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# Acetylglutamate Kinase-Acetylglutamyl-phosphate Reductase Complex of *Neurospora crassa*

EVIDENCE FOR TWO POLYPEPTIDES\*

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Mutations at the *arg-6* locus in *Neurospora crassa* are divided into two complementation groups (A and B) and a third noncomplementing group. There are many suppressible nonsense mutations among mutants in complementation group B and one in the noncomplementing group; no nonsense mutations exist among mutants in complementation group A (Davis, R. H., and Weiss, R. L. (1983) *Mol. Gen. Genet.* 192, 46-50). We show here that the mutants are defective in either or both of two enzymes of arginine biosynthesis, acetylglutamate kinase and/or acetylglutamyl-phosphate reductase. Mutants in complementation group A lack acetylglutamate kinase, those in complementation group B lack acetylglutamyl-phosphate reductase, and those in the noncomplementing group lack both activities. Mutants in group B also have reduced levels of acetylglutamate kinase. The enzymes from purified mitochondria are readily separable by gel filtration and by Blue A dye affinity chromatography. Acetylglutamate kinase appears to be an octamer with a molecular weight of 400,000, whereas acetylglutamyl-phosphate reductase appears to be a dimer with a molecular weight of 93,000. This suggests that the two activities reside on distinct polypeptides. These results are best accommodated by the following model: the *arg-6* locus encodes a single mRNA which is translated into a single polypeptide; the latter is then cleaved post-translationally to yield two physically separable enzymes.

between the three groups; suppressible mutations are found only in group B and the noncomplementing group (1, 3). The latter's suppressibility suggests that a double deficiency can arise by virtue of a nonsense mutation and that suppression restores function to the domain in which the mutation lies and also relieves a translational polarity effect on the second function. The complementation and suppression data suggested that the *arg-6* locus encodes a bifunctional protein. Similar findings have been reported for the *argB-argC* gene cluster of *Saccharomyces cerevisiae* (5, 6). However, subsequent characterization of the *argB-argC* gene products showed that the two activities were separable by either of two different purification steps.

Enzymological analysis of the polypeptide products of the similar *N. crassa arg-6* locus was made in order to gain a better understanding of the organization and expression of the locus. Representatives of each of the three complementation groups of the *arg-6* locus were screened for acetylglutamate kinase and acetylglutamyl-phosphate reductase activities. In addition, gel filtration and Cibacron dye chromatography were performed to determine whether acetylglutamate kinase and acetylglutamyl-phosphate reductase are separable enzymes, as they are in yeast. If so, these two eukaryotic systems could be used to study the mechanism whereby two proteins are produced from a single mRNA, e.g. mRNA processing, internal translation initiation, or post-translational processing.

## EXPERIMENTAL PROCEDURES

**Strains, Media, and Chemicals**—The strains of *N. crassa* used in the experiments described here are shown in Table I. Strain LA16 is an arginine bradytroph having an altered ornithine carbamoyltransferase (EC 2.1.3.3.). The strain has a low arginine pool and is derepressed for the enzymes of arginine biosynthesis (7, 8). The arginine auxotrophic strains have been previously described (1, 3). The medium used for growth was Vogel's minimal medium N (9) containing 1.5% sucrose as the carbon source. Strains were maintained on solid medium containing 2% agar and supplemented with 0.2 g/liter arginine, when appropriate. *Escherichia coli* strain MA13 was obtained from the *E. coli* Genetic Stock Center. Chemicals were obtained from common sources and were reagent grade or equivalent. Catalase and ferritin were obtained from Pharmacia. Thyroglobulin, immunoglobulin G, ovalbumin, myoglobin, and ion exchange resin were obtained from Bio-Rad. Lactate dehydrogenase, bovine serum albumin, pyruvate kinase, glyceraldehyde-3-phosphate dehydrogenase, and carbonic anhydrase were obtained from Sigma. L-[U-<sup>14</sup>C]Glutamate was obtained from ICN. Blue A Dyematrix was obtained from Amicon.

**Growth, Harvesting, and Purification of Mitochondria**—Mycelia were grown from a conidial inoculum (approximately  $5 \times 10^8$  conidia/ml) at 30 °C for approximately 15 h. Growth was performed either in Ehrlenmeyer flasks with rotary shaking or in large carboys with mixing using hydrated air. Mycelia were collected by filtration

Mutations at the complex *arg-6* locus in *Neurospora crassa* have been reported to lack the first two enzymes of the arginine biosynthetic pathway, acetylglutamate kinase (EC 2.7.2.8) and acetylglutamyl-phosphate reductase (*N*-acetyl- $\gamma$ -glutamyl-phosphate reductase, EC 1.2.1.38) (1-3). They are encoded by the nuclear *arg-6* locus but function in the mitochondrial matrix (4). Genetic analysis demonstrated that *arg-6* mutations can be classified into three complementation groups (A, B, and noncomplementing) and that there is a nonuniform distribution of suppressible nonsense mutations

