M NaOH were added and incubated for at least 20 min. They were then passed through columns (3 x 0.7 cm) of AG-50W-H^{+} resin, 200-400 mesh, to remove Na^{+}. The eluate plus 1 ml water wash was collected, and ureidosuccinate (representing the sum of ureidosuccinate + dihydroorotate of the original sample) was determined by the method of Pierson (37). When, as was often the case, greater purity was required, the AG-50 column eluate was loaded onto a column of AG-1-OH^{-} (2 x 0.7 cm). The column was washed with 3 ml water, 10 ml 0.1 M sodium formate, pH 3.5, and 1 ml 1 M formic acid. Ureidosuccinate was then eluted with 4 ml additional 1 M formic acid. Samples were evaporated, redissolved in 1 ml and used for determination of ureidosuccinate (37). Recovery of ureidosuccinate was over 92%. Controls established the complete conversion of dihydroorotate to ureidosuccinate in the early alkali step. Total protein was determined (36) on the sedimentable material from the trichloroacetic acid extract after dissolving it in 1 N NaOH.

Carbamoyl phosphate was determined by a method originated by Williams *et al.* (10), and refined by Pausch *et al.* (38). The purpose was to extract carbamoyl phosphate rapidly, convert it enzymatically to [^{14}C]citrulline, and measure the radioactivity. Samples for determination (50 ml), representing 4 - 15 mg dry weight of mycelium, were taken and washed on membrane filters, and transferred to a 1.5-ml Eppendorf tube containing 0.7 ml 0.4 M $KClO_4$. The suspension was mixed with a spatula, centrifuged for 2 min, and processed immediately. The supernatants were transferred to another Eppendorf tube containing approximately 150 mg of damp AG-50-H^{+} cation exchange beads, 200-400 mesh, and centrifuged for 1 min. This removed most of the endogenous ornithine from the samples. A sample (500 µl) of the supernatant was placed in another Eppendorf tube

containing 140 ul 1.6 M $KHCO_3$, to bring the pH to 8.5. The tube was mixed well and centrifuged for 3 min. Of the supernatant, 550 µl (0.86 of the original sample) was put into 100 µl of ornithine carbamoyltransferase reaction mixture ingredients containing 20 nmoles L-ornithine, 2 µl of L-[1-^{14}C]ornithine (ca 8 x 10^4 cts/min), 1 unit of Streptococcus faecalis ornithine carbamoyltransferase (Sigma Chem. Co., St. Louis, MO), 38 µmol Tris buffer, the whole adusted to pH 8.5. The reaction proceeded for 1 hr at 37° to exhaust carbamoyl phosphate, and was stopped by addition of 295 µl 1M citric acid adjusted to pH 4.0 with NaOH. The final pH was about 5.0; the reaction mixture (1 ml) was loaded on short AG-50-Na^+ columns (0.7 x 3 cm, 200-400 mesh) equilibrated with 0.116 M sodium citrate (adjusted to pH 5.3 with HCl). The flow-through plus 2 additional ml, eluted with the equilibrating buffer, contained the radioactive citrulline. Two ml of this were counted in a scintillation counter.

A further 7 ml was eluted from the column with the 0.116 M sodium citrate buffer. This represented the radioactive ornithine used as a substrate for the enzymatic reaction. It was necessary to determine its specific radioactivity because in many cases where ornithine was allowed to accumulate in cells, it was not fully removed from the cell extracts by treatment with cation exchange beads. It therefore reduced the specific radioactivity of the substrate. Five of the 7 ml of the ornithine fraction were bound on a column (0.7 x 7.5 cm) of AG-50-H^+, which was eluted successively with 5 ml 0.1 M HCl, 10 ml 1.5 M HCl and 5 ml 6 N HCl. The last fraction, containing ornithine, was evaporated to dryness, rehydrated and used for determination of radioactivity by scintillation counting and for colorimetric determination of ornithine by the method of Chinard (39). The specific activity of ornithine was used to convert the citrulline radioactivity to umoles; the latter was the measure of carbamoyl phosphate converted in the reaction mixture.

