Independent Localization and Regulation of Carbamyl Phosphate Synthetase A Polypeptides of Neurospora crassa

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Summary. Carbamyl phosphate synthetase A is a two-polypeptide, mitochondrial enzyme of arginine synthesis in Neurospora. The large subunit is encoded in the \textit{arg}-3 locus and can catalyze formation of carbamyl-P with ammonia as the N donor. The small subunit is encoded in the unlinked \textit{arg}-2 locus and imparts to the holoenzyme the ability to use glutamine, the biological substrate, as the N donor. By using nonsense mutations of \textit{arg}-3, it was shown that the small subunit of the enzyme enters the mitochondrion independently and is regulated in the same manner as it is in wild type. Similarly, \textit{arg}-2 mutations affecting the small subunit, have no effect on the localization or the regulation of the large subunit. The two subunits are regulated differently. Like most polypeptides of the pathway, the large subunit is not repressible and derepresses 3- to 5-fold upon arginine-starvation of mycelia. In contrast, the glutamine-dependent activity of the holoenzyme is fully repressible and has a range of variation of over 100-fold. In keeping with this behavior, it is shown here that the small polypeptide, as visualized on two-dimensional gels, is also fully repressible. We conclude that the two subunits of the enzyme are localized independently, controlled independently and over different ranges, and that aggregation kinetics cannot alone explain the unusual regulatory amplitude of the native, two-subunit enzyme. The small subunit molecular weight was shown to be approximately 45,000.

Introduction

In Neurospora, the carbamyl phosphate used for arginine synthesis is made in mitochondria by an arginine-specific carbamyl phosphate synthetase A (Davis 1972; Weiss and Davis 1973). A second enzyme, carbamyl-P synthetase P, is located in the nucleolus and serves pyrimidine synthesis (Bernhardt and Davis 1970). Carbamyl-P synthetase A contains two polypeptides, the large and small subunits (Davis et al. 1980). The large subunit carries out most of the steps of carbamyl-P synthesis and, in fact, can catalyze the non-biological formation of carbamyl-P from bicarbonate, two molecules of ATP-Mg, and ammonia, the last being the N donor. The small subunit, when aggregated with the large subunit, imparts to the native enzyme the ability to use the amide N of glutamine as an N donor. Free Mg$^{2+}$ and K$^+$ are required in these reactions.

The large and small subunits are encoded in unlinked genes, the \textit{arg}-3$^+$ and the \textit{arg}-2$^+$ loci, respectively (Davis 1967). The large subunit has a molecular weight of approximately 125,000; the small subunit is estimated by the difference in weight of native enzyme and large subunit at approximately 30,000 to 40,000 in molecular weight (Davis et al. 1980). The two polypeptides ultimately form the native enzyme in the mitochondrion. The native enzyme is reasonably stable when in the mitochondrion, but the glutamine-dependent reaction is extremely labile ($t_{1/2}=35$ min at 25°C) upon extraction, presumably reflecting the dissociation of subunits in dilute buffers. The ammonia-dependent reaction is stable upon extraction.

The interest in this enzyme lies in its unusual regulatory properties and the many possible points at which the realization of the glutamine-dependent reaction might be controlled. The ammonia-dependent activity of the large subunit, like that of all other enzymes of the pathway, is at its basal level in mycelia grown on minimal medium and cannot be further repressed by addition of arginine. This activity, however, rises 3- to 5-fold upon arginine starvation of the organism. In contrast, the glutamine-dependent reaction, an attribute of the heteropolymERIC native enzyme, is almost wholly repressible. Moreover, this activity in arginine-starved cells becomes elevated 10- to 20-fold over levels characteristic of cultures grown in minimal medium (Cybis and Davis 1975). The high level of variation (about 40- to 200-fold) of this activity might be presumed to reflect a large variation in the rate of synthesis of the small subunit. This hypothesis has not been tested because the native enzyme has not been wholly purified, and all attempts to visualize or to measure accurately the amount of the small subunit have hitherto failed. Other possible points of control of the glutamine-dependent reaction, following synthesis of the two subunits, are (1) subunit modification; (2) entry of polypeptides into the mitochondrion; (3) aggregation of subunits, before or after entry into the mitochondrion; (4) modification, "maturation", or turnover of the heteropolymERIC native enzyme once it is present within the mitochondrion. With the exception of mechanistic considerations apply to the carbamyl-P synthetase A of yeast, which is cytosolic, but which has a structure and regulation similar to that of the \textit{Neurospora} enzyme (Thuriaux et al. 1972; Urrestarazu et al. 1977; Piérdar and Schröter 1978; Piérdar et al. 1979).