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TABLE I  
Strains of *N. crassa*

Strain	Genotype	Allele	Group
LA1	Wild type		
LA16	<i>arg-12</i> <sup>a</sup>		
LA54	<i>arg-6</i>	CD29	A
LA55	<i>arg-6</i>	CD63	Noncomplementing
LA56	<i>arg-6</i>	CD68	Noncomplementing
LA59	<i>arg-6</i>	CD25	B
LA302	<i>arg-6</i>	CD34	B
LA304	<i>arg-6</i>	CD44	B
LA305	<i>arg-6</i>	CD46	B
LA310	<i>arg-6</i>	CD65	A
LA317	<i>arg-6</i>	CD92	B
LA318	<i>arg-6</i>	CD111	B
LA319	<i>arg-6</i>	CD116	B
LA320	<i>arg-6</i>	CD121	Noncomplementing
LA322	<i>arg-6</i>	CD136	A
LA323	<i>arg-6</i>	CD174	A

through two layers of cheesecloth, washed thoroughly with cold water, and then squeezed to remove excess water.

The mycelia were then suspended in three times their wet weight of cold Hepes<sup>1</sup> extraction buffer (50 mM Hepes, pH 7.4, 1 mM EDTA, 0.2 mM dithiothreitol, 70 mM sucrose, 220 mM mannitol, 0.5 g/liter bovine serum albumin, 1 mM phenylmethylsulfonyl fluoride). The suspension was centrifuged at 3000 × *g* for 5 min. The supernatant was discarded, and the washed mycelia were used to isolate mitochondria by a modification of the method of Cramer *et al.* (10).

The wet mycelial pad was added to a mixing chamber (the Bead Beater, Biospec Products) one-third filled with glass beads (0.3–0.5 mm in diameter). The chamber was then filled with Hepes extraction buffer. The chamber was cooled with an ice-water jacket. The mycelia were broken by 3 × 30-s pulses of mixing, with cooling between pulses to minimize heating. The homogenate was decanted from the glass beads, and mycelial debris was removed by centrifugation at 3000 × *g* for 5 min, followed by filtration through Miracloth (glass fiber filters may be substituted).

A crude organellar fraction was prepared by centrifugation at 30,000 × *g* for 20 min. The mitochondria were separated from other organelles on a sucrose step gradient. The gradient was prepared by layering 20 ml of 1.2 M sucrose in Hepes buffer (50 mM Hepes, pH 7.4, 1 mM EDTA, 0.2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride) on top of 10 ml of 1.6 M sucrose in Hepes buffer. Approximately 6 ml of the organellar fraction, resuspended in 3 volumes of Hepes extraction buffer, was layered on the gradient. It was then centrifuged for 75 min at 70,000 × *g* in a Beckman SW 27 rotor. The mitochondria banded at the interface between the two layers. Fifteen ml of the top layer was removed with an aspirator and discarded. The band at the interface was collected with a Pasteur pipette. This suspension was diluted 1:3 with Hepes extraction buffer, and the purified mitochondria were collected by centrifugation at 30,000 × *g* for 20 min.

**Chromatography of Mitochondrial Matrix Proteins**—Purified mitochondria were suspended in 3 pellet volumes of phosphate buffer (50 mM potassium phosphate, pH 7.5, 0.1 mM EDTA, 0.2 mM dithiothreitol, 10 mM acetylglutamate, 1 mM MgCl<sub>2</sub>, 1 mM phenylmethylsulfonyl fluoride) and sonicated (3 × 10-s pulses) to lyse the mitochondria. Mitochondrial membrane fragments were removed by centrifugation at 30,000 × *g* for 40 min. The resulting preparation of mitochondrial matrix proteins was used for all chromatographic separations (see below).

The mitochondrial matrix preparation was fractionated by gel filtration chromatography using Sephadex G-200. The column (2.6 × 65 cm) was equilibrated with phosphate buffer. A sample of the matrix proteins was applied to the column and subsequently eluted with phosphate buffer. Fractions were assayed for acetylglutamate kinase and acetylglutamyl-phosphate reductase.

