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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 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 nucleus (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). There is no evidence that the synthesis of carbamoyl phosphate depends upon the integrity of ornithine synthetic enzymes (13). 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 intramitochondrial 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 arginine, 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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† To whom reprint requests should be addressed.

1 J. J. DiGangi and R. H. Davis, unpublished observations.


TABLE I

<table>
<thead>
<tr>
<th>Strain (genotype)</th>
<th>Condition*</th>
<th>Carbamoyl-P synthetase A activity</th>
<th>Carbamoyl phosphate content</th>
<th>Synthetic rate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>units/mg mitochondrial protein</td>
<td>nmol/mg cell protein</td>
<td>US'</td>
</tr>
<tr>
<td>A. Steady states</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pyr-3M pyr-1</td>
<td>Ur + Arg</td>
<td>0.3-1.0</td>
<td>0.01</td>
<td>2.6</td>
</tr>
<tr>
<td>pyr-3M pyr-1 arg-1</td>
<td>Ur + Arg</td>
<td>0.5-1.2</td>
<td>0.01</td>
<td>0.03</td>
</tr>
<tr>
<td>pyr-3M pyr-1 arg-1 sup-3</td>
<td>Ur + Arg</td>
<td>0.3-1.3</td>
<td>0.02</td>
<td>0.25</td>
</tr>
<tr>
<td>pyr-3M pyr-1 arg-12</td>
<td>Ur + Arg</td>
<td>0.6-2.2</td>
<td>0.06</td>
<td>0</td>
</tr>
<tr>
<td>pyr-3M pyr-1 arg-12*</td>
<td>Ur</td>
<td>35.0</td>
<td>0.9</td>
<td>1.28</td>
</tr>
</tbody>
</table>


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           |
| pyr-3M pyr-1 arg-12| U (starved)| 6-2                               | 0.6 (pk)                    | 0             |
| pyr-3M pyr-1 arg-12* | U       | 3-6 (pk)                          | 0.9                         | 0.25          |

*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.

**Cit**, citrulline; **pk**, peak value; **ND**, not determined.

**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

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 dihydroorotate 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 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
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-12" 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-12" strain, was grown in arginine-free medium. Its calculated rate of citrulline synthesis was about one-half normal (Table I, part A). This strain was treated 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-12" 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-12" (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 mmol/min/mg of cell protein, compared to the prior rate of citrulline synthesis (before addition of arginine) of 2.6 mmol/min/mg (Table I, part A). The carbamoyl phosphate pool at its peak, 0.45 mmol/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 in 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
A culture of the strain was grown in arginine- and uridine-supplemented medium until time 3. It was harvested, washed, and resuspended in uridine-supplemented, arginine-free medium at time 0. At 3 h (right arrow), arginine was added to a portion of the culture (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 carbamoyl phosphate and is much less than the carbamoyl phosphate-forming activity in the experiment with the pyr-3M pyr-1 strain in minimal medium (Table I, part A). Data from this culture in terms of protein are reported in Table I.

The experiments show that after arginine is added to cells, carbamoyl phosphate is synthesized 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 the rate of citrulline synthesis in the control. Only about 25% more carbamoyl phosphate was accumulated than the rate of citrulline synthesis in the absence of arginine (23). The amount of ureidosuccinate accumulated (0.26–0.5 nmol/min/mg of protein) is much less than the carbamoyl phosphate-forming potential. In previous work, addition of ornithine to an arginine- and arginine-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).

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 seem 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 Carbamoyl 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. 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 admittance small overflow to the pyrimidine pathway. The amount of ureidosuccinate accumulated (~0.26–0.5 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 instantaneous 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. This is therefore possible that carbamoyl phosphate inhibits its own synthesis. Mitochondria occupy about 15% 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. 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 ornithine carbamoyltransferase activity.

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.)

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 rate: 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.

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 turnover is very rapid, at least when it is 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”). 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 mutual 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 ornithine 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 Mini-print.

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REFERENCES

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CONTAINING 14C-BL GLYCOLYTIC ACIDS, TO BRING THE pH TO 8.2. THE TUBE WAS MIXED WELL AND CENTRIFUGED FOR 3 MIN. OF THE SUPERNUCLEUS, 150 ML (3.45 OF THE ORIGINAL SAMPLE) WAS TAKEN OFF AND DETERMINED SOLUTIONS TESTING FOR CARBAMOYL PHOSPHATE SCAFFOLD, 92 ML OF THE SOLUTION CONTAINING 2 AMOUNTS OF PHOSPHATE-DEPENDENT ACTIVITY AND 1.44 ML OF CARBAMOYL PHOSPHATE AS SUBSTRATE, TAKEN TO THE PHOSPHATE ASCORBATE BUFFER, THE TUBE WAS INCUBATED AT 37° FOR 1 HR TO ENSURE CARBAMOYL PHOSPHATE DIHYDROOROTASE ACTIVITY. THE TUBE WAS THEN CENTRIFUGED AT 5000 RPM FOR 1 HR. DIHYDROOROTASE ACTIVITY WAS DETERMINED ON A LID, WHICH WAS 1000 TIMES GREATER IN SCALE-PHASED COLUMNS (7.5 X 1 CM) 250-800 METRES IN MEDIUM SCAFFOLDED (5.5 WITH HC). THE 2-HOUR PHASE 2 ADDITIONAL ML, THE 2-HOUR PHASE 2 WITH THE 2-HOUR PHASE 2 CONTAINED THE RADIOACTIVE CIRCULATION. 2 ML OF THIS WAS COUNTED IN A SCINTILLATION COUNTER.

