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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 *arg-3* locus and can catalyze formation of carbamyl-P with ammonia as the N donor. The small subunit is encoded in the unlinked *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 *arg-3*, it was shown that the small subunit of the enzyme enters the mitochrondrion independently and is regulated in the same manner as it is in wild type. Similarly, *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, carbamyl-P 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 *arg-3*$^{+}$ and the *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 in 50,000 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 mechanisms of localization, the same 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 *Neurospora* enzyme (Thuriaux et al. 1972; Urrestarazu et al. 1977; Piérard and Schröter 1978; Piérard 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 *arg-2* and *arg-3*

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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	wild type (X)	Beadle and Tatum (1945)
		MEP-7, 12, 37, 41	*arg-12*^{s} (UV)	Pleskacz and Davis (unpublished)
		UM-609	wild type (UV)	Davis (unpublished)
		CD-4, 18, 43, 71, 75, 79, 80, 103, 215, 163	*pyr-3, arg-12*^{s} (UV)	Davis (1979)
arg-3	Carbamyl-P synthetase A (large subunit)	MEP-6, 9, 21	*arg-12*^{s} (UV)	Pleskacz and Davis (unpublished)
		UC-2, 9, 29	wild type (UV)	Davis (unpublished)
		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	*pyr-3, arg-12*^{s} (UV)	Davis (1979)
arg-5	Acetylornithine acetyltransferase	CD-6	*pyr-3, arg-12*^{s} (UV)	Davis (1979)
arg-6	Acetylglutamate kinase and acetylglutamyl phosphate reductase	CD-63	*pyr-3, arg-12*^{s} (UV)	Davis (1979)
pyr-3	Carbamyl-P synthetase P and aspartate transcarbamylase	DFC-3	wild type (NG)	Caroline (1969)
ssu-1		Y319-44	*arom* 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, 74*A* and 73*a* 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 catalase were purchased from Boehringer-Mannheim. Materials for preparation of polyacrylamide 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 [^{14}C]bicarbonate used for 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-day cultures with conidia of the opposite mating type. After three to four 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 (approx. 1×10^6 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 growth 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 discontinous 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 $44{,}000 \times g$. Mitochondria were collected from the 1 6 M–1.2 M interface and diluted 1:1 with the 1 M sorbitol buffer above. Fresh phenylmethylsulfonylfluoride (PMSF) was added (1 mM, final concentration) and the suspension was recentrifuged at $44{,}000 \times g$ 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 polyacrylamide 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 × 16 mm) were used with a five-percent stacking gel in an apparatus

described by Studier (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-demensional 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

Gels were run as described by O'Farrell (1975) with the exceptions that (a) DNase and RNase treatments were omitted; (b) the first dimension gel mixture contained the ampholine mixture described above; and (c) the detergent Triton X-100 was used in place of NP-40.

Results

Identification of Nonsense Mutants. By means of crosses to a strain carrying the nonsense suppressor *ssu-1*, a search for suppressible *arg-2* (LG IVR) and *arg-3* (LG IL) nonsense mutations was undertaken. The suppressor, on LG VII, has a broad spectrum of action and is unlinked to either *arg* gene (Seale 1976). Two features of the system improved the rigor of the search. First, *ssu-1* progeny were identifiable by slow growth and their less fluffy conidial morphology; second, markers tightly linked to *arg-2* (*pyr-3*, 1 map unit from *arg-2*) and *arg-3* (mating type, 10 map units from *arg-3*) allowed identification of progeny carrying the *arg* parental chromosome in *ssu-1* strains even if the arginine mutation was suppressible.

For *arg-2*, matings of the form *arg-2, pyr-3; ssu-1*$^+$ (normal inactive allele of suppressor) × *arg-2*$^+$, *pyr-3*$^-$; *ssu-1* (active suppressor allele) were made The Ssu$^-$ progeny were surveyed for uridine and arginine requirements. In all 16 crosses, all Pyr$^-$ Ssu$^-$ progeny were also arginine auxotrophs, thus demonstrating that no *arg-2* mutation was suppressible. A similar survey of seven more alleles isolated by Dr. I.B Barthelmess was similarly negative.