**Blue A Dyematrix Chromatography**—A sample of mitochondrial

matrix proteins was partially purified by ammonium sulfate fractionation. The fraction precipitating between 30 and 65% saturation was resuspended in start buffer (10 mM potassium phosphate, pH 7.5, 0.5 mM EDTA, 0.2 mM dithiothreitol, 5 mM acetylglutamate, 5 mM MgCl<sub>2</sub>, 1 mM phenylmethylsulfonyl fluoride), and 0.1-ml aliquots were desalted on four Penefsky columns (11). These desalted fractions were pooled and applied to a 9 × 32-mm Blue A Dyematrix column equilibrated with start buffer. Following a 30-min no-flow equilibration period to allow binding, the column was washed with 10 ml of start buffer. Bound proteins were then eluted with 10 ml of start buffer containing 1 mM NADP<sup>+</sup> followed with 10 ml of 100 mM potassium phosphate, pH 8.0, containing 5 mM acetylglutamate, 0.2 mM dithiothreitol, 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride. The effluents were assayed for acetylglutamate kinase and acetylglutamyl-phosphate reductase.

**Synthesis of *N*-Acetylglutamate 5-Semialdehyde**—*N*-Acetylglutamate 5-semialdehyde was synthesized enzymatically from acetylornithine and  $\alpha$ -ketoglutarate by a method based on that of Vogel and McLellan (12) and modified as described below. The enzyme, acetylornithine transaminase, was isolated from *E. coli* strain MA13 (*argE25*, *argR63*), derepressed for arginine biosynthesis and lacking acetylornithinase.

Cells were grown at 37 °C for 24 h in M9 medium (13) and harvested using a Sharples Model T-1 centrifuge. Cells were resuspended in phosphate buffer (0.1 M potassium phosphate, pH 7.0, 0.2 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride) to give approximately 0.15 g of cells/ml. Cells were disrupted in the Bead Beater (see above) by 5 × 1-min pulses. The crude extract was clarified by centrifugation at 20,000 × *g* for 30 min, and the supernatant was fractionated by ammonium sulfate precipitation. Acetylornithine transaminase was precipitated between 30 and 40% ammonium sulfate. The precipitate was resuspended in phosphate buffer and desalted by passage through a Sephadex G-25 column (2.6 × 44 cm). The pooled fractions containing activity could be frozen and stored at –20 °C or used directly for the enzymatic synthesis of acetylglutamate 5-semialdehyde.

Acetylglutamate 5-semialdehyde was synthesized in a reaction mixture containing 0.14 M  $\alpha$ -ketoglutarate, 0.12 M acetylornithine, 0.8 mM pyridoxal phosphate (all in 0.1 M potassium phosphate, pH 7.0 (total volume of 5 ml)), and 5 ml of desalted enzyme. The pH of the reaction mixture was adjusted to 7.0 just prior to the addition of the enzyme. The reaction was carried out for 18–24 h at 30 °C and was stopped by boiling for 3 min. The precipitated protein was removed by centrifugation, and the supernatant was acidified with 0.4 ml of 12 N HCl. One-ml aliquots were applied to AG50W-X8 ion exchange columns (H<sup>+</sup> form, 0.7 × 12 cm), which were equilibrated with 0.1 N HCl. Acetylglutamate 5-semialdehyde was eluted with 10 ml of H<sub>2</sub>O. Fractions were evaporated and pooled, and the pH was adjusted to 7.0 with 50% NaOH. The yield was 40–50%.

The amount of *N*-acetylglutamate 5-semialdehyde was determined as follows (14). An aliquot of the sample was diluted to 0.5 ml with water. The acetyl group was removed by boiling for 30 min following the addition of 0.3 ml of 6 N HCl. The reaction mixture was cooled and stopped by the addition of 1.0 ml of 3.6 M sodium acetate. The product, glutamate 5-semialdehyde, was converted to a colored product by reaction with 0.2 ml of 33 mM *o*-aminobenzaldehyde for 15 min at room temperature. An absorbance of 0.1 at 440 nm corresponds to 86 nmol of glutamate 5-semialdehyde (14). The concentration of the remaining sample was adjusted to the desired concentration (0.01 M) with water and was stored in aliquots at –20 °C.

**Enzyme Assays**—Acetylglutamate kinase was assayed using a radioactive assay as previously described (2). One unit of enzyme is defined as the amount required to produce 1  $\mu$ mol of product in 1 min at 30 °C. Acetylglutamyl-phosphate reductase was assayed by following the increase in fluorescence as NADP<sup>+</sup> was converted to NADPH (12). The reaction mixture contained 0.1 M glycine, pH 9.3, 1.33 mM acetylglutamate 5-semialdehyde, 25 mM potassium phosphate, pH 9.3, 0.67 mM NADP<sup>+</sup> (freshly prepared) and 1.0 ml of appropriately diluted enzyme in a final volume of 3.0 ml. The reaction was initiated by the addition of enzyme, and the increase in fluorescence was followed using a Gilson Spectra/Glo filter fluorometer (excitation filter, 330–380 nm, and emission filter, 430–600 nm). The

<sup>1</sup> The abbreviation used is: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

reactions were carried out at 25 °C. The activity is expressed as the change in fluorescence/min.