The present paper seeks to exclude certain classes of these hypotheses by demonstrating mutual independence of the two polypeptides' entry into the mitochondrion and, in qualitative terms, mutual independence of their regulation. In addition, the molecular weight and regulatory behavior of the small subunit are shown to be consistent with inferences in previous work.

Materials and Methods

Strains. All strains were from the collection of R H Davis and are listed in Table 1. They include many alleles of the \textit{arg}-2 and \textit{arg}-3 loci.
Table 1. Mutations used in this study

| Locus | Enzyme deficiency | Mutation(s) | Origin | Source or reference |
|-------|-------------------|-------------|--------|---------------------|
| arg-2 | Carbamyl-P synthetase A (small subunit) | 33442 MEP-7, 12, 37, 41 UM-609 CD-4, 18, 43, 71, 75, 79, 80, 103, 215, 163 | wild type (X) arg-12 (UV) arg-12 (UV) | Beadle and Tatum (1945) Davis (1979) Davis (1979) | Pleskacz and Davis (unpublished) |
| arg-3 | Carbamyl-P synthetase A (large subunit) | MEP-6, 9, 21 UC-2, 9, 29 CD-10, 26, 31, 32, 38, 45, 56, 58, 77, 88, 90, 102, 106, 107, 108, 110, 114, 148, 149, 152, 153, 159, 160, 165, 178, 186, 188, 192, 193, 196, 200, 202, 206, 214, 216, 221, 225, 232, 234 | wild type (UV) pyr-3, arg-12 (UV) pyr-3, arg-12 (UV) | Davis (1979) Davis (1979) | Davis (1979) |
| arg-5 | Acetylornithine acetyltransferase | CD-6 | pyr-3, arg-12 (UV) | Davis (1979) |
| arg-6 | Acetylglutamate kinase and acetylglutamyl phosphate reductase | CD-63 | pyr-3, arg-12 (UV) | Davis (1979) |
| pyr-3 | Carbamyl-P synthetase P and aspartate transcarbamylase | DFC-3 | wild type (NG) | Caroline (1969) |
| ssu-1 | Y319-44 | aron M54 (UV) | Case and Giles (1974) |

Mutagens used were X rays (X), ultraviolet light (UV), or nitrosoguanidine (NG)

loci, and typical alleles of the arg-5 (CD-6) and arg-6 (CD-63) loci drawn from a previous study (Davis 1979). In addition, mutations of the pyr-3 (DFC-3) and ssu-1 (Y319-44; Case and Giles 1974) loci, and the wild type strains, 74A and 73a were used. The pyr-3 mutation used eliminates activity of carbamyl-P synthetase P and aspartate transcarbamylase (Caroline 1969).

Materials. Most non-isotopic chemicals were obtained from Sigma Chemical Co., and were of reagent grade. ATP, β-galactosidase, and catalse were purchased from Boehringer-Mannheim. Materials for preparation of polycyclamide gels were obtained from Bio-Rad. Ampholines were purchased from LKB; ultrapure urea was purchased from Schwarz-Mann. Triton X-100 used for scintillation counting was purchased from Research Products International. The [14C]bicarbonate used as enzyme assays was obtained from New England Nuclear.

Media. The minimal medium N of Vogel (1964) was used as the basis of all culture media except for crosses. Arginine (200 μg/ml) and uridine (100-300 μg/ml) were used for supplements as necessary.

Genetics. Genetic techniques used are described in Davis and de Serres (1970). Crosses were performed on corn meal agar by fertilizing four-week day cultures with conidia of the opposite mating type. After three weeks, spores were activated and plated on plating medium, isolated into small tubes, and spot-tested on appropriate medium when fully grown.