The standards used in the determinaton of carbamoyl phosphate were a set of mycelial pads of the carbamoyl phosphate synthetase A- and carbamoyl phosphate synthetase P-deficient strain, pyr-3M pyr-1 arg-3, in 700 µl $HClO_4$, to which 0, 1, 2, or 5 nmol freshly dissolved dilithium carbamoyl phosphate monohydrate had been added. These standards were always processed in parallel with the experimental samples. Recovery of carbamoyl phosphate was consistently about 40% in the range of values encountered in our samples, and experimental values were corrected accordingly. The major loss occurred in the step in which endogenous ornithine was removed from the extracts by ion exchange.

In Arg^+ strains, which do not accumulate intermediates before a genetic block, calculation of the rate of citrulline and carbamoyl phosphate synthesis was based on parameters of the steady-state cultures. The pertinent determinations are the size of the arginine pool and the amount of protein arginine per mg dry weight or protein; the citrulline and argininosuccinate pools are insignificant in such cultures. The calculation used the exponential growth equation and the generation time in order to determine the rate of carbamoyl phophate (and citrulline) synthesis per minute required to maintain steady state. This is explained in detail in Ref. 11.

Trapping of carbamoyl phosphate in the aspartate carbamoyltransferase reaction in vivo. The strains used all carried the pyr-3M and pyr-1 mutations, which imposed deficiencies for carbamoyl phosphate synthetase P and dihydroorotate oxidase, respectively (Fig. 1, main text). Because the strains retained aspartate carbamoyltransferase and dihydroorotase, carbamoyl phosphate diverted from the mitochondrion could be trapped by these enzymes, the first of which is normally situated in the nucleus (9). We took the accumulation of ureidosuccinate and dihydroorotate to signify escape of carbamoyl phosphate from the mitochondrion, but we do not know the efficiency of this trapping system. Several pertinent points can be made, however. First, in strains derepressed for carbamoyl phosphate synthetase A and impaired in ornithine carbamoyltransferase (e.g., pyr-3M pyr-1 arg-12^S), substantial diversion of carbamoyl phosphate was seen. Derepression of aspartate carbamoyltransferase in these strains had no effect on the amount of ureidosuccinate and dihydroorotate accumulated. Second, the addition of aspartate to the medium had no effect on metabolite accumulation in the same strains. Finally, the amount of ureidosuccinate and dihydroorotate accumulated in strains derepressed for carbamoyl phosphate synthetase A was several fold that seen in our experiments, which concentrated on situations in which carbamoyl phosphate was made (by carbamoyl phosphate synthetase A in the mitochondrion) at modest levels. In this work, therefore, we have assumed that the amount of ureidosuccinate and dihydroorotate accumulated is positively correlated with, but not necessarily equal to, escape of carbamoyl phosphate from the mitochondrion.

Materials--Most biochemical reagents and ion exchange materials were obtained from Sigma Chemical Co., St. Louis, MO. L-[1-^{14}C]-L-ornithine was obtained from Amersham Corporation, Arlington Heights, Illinois.

RESULTS

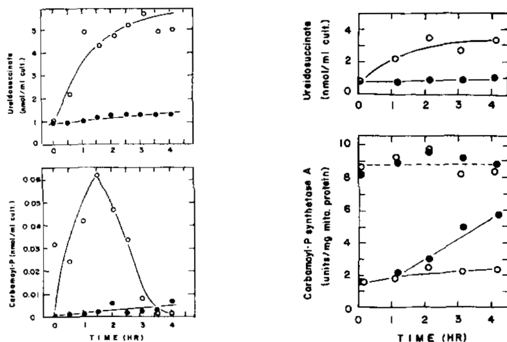

FIG. 2 (Left). Ureidosuccinate synthesis and carbamoyl phosphate content of the pyr-3M pyr-1 strain. The mycelium was grown in the presence of uridine to 0.4 mg dry weight per ml, and the culture was divided into two portions at zero hours. One continued growth in the same medium (closed circles). The other received arginine at a final concentration of 1 mM (open circles). Ureidosuccinate (top) and carbamoyl phosphate (bottom) were measured periodically thereafter. The generation time of both cultures was approximately 3 hr, and the cellular protein was approximately 0.25 mg per mg dry weight. Data from this experiment in terms of protein are included in Table 1.