A FURTHER 7 ML WAS ELUTED FROM THE COLUMN WITH THE 0.166 M UREA CITRATE BUFFER. THIS WAS THE SOLUTION WHICH RADIOLYSIS OF THE CARBAMOYL PHOSPHATE SYNTHETASE AS SUBSTRATE FOR THE PHOSPHATE SYNTHETASE. IT WAS NEEDED TO DETERMINE ITS SPECIFIC RADIOACTIVITY BECAUSE IN MANY CASES ATOMIC WERE ABSORBED. THESE ASPECTS WERE ALSO PROCESSED IN PARALLEL WITH THE EXPERIMENTAL SAMPLING. CARBAMOYL PHOSPHATE WAS THERMOGENIC IN A RANGE OF VALUES ACCORDING TO OUR SAMPLES, AND EXPERIMENTAL VALUES WERE CORRECTED ACCORDINGLY. THE MAJOR LOW SENSITIVITY TO UREA CITRATE WERE EXTRACTED FROM THE PROPER AS ION EXCHANGE.

Control of Carbamoyl Phosphate Metabolism

Carbamoyl Phosphate Metabolism in Saccharomyces cerevisiae—It is illuminating to compare carbamoyl phosphate metabolism in S. cerevisiae and E. coli (reviewed in Ref. 1). In the latter organism, the pyrimidine enzymes are similar in location to those of S. cerevisiae (24). However, carbamoyl phosphate synthetase I and ornithine carbamoyltransferase in S. cerevisiae are both cytosolic, not mitochondrial (41). This is correlated with the ability of carbamoyl phosphate synthetase I to satisfy nearly all demands of the pyrimidine pathway as well as those of the arginine pathway in unsupplemented medium (43). Repression (mediated by both the arginine and general amino acid systems) is the only negative control mechanism known for carbamoyl phosphate synthetase I in S. cerevisiae (23). Arginine does not inhibit the enzyme itself (43). Arginine-mediated repression of carbamoyl phosphate synthetase I is correlated with the ability to generate ammonia in S. cerevisiae (40). The difference is correlated with the fact that the product of carbamoyl phosphate synthetase I can easily bypass feedback inhibition of carbamoyl phosphate synthetase I in S. cerevisiae. However, the product of carbamoyl phosphate synthetase I, carbamoyl phosphate, cannot do so in E. coli.

Relation to ureotelic organisms—In ureotelic organisms, the diet is nitrogen-rich. Carbamoyl phosphate is consumed in the urea cycle and discharged in the form of the secretory product, urea. In such systems, the cycle enzymes are localized as they are in S. cerevisiae. However, unlike S. cerevisiae, ureases allow urea to pass across the mitochondrial membrane, and this is only weakly inhibited by ATP (44). As in S. cerevisiae, arginine and other amino acids stimulate carbamoyl phosphate synthetase I by their ability to generate ammonia. However, unlike S. cerevisiae, arginine does not inhibit carbamoyl phosphate synthetase I (45), and more slowly in certain circumstances by stimulating the synthesis of carbamoyl phosphate synthetase I (46). Clearly, feedback inhibition by arginine would be negligible in ureasates. Despite the differences between E. coli and S. cerevisiae, the basic mechanism of carbamoyl phosphate synthetase I is probably the same in both. Feedback inhibition by arginine must be considered. However, the degree of feedback inhibition cannot be judged. For example, if arginine is insufficient, or if carbamoyl phosphate is made rapidly, the enzyme is derepressed to the pyrimidine pathway in intact organisms and in cell cultures (46). It is not possible to judge what fraction of the carbamoyl phosphate synthetase I is actually realized in terms of urea/acidic ornithine, but the phenomenon resembles the use of the urea/acidic ornithine-deficient auxotrophic strain of E. coli (47).

A more pertinent parallel can be made between S. cerevisiae and ureotelic systems. In both, the mitochondrial membrane is a partial barrier to the flux of carbamoyl phosphate into the cycle. Therefore, feedback inhibition by UTP leads to a temporary rise in intramitochondrial carbamoyl phosphate when it is not used for citrulline synthesis. The rise of feedback inhibition by UTP is correlated with the fact that carbamoyl phosphate made in the absence of ornithine was 1.5-fold less than when ornithine was added to trap carbamoyl phosphate. Carboxylase controversy has surrounded this observation for some time. Three explanations have been considered. First, carbamoyl phosphate might inhibit the urea synthesis. This explanation is inadequate, because carbamoyl phosphate synthetase 1, like the carbamoyl phosphate synthetase I of E. coli, is only weakly inhibited by carbamoyl phosphate (47), and the intramitochondrial carbamoyl phosphate concentrations, at most, are not enough to explain the inhibition (47, 50). 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., 1.5-fold with ornithine added) is not correlated, at steady state, with the carbamoyl phosphate synthetase I concentration (51). All considerations of the product-inhibition hypothesis, however, are compromised by our ignorance of the behavior of ornithine synthetase in the peculiar environment of the mitochrondial 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 proposed. One is that the 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 supported by our own studies through analysis of the flux to citrulline in relation to intramitochondrial carbamoyl phosphate concentrations. Evidence of ornithine synthesis (17) is accounted for in any case by other laboratories by measurement of ATP synthesis from carbamoyl phosphate or by oxygen consumption. A more plausible hypothesis is that a catalytic 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 activity was obtained (24), and we favor this mechanism as a possible explanation of our results.

A third hypothesis has been provided by the inadequacies of those offered previously. It was suggested that ornithine synthetase requires an auxiliary phosphatase synthetase I, as effect mediated by the ornithine synthetase I and ornithine carbamoyltransferase systems (24). Evidence in support of this hypothesis is as yet inadequate to prove it (23). Our data virtually eliminate this hypothesis to the case of S. cerevisiae, where the ornithine synthetase I and ornithine carbamoyltransferase systems would greatly aid in assessing this notion in the case of mammalian mitochondria.