Growth, Harvesting, and Mitochondrial Purification. Growth for nutritional tests was done in 10 ml medium N with 1.5 percent sucrose. Mycelia were collected after 48 h growth at 33°C, dried and weighed.

Growth for enzyme assays was done by inoculating conidia (appro. 1 x 10^6 per ml medium) into low-form, 2.500-ml culture flasks containing 700 ml medium N (Davis and de Serres 1970). In most cases, arginine-starvation was imposed by growing in 100 μg L-arginine HCl per ml for 36 h. Growth, as limited by arginine, was complete in 24 h, the remaining time led to derepression of arginine enzymes. Where arginine-repressed cultures were desired, mycelia were grown for no more than 24 h in media supplemented with 400 μg arginine-HCl per ml.

Mycelia destined for acetone drying were harvested in Buchner funnels with Whatman No. 1 filter circles and immediately dried in the funnels with acetone (Davis and de Serres 1970). They were ground later to a powder in cold acetone in an Omni-Mixer (Sorvall Co.), and the powder was air-dried after collection in a Buchner funnel.

Mycelia destined for extraction of mitochondria were grown as above, harvested on cheesecloth, and extracted as described previously (Davis et al. 1980). Mitochondria were isolated by differential centrifugation (Davis et al. 1980), and then further purified to remove vacuoles and their associated proteases. This was accomplished by suspending the organellar pellet in 1M sorbitol containing 10 mM TES-NaOH buffer, pH 7.3 and 1 mM EDTA, layering 5-10 ml of this on a discontinuous gradient consisting of 10 ml 1.6 M sucrose and 10 ml 1.2 M sucrose in a 50 ml polycarbonate tube, and centrifuging for 2 h at 44,000 x g. Mitochondria were collected from the 1.6 M-1.2 M interface and diluted 1:1 with the 1M sorbitol buffer above. Fresh phenyl-methylsulfonylfluoride (PMSF) was added (1 mM, final concentration) and the suspension was recentrifuged at 44,000 x g for 20 min. The mitochondrial pellet was collected in the same buffer with PMSF. The resulting mitochondrial suspensions were quite stable thereafter upon freezing (-70°C) with respect to most enzyme activities and denaturing polycyclamide gel patterns. Little protease activity could be detected by the azocoll method of Lampkin et al. (1976).

Enzyme Assay. Mitochondria were extracted by shaking with glass-beads; after high-speed centrifugation of the extract, the supernatants were assayed by the method of Davis et al. (1980). In many cases, whole mitochondria were also assayed by the same method without extraction. Specific activities are given as milliunits (nmol per min) per mg protein. Protein was determined according to Lowry et al. (1951).

Gels. One-dimensional, 1.5 mm SDS-polyacrylamide slab gels were used as described by Laemmli (1970). Twelve-percent gels (13 mm x 16 mm) were used with a five-percent stacking gel in an apparatus
described by Studler (1973). Gels were run at 50 V for 1 h and 150 V for another 2 h. They were stained overnight in 0.025% Coomassie Blue in methanol-acetic acid and destained for 24 h in methanol-acetic acid.

For two-dimensional gels, purified mitochondrial pellets, isolated as described above, were resuspended in a lysis buffer such that the final protein concentration was 10 mg/ml. The lysis buffer contained 9.5 M urea, 4% Triton X-100, 5% β-mercaptoethanol and 2% Ampholines comprising 40% pH range 5–7, 40% pH range 5–8, and 20% pH range 3–10. The suspension was subjected to four cycles of freezing and thawing. The lysed mixture was either used immediately or was stored at −70°C for 2-4 weeks.

Biochemical evidence for nonsense suppression was sought by measurements of the ammonia-dependent carbamyl-P synthetase A activity, and by visualization of the large subunit on gels. Activity measurements were done on pyr-3 derivatives of the arg-3 mutants in order to eliminate any possible contribution of carbamyl-P synthetase P. Further, mitochondria were highly purified before assay in order to amplify the activity (especially of suppressed mutants) and to simplify the polyacrylamide gel patterns.