For *arg-3*, crosses of the form *arg-3, A; ssu-1*$^+$ × *arg-3*$^-$ *a; ssu-1* were done. The Ssu$^-$ progeny were tested for mating type and arginine requirement. Where Ssu$^-$ progeny were uniformly prototrophic, they were tested for mating type to ascertain the presence of the chromosome carrying the *arg-3* mutation. Backcrosses of presumed *arg-3, A; ssu-1* strains to wild type were then done to verify the presence of the *arg-3* mutation. By these genetic criteria, three *arg-3* alleles (CD-186, CD-192 and CD-214), of some 41 tested, were suppressible by *ssu-1*.

Nutritional tests of suppressible nonsense mutants, with or without the suppressor mutation, showed all of them to have some impairment of arginine metabolism (Table 2) The different degrees of suppression of the *arg-3* alleles by the suppressor mutation indicates that CD-192, at least, is different from the other two.

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 was clearest in whole mitochondria, and was usually lost upon extraction of mitochondria. The loss of the glutamine-dependent activity was expected in view of the extreme instability of the wild-type glutamine-dependent activity. Nevertheless, the glutamine-dependent activity in vivo is evidently sufficient to support growth of the suppressed mutants. In addition, the most weakly suppressed mutation on nutritional grounds, CD-192, is the most weakly suppressed on enzymic grounds.

The large subunit of the enzyme, encoded by *arg-3*$^+$, is the largest visible polypeptide of extracts of purified mitochondria on denaturing polyacrylamide gels. This was established previously by noting the absence of this polypeptide in a strain carrying the nonsense mutation CD-186 (Davis et al. 1980). An SDS-polyacrylamide gel of the mitochondrial extracts of derepressed cultures of the three nonsense mutants (*arg-3, ssu-1*$^-$) and the corresponding suppressed mutants (*arg-3, ssu-1*) demonstrates the restoration of the large polypeptide in the latter (Fig. 1). The restoration of the large polypeptide by the action of the suppressor is not complete (in terms of staining intensity) in comparison with the arginine-starved *arg-3*$^+$ strain. This is probably not wholly due to the fact that a strict starvation

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

Strain	mg dry weight per 10 ml minimal medium supplemented with:		Dry weight ratio:
	Uri	Uri + Arg	−Arg/+Arg
pyr-3	22.9	23 9	0 96
pyr-3, ssu-1	16.1	12 6	1.28
pyr-3, CD-186	0	20.0	0
pyr-3, CD-186, *ssu-1*	12.4	14 4	0.86
pyr-3, CD-192	0	20.8	0
pyr-3, CD-192, *ssu-1*	1.1	14.2	0.08
pyr-3, CD-214	0	22.2	0
pyr-3, CD-214, *ssu-1*	13 3	18 0	0.74

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

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			
	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	0	0	0
pyr-3, CD-186, *ssu-1*	0.07	0 68	0	2 68
pyr-3, CD-192	0	0	0	0
pyr-3, CD-192, *ssu-1*	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 $min^{-1} \cdot mg\ protein^{-1}$. Extraction of mitochondria effects a 3–4 fold purification of stable matrix enzymes