FIG. 3 (Right). Carbamoyl phosphate synthetase A activities and ureidosuccinate synthesis in the pyr-3M pyr-1 strain and their response to arginine. At zero hours, a uridine-supplemented culture of pyr-3M pyr-1 was divided into two portions. One continued growth in the presence of uridine alone (closed circles). Arginine was added to the other portion at a final concentration of 1 mM (open circles). Ureidosuccinate (top) and carbamoyl phosphate synthetase A activities in mitochondria (bottom) were measured thereafter. In the bottom panel, the solid lines show the glutamine-dependent activity of the enzyme; the broken lines show the ammonia-dependent activity of the enzyme, catalyzed by its large subunit.

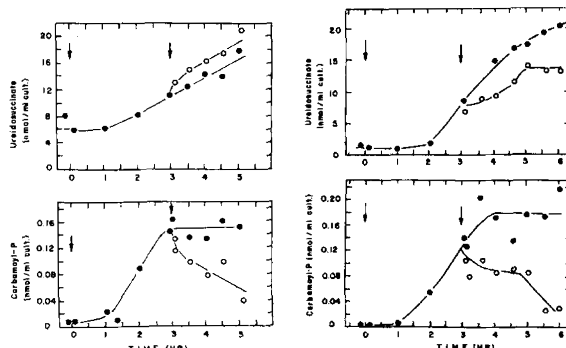

FIG. 5. (Left) Ureidosuccinate synthesis and carbamoyl phosphate content of the pyr-3M pyr-1 arg-12 strain. A culture of the strain was grown in arginine- and uridine-supplemented medium until time zero. It was harvested, washed and resuspended in uridine-supplemented, arginine-free medium (closed circles) at time zero (left arrow). At three hours (right arrow), arginine was added to a portion of the culture (open circles) to a final concentration of 1 mM. Ureidosuccinate (top) and carbamoyl phosphate (bottom) were measured in samples of culture and expressed as nmol per ml culture volume. The dry weight of the culture at 3 hours was 0.6 mg per ml, and protein was 0.18 mg per ml culture volume. Increase of protein stopped at 3.5 hr in the arginine-free culture, and continued in the arginine-supplemented culture. Data from this culture in terms of protein are also reported in Table 1.

FIG. 6 (Right). Ureidosuccinate synthesis and carbamoyl phosphate content of the pyr-3M pyr-1 arg-6 strain. The conditions and symbols are the same as in Fig. 5. The dry weight of the culture at 3 hours was 0.7 mg per ml, and protein was 0.18 mg per ml culture volume. Increase of protein stopped at 3.1 hr in the arginine-free culture, and continued in the arginine-supplemented culture. Data from this culture in terms of protein are also reported in Table 1.

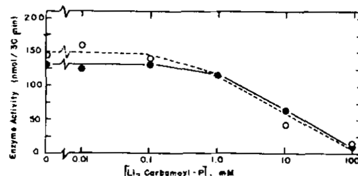

FIG. 7. Response of carbamoyl phosphate synthetase A to carbamoyl phosphate added to the reaction mixture. Mitochondria were assayed for carbamoyl phosphate synthesis by incorporation of radioactive label into the product, carbamoyl phosphate, from labelled substrate, $H^{14}CO_3^-$. Open circles, glutamine-dependent activity; solid circles, ammonia-dependent activity.