Activities of normal, mutant and suppressed mutant strains under derepressing conditions are reported in Table 3; it is clear that neither the glutamine-dependent nor the ammonia-dependent activity of the enzyme is detectable in the mutants. This is consistent with the presumed loss of the large subunit of the enzyme. The suppressed mutants display a definite ammonia-dependent activity (2 to 7% of normal) and a slight, erratic, and barely detectable glutamine-dependent activity. The latter is consistent with the presumed loss of the large subunit of the enzyme. The suppressed mutants display a definite ammonia-dependent activity (2 to 7% of normal) and a slight, erratic, and barely detectable glutamine-dependent activity. The latter is consistent with the presumed loss of the large subunit of the enzyme.

### Table 2. Effect of ssu-1 on growth of suppressible arg-3 mutants

| Strain | Specific activity | Dry weight ratio |
|--------|-------------------|-----------------|
|        | Whole mitochondria | Mitochondrial extracts |
|        | Gln-dep. | Amm-dep. | Gln-dep. | Amm-dep. |
| pyr-3 | 10.18 | 11.13 | 4.17 | 31.80 |
| pyr-3, CD-186 | 0.07 | 0.68 | 0 | 2.68 |
| pyr-3, CD-192 | 0.03 | 0.22 | 0 | 0.75 |
| pyr-3, CD-214 | 0 | 0 | 0 | 0 |
| pyr-3, CD-214, ssu-1 | 0.37 | 0.48 | 0.10 | 1.68 |

Glutamine- and ammonia-dependent activities are expressed as nmoles carbamyl-P mm⁻¹ mg protein⁻¹. Extraction of mitochondria effects 3-4 fold purification of stable matrix enzymes.

### Table 3. Effect of ssu-1 on carbamyl-P synthetase A activities of mitochondria and mitochondrial extracts of suppressible arg-3 mutants

| Strain | Specific activity | Dry weight ratio |
|--------|-------------------|-----------------|
|        | Whole mitochondria | Mitochondrial extracts |
|        | Gln-dep. | Amm-dep. | Gln-dep. | Amm-dep. |
| pyr-3, arg-5 | 10.18 | 11.13 | 4.17 | 31.80 |
| pyr-3, CD-186 | 0.07 | 0.68 | 0 | 2.68 |
| pyr-3, CD-192 | 0.03 | 0.22 | 0 | 0.75 |
| pyr-3, CD-214 | 0 | 0 | 0 | 0 |
| pyr-3, CD-214, ssu-1 | 0.37 | 0.48 | 0.10 | 1.68 |

Uridine (Uri) was added to a final concentration of 100 μg/ml: Arginine (Arg) was added at 200 μg/ml. Growth was in 10 ml medium at 32°C for 48 h.

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Activities of normal, mutant and suppressed mutant strains under derepressing conditions are reported in Table 3; it is clear that neither the glutamine-dependent nor the ammonia-depen-
Fig. 1. Action of ssu-1 on phenotype of arg-3 nonsense mutations, using one-dimensional SDS-polyacrylamide gel resolution of mitochondrial extracts. The CD-186, -192, and -214 mutants are shown, with and without the ssu-1 mutation, together with control (con) preparation of the arg-2 mutant CD-80. The large subunit is the uppermost band of the control. In CD-214, ssu-1, a faint band appears under the large-subunit band; this is a mitochondrial membrane protein, incompletely removed by centrifugation of broken mitochondria.

Table 4. Carbamyl-P synthetase A activities of whole mitochondria of arginine mutants

| Locus | Mutation | Growth condition | Specific activity |
|-------|----------|------------------|------------------|
|       |          |                  | Gln-dep | Amm-dep |
| arg-5 | CD-6     | derep.           | 14.80   | 11.30   |
| arg-6 | CD-63    | derep.           | 16.70   | 8.43    |
| arg-2 | CD-80    | rep.             | <0.02   | 16.80   |
| arg-3 | CD-10    | derep.           | 0.03    | 0.10    |
|       | CD-88    | derep.           | 0       | 0.03    |
|       | CD-202   | derep.           | 0       | 0.17    |
|       | CD-232   | derep.           | 0.52    | 0.42    |
|       | all others | derep.       | <0.02   | <0.02   |

Conditions of growth are given in Materials and Methods. The additional arg-3 mutants tested were: CD-9, -31, -38, -45, -91, -102, -152, -153, -165, -186, -192, -214, -196, -234. Specific activity is expressed as in Table 4.

for arginine cannot be imposed upon the suppressed mutants; the latter still require arginine for optimal growth (Table 2).