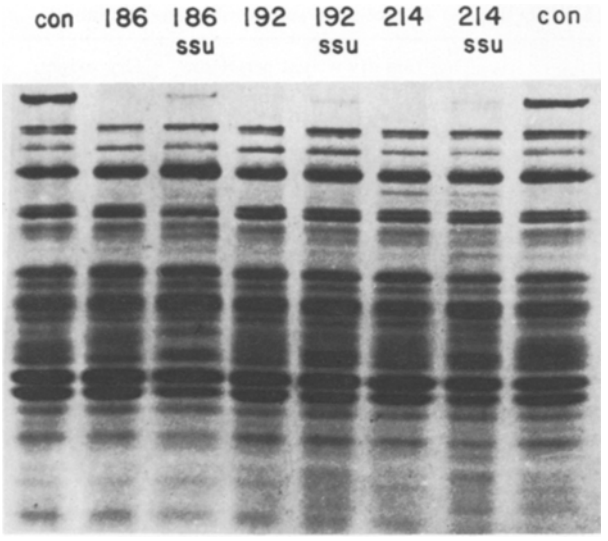

Fig. 1. Action of *ssu-1* on phenotype of *arg-3* nonsense mutations, using one-dimensional SDS-polyacrylamide gel resolution of mitochondrial extracts. The CD-186, -192, and -214 mutants are shown, with and without the *ssu-1* mutation, together with control (con) preparation of the *arg-2* mutant CD-80. 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
	CD-63	rep.	< 0 08	4.82
arg-2	CD-80	derep.	< 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).

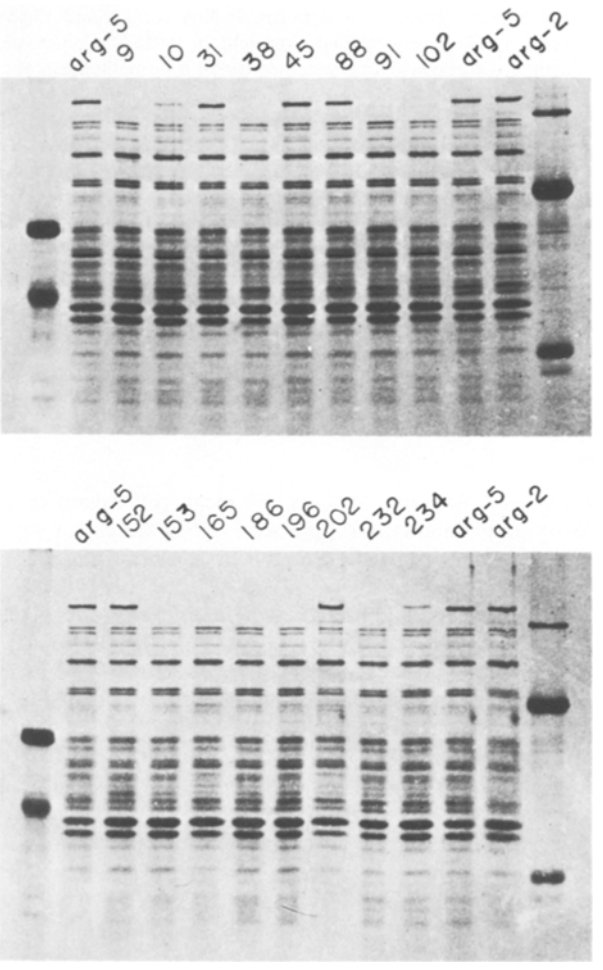

Fig. 2. Survey of mitochondrial extracts of *arg-3* mutants on one-dimensional SDS-polyacrylamide gels The *arg-3* mutantions are designated by their "CD" numbers; control *arg-5* (CD-6) and *arg-2* (CD-80) strains are also shown. Molecular weight standards in descending order of subunit size are, on right, β-galactosidase (mol. wt 116,000), bovine serum albumin (68,000) and lactate dehydrogenase (35,000); on left, catalase (60,000) and ovalbumin (43,000)

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*.