DISCUSSION

Related Studies in N. crassa--Recently, Goodman and Weiss (29), using radioactive tracer techniques, reported that ornithine could be made by their feedback-resistant strain, growing exponentially in arginine-containing medium, at about 60 - 70% of the rate seen in minimal medium. The extent of inhibition was consistent with the residual sensitivity of the mutant acetylglutamate kinase to arginine. (The feedback-resistance mutation was the same allele as the one we have used here.) While arginine synthesis was not specifically measured, Goodman and Weiss' report suggests that carbamoyl phosphate was being used at about 60 - 70% of the rate normally seen in minimal medium. This was a higher figure than we have determined in the present work (10%). The discrepancy may be owing to the different physiological ages of the cultures used by us and by Goodman and Weiss (29), or to the temperature of growth (25 and 30°C, respectively). Goodman and Weiss' experiments, together with the fact that carbamoyl phosphate synthetase A repression diminishes as cultures mature (Table 1), suggest that carbamoyl phosphate synthesis and turnover will increase with time in normal, feedback-sensitive cultures grown in the presence of arginine.

The continued synthesis of ornithine by strains carrying the feedback-resistance mutation, sup-3, is unexpected. This is because there is another feedback-sensitive step in the pathway, acetylglutamate synthase (EC 2.3.1.1) (6). This enzyme catalyzes the anaplerotic contribution of acetyl groups to the cyclic pathway. The enzyme should be vital to long-term ornithine synthesis, even if the requirement were not immediately asserted upon exposure of cells to arginine. In fact, mutants of the arg-14 locus that lack this enzyme activity are tight auxotrophs (6). One might speculate that the initial continuation of arginine-resistant ornithine synthesis in the sup-3 strains interferes with the inhibition by arginine of the synthase; possibly the synthase and kinase are coordinated physically in their reaction to arginine. Whatever the reason, it appears that the sup-3 mutation, genetically inseparable from the kinase-determining locus, arg-6, is sufficient to confer feedback resistance upon ornithine synthesis.

Carbamoyl Phosphate Metabolism in Saccharomyces cerevisiae-- It is illuminating to compare carbamoyl phosphate metabolism in N. crassa and S. cerevisiae (reviewed in Ref. 1). In the latter organism, the pyrimidine enzymes are similar in location to those of N. crassa (40). However, carbamoyl phosphate synthetase A and ornithine carbamoyltransferase in S. cerevisiae are both cytosolic, not mitochondrial (41). This is correlated *with the ability of carbamoyl phosphate synthetase* A to satisfy easily the demands of the pyrimidine pathway as well as those of the arginine pathway in unsupplemented medium (42). Repression (mediated by both the arginine and general amino acid systems) is the only negative control mechanism known to act on carbamoyl phosphate synthetase A of S. cerevisiae (1); arginine does not inhibit the enzyme itself (43). Arginine-mediated repression of carbamoyl phosphate synthetase A in S. cerevisiae is severe enough to block growth if the pyrimidine pathway is made dependent (through mutation of carbamoyl phosphate synthetase P) upon the arginine-controlled synthetase (42).

An interesting difference in pyrimidine enzyme control prevails between the two organisms: in S. cerevisiae aspartate carbamoyltransferase, like carbamoyl phosphate synthetase P, is feedback-inhibited by UTP (44), whereas in N. crassa, aspartate carbamoyltransferase is not feedback inhibited (8). The difference is correlated with the fact that the product of carbamoyl phosphate synthetase A can easily bypass feedback inhibition of carbamoyl phosphate synthetase P in S. cerevisiae, but the product of carbamoyl phosphate synthetase A, confined in mitochondria, cannot do so in N. crassa.