The data are consistent in showing that at least three mutants carry nonsense mutations suppressible by ssu-1 by three criteria: nutritional requirement, enzyme activity, and visualization of the arg-3 + product.

Diversity of arg-3 Alleles. A survey of arg-3 mutants was undertaken to determine, first, what mutational variation of activity and SDS gel patterns there might be among arg-3 mutants, and, second, whether any arg-3 mutant displayed ammonia-dependent activity in whole-cell extracts, but failed to localize this activity in its mitochondria.

Acetone powders of 41 mutants were prepared and extracted for assay of whole-cell activity. Four mutants (CD-10, CD-88, CD-202, and CD-232) showed a slight (over 2 per cent of normal) ammonia-dependent activity. Assays of mitochondria isolated from these strains showed that the ammonia-dependent activity was properly localized (Table 4).

These, and a number of enzymically inactive mutants, were surveyed for the existence and band-intensity of the large subunit on SDS-polyacrylamide gels (Fig. 2). The four mutants with residual activity were diverse: two, CD-88 and CD-202, had bands of normal intensity for derepressed strains; CD-10 had a rather weak band, and CD-232 showed no band at all. The CD-232 mutant was unusual in that it consistently had the greatest residual ammonia-dependent activity, as well as equal amounts of glutamine-dependent activity (Table 4). The mutant has not been pursued further, but may be a “low-promoter” type in which an unimpaired large subunit is synthesized in very small amounts.

The enzymically inactive mutants showed similar diversity: eight lacked detectable bands corresponding to the large subunit; one had a faint band; and three had bands of normal intensity. It is possible that some of those lacking visible bands are nonsense mutants which are not suppressible at the nutritional level by ssu-1.
Fig. 3. Two-dimensional gels of whole mitochondria of derepressed arg-2+ (CD-6, top) and arg-2 (CD-80, bottom) strains. The first dimension (horizontal) was isoelectric focusing of urea-denatured material, the second (vertical) was an SDS-denaturing gel. The arrow on the top gel points to the small carbamyl-P synthetase A subunit missing in the arg-2 strain. The large subunit of the enzyme is the uppermost, central spot as determined by comparison with gels of the arg-3 nonsense mutant, CD-186. Approximate positions of molecular-weight standards are shown in the top gel, as follows: 1, myosin (200,000); 2, β-galactosidase (116,000); 3, phosphorylase B (92,500); 4, bovine serum albumin (68,000); 5, catalase (60,000); 6, ovalbumin (43,000); 7, lactate dehydrogenase (35,000).

Visualization of the Small Polypeptide. Despite variation of conditions of one-dimensional SDS-gel separations, including gradient gels, no arg-2 mutant displayed a deficiency in any band seen in arg-2+ mitochondria. Two-dimensional gels, however, revealed such a deficiency in a strain carrying the arg-2 allele CD-80 in comparison to the control arg-5 strain (Fig. 3). The same polypeptide was missing in two other arg-2 mutants which were tested, CD-79 and CD-103. While none of these three mutations is suppressible by ssu-1, it is possible that a mutant form of the small subunit is still present in all arg-2 mutants. Therefore, it is not rigorously proven that the large subunit enters without participation of a full-length arg-2 product.

In the case of the small subunit, its entry into mitochondria is unimpaired in all arg-3 mutants tested. This is true in particular of strains carrying the nonsense mutations CD-186, CD-192, and CD-214. Thus, if an arg-3 product is essential for entry of the small polypeptide, N-terminal fragments of the large subunit will suffice.

Qualitative Observations on Subunit Regulation. The ammonium-dependent activity (an attribute of the large subunit) is regulated over a two- to five-fold range. In contrast, the glutamine-dependent activity varies over a 40- to 200-fold range, and is the only known protein of the pathway which is almost wholly repressible (Table 4; Cybis and Davis 1975). One question, answerable with the present data, is whether this large "repression" ratio is correlated with similar behavior of the small polypeptide as visualized on gels. The gels in Fig. 4 are from mitochondria of derepressed and repressed strains of arg-6 (CD-63). In the case of the repressed mycelium, mitochondria lack any visible trace of the small subunit in its characteristic position. This is in contrast to the large subunit in the same gel, which is not fully repressible by arginine.