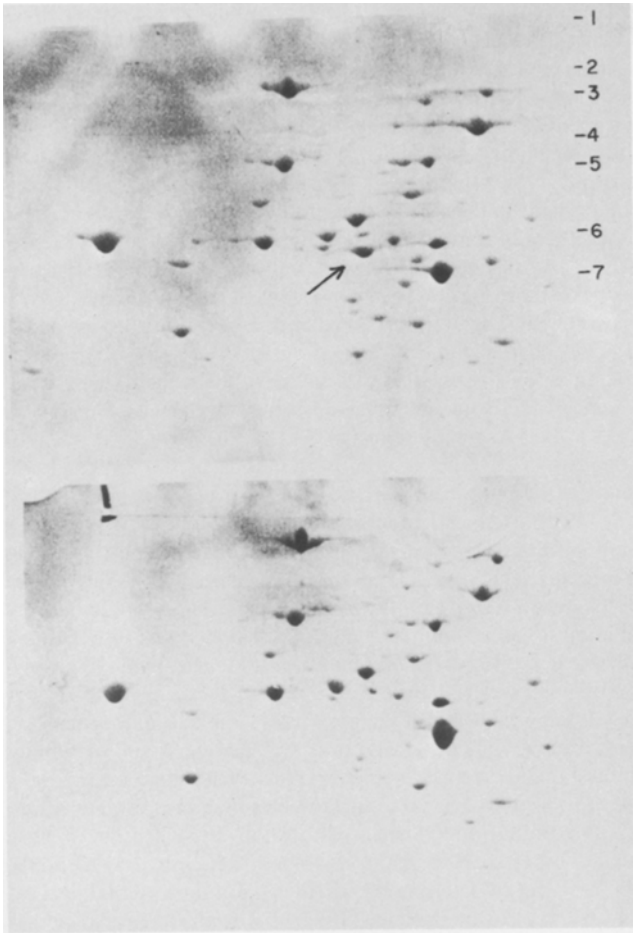

Fig. 3. Two-dimensional gels of whole mitochondria of derepressed *arg-2*$^+$ (CD-6, top) and *arg-2* (CD-80, bottom) strains. The first dimension (horizontal) was isoelectric focussing of urea-denatured material, the second (vertical) was an SDS-denaturing gel. The arrow on the top gel points to the 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 one or more of them is a nonsense mutation.

Molecular Weights of the Polypeptides. By comparison with standards on the second dimension (SDS) of two-dimensional gels, the molecular weight of the large polypeptide is estimated to be approximately 125,000, in agreement with a previous determination. On the same gels, the small polypeptide has a mobility suggesting a molecular weight of 45,000. (In Fig. 3, the scale

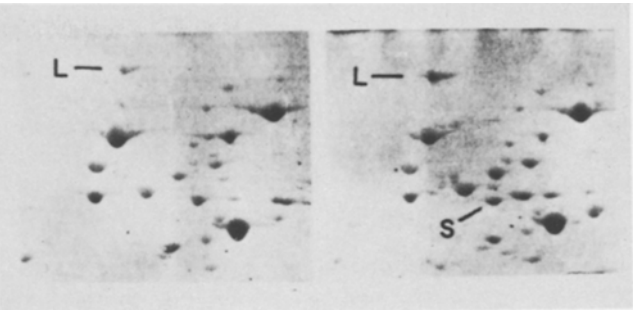

Fig. 4. Absence of the small subunit of carbamyl-P synthetase A in conditions of repression The *arg-6* (CD-63) mutant was grown under conditions of repression (left) or derepression (right). Purified whole mitochondria were analyzed as in Fig. 3. Portions of gels including the large (L) and small (S) subunits are shown, and illustrate the difference in their response to excess arginine during growth. (A polypeptide present in Fig 3 to the left of the small subunit, and missing in both gels here, is probably encoded by the *arg-6* locus)

used is an average based on 3–5 separate determinations; the small subunit molecular weight is based on averaging 6 determinations.)

Independent Entry of the Polypeptides into Mitochondria. The two subunits of the enzyme appear to enter the mitochondria independently of one another. In the case of the large subunit, this is shown by the fact that the activities and band-intensities (on gels) of the *arg-3*$^+$ product are normal in mitochondria of all ten *arg-2* mutants tested (data not shown) This is true of strains carrying the *arg-2* alleles CD-80, CD-79, and CD-103, all of which lack visible small subunits on two-dimensional gels. However, because we have no bona fide *arg-2* nonsense mutants, it is conceivable 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 Piérard 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 (Piérard 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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