Relation to ureotelic organisms-- In ureotelic organisms, the diet is nitrogen-rich. Carbamoyl phosphate is consumed in the ornithine cycle and discharged in the form of the excretory product, urea. In such systems, the cycle enzymes are localized as they are in N. crassa. However, unlike N. crassa, ureoteles allow ornithine to pass across the mitochondrial membrane, and this is only weakly inhibited by arginine (45). Arginine and other amino acids stimulate carbamoyl phosphate synthetase I by their ability to generate ammonia (the substrate of carbamoyl phosphate synthetase I) (46), and more crudely in certain circumstances by stimulating the synthesis of acetylglutamate, the cofactor of the enzyme (47, 48; see, however, 46, 49). Clearly, feedback inhibition by arginine would be maladaptive in ureoteles. Despite the differences between N. crassa and ureoteles, some interesting comparisons can be made. For instance, if ornithine is insufficient, or if carbamoyl phosphate is made in excess, the latter is diverted copiously to the pyrimidine pathway in intact organisms and in cell cultures (50). It is not possible to judge what fraction of the carbamoyl phosphate-forming potential is actually *realized in terms of ureidosuccinate synthesis*, but the phenomenon resembles the case of the ornithine carbamoyltransferase-deficient arg-12[s] strain of N. crassa (7).

A more pertinent parallel can be made between N. crassa and ureotelic systems: in both, the mitochondrial membrane is a partial barrier to carbamoyl phosphate escape. In tests with isolated mitochondria, this led to a temporary rise in intramitochondrial carbamoyl phosphate when it was synthesized in the absence of ornithine (51). The amount of carbamoyl phosphate made in the absence of ornithine was 12-fold less than when ornithine was added to trap carbamoyl phosphate as citrulline. Controversy has surrounded this observation for some time. Three explanations have been considered. First, carbamoyl phosphate might inhibit its own synthesis. This explanation is inadequate, because carbamoyl phosphate synthetase I, like the carbamoyl phosphate synthetase A of N. crassa, is only weakly inhibited by carbamoyl phosphate (47), and the intramitochondrial carbamoyl phosphate concentrations, at most, are not enough to explain the inhibition (23, 52). Another inadequacy of the product-inhibition idea is that the increase in the rate of carbamoyl phosphate synthesis when it was trapped as citrulline (i.e., 12-fold with ornithine added) *is not correlated, at steady state, with a reduced* intramitochondrial carbamoyl phosphate concentration (51). All considerations of the product-inhibition hypothesis, however, are compromised by our ignorance of the behavior of carbamoyl phosphate synthetase in the peculiar environment of the mitochodrial matrix.

A second explanation of the slow rate of carbamoyl phosphate production in mammalian mitochondria when it is not used for citrulline synthesis is that it turns over rapidly. Two mechanisms have been suggested. One is that the carbamate kinase activity of carbamoyl phosphate synthetase I would use one mole of carbamoyl phosphate for synthesis of one mole of ATP, thus dissipating the additional mole of ATP needed to synthesize each mole of carbamoyl phosphate (25). This view is not consistent with a more thorough analysis of the flux to citrulline in relation to intramitochondrial carbamoyl phosphate concentrations. Idling of carbamoyl phosphate synthetase I is not accounted for in any case in other laboratories by measurements of ATP synthesis from carbamoyl phosphate or by oxygen consumption[4]. A second form of this hypothesis is that a catabolic activity in mitochondria, with a low affinity for carbamoyl phosphate, might degrade carbamoyl phosphate more rapidly as its intramitochondrial concentration rises (24). Preliminary evidence for such an activity was obtained (24), and we favor this mechanism as a possible explanation of our results.

A third hypothesis has been provoked by the inadequacies of those offered previously. It was suggested that ornithine stimulates carbamoyl phosphate synthetase A, an effect mediated by the ornithine carbamoyltransferase protein (22). Evidence in support of this hypothesis is as yet inadequate to prove it (23). Our own data virtually eliminate this hypothesis in the case of N. crassa. Mitochondria lacking the ornithine carbamoyltransferase protein would greatly aid in assessing this notion in the case of mammalian mitochondria.

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[4] L. Raijman, personal communication.