The regulation of the small subunit in the arg-3 nonsense strain, CD-186, is the same as in arg-6 strains (data not shown). Thus, the regulation of the small subunit appears to be independent of the large subunit.
Discussion

The results reported here have led to a visualization of the two subunits of carbamyl-P synthetase A by use of mutations and suppressor action. In mutants of each locus lacking detectable amounts of the corresponding subunit, it was inferred that the localization and regulation of the other subunit was normal. Thus, localization and regulation are undergone independently by each subunit.

The molecular weight estimate of the large polypeptide, of approximately 125,000 (Davis et al. 1980), was confirmed, and the visualization of the small subunit allowed us to estimate its molecular weight at approximately 45,000. The latter figure is a much more precise estimate than the estimate of 30,000 to 50,000 obtained previously (Davis et al. 1980). The sum of the molecular weights of the two subunits (170,000) is very close to the previous estimate for the undenatured native enzyme (176,000), based upon gel filtration and density-gradient centrifugation. The correspondence allows us to conclude that the native enzyme is a stoichiometric aggregate of one large and one small subunit. This conclusion is consistent with the lack of intragenic complementation among arg-2 mutations or among arg-3 mutations (Davis 1979).

Because mutations affecting one subunit of carbamyl-P synthetase A do not affect the entry of the other subunit into mitochondria, we may conclude, first, that the aggregation of subunits to form the native enzyme occurs in the mitochondrion (or, at the very least, that aggregation of subunits is not required for their entry into the organelle). Second, because regulation of each subunit is normal in mutants which lack detectable amounts of the other subunit, we may conclude that neither polypeptide appears to participate in the determination of the steady-state levels of the other. Both respond relatively independently to the arginine status of the cell. Finally, the two subunits, as visualized on gels, are regulated quite differently: the large subunit is modulated over a small range, while the small subunit spot ranges from very prominent (derepression) to undetectable (repression). The behavior of the two subunits on gels is consistent with enzyme activity measurements where the glutamine-dependent activity, requiring the small subunit, has a very high amplitude of regulation, and is virtually absent in conditions of repression (Cybis and Davis 1975). It is not known whether the unusual control of the small subunit is pre- or post-translational: for instance, its absence from mitochondrial extracts of repressed mycelia could reflect interference with small subunit insertion into mitochondria, or modification (or lack of it) once the subunit entered. Only antibody tests can answer this question more closely, and only measurement of arg-2 "mRNA will offer more definitive tests of transcriptional regulation.

The present data allow us to eliminate a major possibility as the sole cause of the high amplitude of the glutamine-dependent activity in extracts. Until now, the possibility existed that neither subunit is fully repressible. The subunits could both be regulated over a small range (approx. 3- to 5-fold), but the large range of catalytic activity is the result of mass action (or other factors) governing the aggregation of two subunits with low affinity for one another. While the bimolecular aggregation process can still underly the large range of glutamine-dependent activity in part, it is not the entire cause. It is clear that the small subunit is the limiting factor when the cells have low glutamine-dependent activity. As the activity rises upon derepression, the large subunit maintains an excess or equivalence over at least part of the derepression range. It will be important to know whether, in more extreme states of derepression, the synthesis of the two polypeptides is coordinated such that neither is made in excess.

This point has been addressed recently by Pïrard et al. (1979) in the case of yeast carbamyl-P synthetase A. In yeast, it appears that the large subunit is under the control of the "general amino acid" regulatory system, while the small subunit responds both to the general system and to a specialized arginine-specific control mechanism devoted to this subunit. The two circuits behave in such a way that the small subunit is never in excess under normal circumstances. However, a considerable amount of cryptic large subunit is present in conditions of arginine excess, assayable only in a glutamine-dependent assay using excess small subunit. This may reflect activation or processing of the large subunit upon aggregation, phenomena for which there is no evidence, and some counter-indication in Neurospora (Davis et al. 1980). A second unusual feature of the yeast system is a depressive effect of nonsense mutations of the large subunit upon the amplitude of variation of the small subunit (Piïrard et al. 1979). This might be the result of inactivation of free small subunit, or of an impairment in small subunit synthesis in mutants lacking the large subunit. Again, no indication of this is seen in Neurospora, either by the present two-dimensional gel analysis or by complementation assays (Davis and Ristow, unpublished observations). Our current studies of the two polypeptides will seek to confirm or eliminate these apparent regulatory differences between the enzymes of the two organisms.

Antibodies will be needed to study the molar ratio of subunit synthesis, and in vitro complementation studies of the aggregation process will be necessary to determine intersubunit affinities and to test for further maturation steps of the enzyme. Finally, other studies, again involving immunological methods, will be useful in determining whether mutations of the localization process can be detected. If so, it will allow a fuller understanding of the many steps needed in the realization of this complex enzyme in vivo.

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References

Beadle GW, Tatum EL (1945) Neurospora. II. Methods of producing and detecting mutations concerned with nutritional requirements. Am J Bot 32: 678-686

Bernhardt SA, Davis RH (1969) Carbamoyl phosphate compartmentation in Neurospora histochemical localization of aspartate and ornithine transcarbamoylases. Proc Natl Acad Sci USA 69: 1868-1872

Caroline DF (1969) Pyrimidine synthesis in Neurospora crassa gene-enzyme relationships. J Bacteriol 100: 1371-1377

Case ME, Giles NH (1974) Revertants and secondary auxotroph mutants. J Bacteriol 123: 196-202

Cybis JJ, Davis RH (1975) Organization and control in the arginine biosynthetic pathway of Neurospora. J Bacteriol 123: 196-202

Davis RH (1967) Channeling in Neurospora metabolism. In: Vogel BJ, Lampen JO, Bryson V (eds) Organizational biosynthesis. Academic Press Inc., New York, p 303

Davis RH (1972) Metabolite distribution in cells. Science 178: 835-840

Davis RH (1979) Genetics of arginine biosynthesis in Neurospora crassa. Genetics 93: 557-575

Davis RH, de Serres FJ (1970) Genetic and microbiological research techniques for Neurospora crassa. In: Tabor H, Tabor CW (eds)
Methods in enzymology. Vol XVIIA. Amino acids and Amines. Academic Press Inc., New York, p.79

Davis RH, Ruslow JL, Hanson BA (1980) Carbamyl phosphate synthetase A of Neurospora crassa. J Bacteriol 141:144-155

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) New Biol 227:680-685

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193:265-275

O'Farrell PH (1975) High resolution two-dimensional electrophoresis of proteins. J Biol Chem 250:4007-4021

Pérard A, Schröter B (1978) Structure-function relationships in arginine pathway carbamoyl phosphate synthase of Saccharomyces cerevisiae. J Bacteriol 134:167-176

Pérard A, Messenguy F, Feller A, Hilger F (1979) Dual regulation of the synthesis of the arginine pathway carbamoylphosphate synthase of Saccharomyces cerevisiae by specific and general controls of amino acid biosynthesis. Mol Gen Genet 174:163-171

Seale TW (1976) Super suppressor action spectrum in Neurospora. Molec Gen Genet 148:105-108

Studier FW (1973) Analysis of bacteriophage T7 early RNAs and proteins on slab gels. J Mol Biol 79:237-248

Thuriaux P, Ramos F, Pérard A, Grenson M, Waame J-M (1972) Regulation of the carbamoyl phosphate synthetase belonging to the arginine biosynthetic pathway of Saccharomyces cerevisiae. J Mol Biol 67:277-287

Urrestarazu LA, Vissers S, Waame J-M (1977) Changes in location of ornithine carbamoyl transferase and carbamoyl phosphate synthetase among yeasts in relation to the arginase/ornithine carbamoyl transferase regulatory complex and the energy status of the cells. Eur J Biochem 79:473-481

Vogel HJ (1964) Distribution of lysine pathways among fungi: evolutionary implications. Am Nat 98:435-446

Weiss RL, Davis RH (1973) Intracellular localization of enzymes of arginine metabolism in Neurospora. J Biol Chem 248:5403-5408

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