**Determination of Native Molecular Weights**—A 7.5 × 300-mm LKB TSK-3000 high pressure liquid chromatography gel filtration column was used to determine the native molecular weights of acetylglutamate kinase and acetylglutamyl-phosphate reductase. The column was equilibrated with running buffer (30 mM potassium phosphate, pH 7.0, 5 mM acetylglutamate, 2 mM MgCl<sub>2</sub>, 0.2 mM dithiothreitol, 0.5 mM EDTA, 100 mM potassium chloride). The following molecular weight protein standards were used to calibrate the column: thyroglobulin (670,000), ferritin (440,000), catalase (232,000), immunoglobulin G (150,000), lactate dehydrogenase from rabbit muscle (140,000), ovalbumin (45,000), and myoglobin (17,500). The protein standards were 5 mg/ml in running buffer, and 20–30 μg of each standard was applied to the column in separate trials. The flow rate was 1 ml/min, and chromatography was monitored by absorbance at 280 nm. The void volume of the column was determined to be 6.55 ml using a 5-μg sample of blue dextran. The molecular weight was plotted versus  $K_{av}$ . The  $K_{av}$  was determined using the following formula:  $K_{av} = (V_e - V_0)/(V_t - V_0)$ , where  $V_e$  is elution volume of the protein,  $V_0$  is void volume, and  $V_t$  is bed volume of the column. Partially purified preparations of acetylglutamate kinase and acetylglutamyl-phosphate reductase were applied to the column in separate trials under conditions described for the standards. One-ml fractions were collected and assayed for activity as described above.

**Determination of Subunit Molecular Weights**—Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed according to Laemmli (15), using 11 × 14-cm running gels (10% total acrylamide, 0.26% *N,N'*-methylenebisacrylamide or 12.5% total acrylamide, 0.34% *N,N'*-methylenebisacrylamide) and 2 × 14-cm stacking gels (3.75% total acrylamide). Partially purified preparations of acetylglutamate kinase and acetylglutamyl-phosphate reductase were mixed with sample buffer to give final concentrations of 1% sodium dodecyl sulfate, 30 mM Tris, pH 6.8, 5% glycerol, 2.5% 2-mercaptoethanol, and 0.0005% bromphenol blue and boiled for 3 min. Molecular weight standards (66,000–30,000) obtained from Sigma were treated in the same manner. The gels were stained with Coomassie Brilliant Blue R or silver-stained according to Goldman *et al.* (16).

## RESULTS

**Activities in Mutant Strains**—The genetic characterization of the *arg-6* locus suggested that the locus encodes both acetylglutamate kinase and acetylglutamyl-phosphate reductase. Representative members of the three complementation groups were assayed for each of these enzymes. As shown in Table II, members of complementation group A had little or no acetylglutamate kinase activity but contained elevated activities for acetylglutamyl-phosphate reductase. Members belonging to complementation group B had acetylglutamate kinase activity but lacked acetylglutamyl-phosphate reductase. Acetylglutamate kinase activity varied from barely detectable (strains LA59 and LA305) to approximately 50% (LA302) of that observed in the wild-type strain. Mutations belonging to the noncomplementing group had little or no kinase activity and lacked reductase activity.

**Separation of the Kinase and Reductase**—Since the *arg-6* locus encodes both the kinase and the reductase, it was of interest to determine whether these activities reside on a single bifunctional polypeptide or whether they represent separable enzymes as is the case in *S. cerevisiae* (6). Several different approaches were used to address this question. Mitochondria were freed of vacuoles by extensive purification in order to remove contaminating protease activity. In addition, purification was carried out in the presence of a protease inhibitor. A crude mitochondrial matrix fraction was prepared, and aliquots were subjected to gel filtration chromatography, Dyematrix chromatography, and ammonium sulfate fractionation.

As shown in Fig. 1, gel filtration chromatography on Sephadex G-200 yielded two well-separated peaks of activity, representing 100% of the acetylglutamate kinase activity and 54% (unstable in dilute form) of the acetylglutamyl-phosphate reductase activity applied to the column. Variations in the extraction technique including digitonin lysis of the mitochondria or sonication under conditions minimizing proteolytic damage failed to yield any evidence of a larger species having both activities.

TABLE II  
Enzyme activities in various strains of *N. crassa*  
All strains were grown in Vogel's minimal medium supplemented with 0.1 g/liter arginine. Mitochondria were purified from the indicated strains, lysed, and used to assay for the activities of acetylglutamate kinase and acetylglutamyl-phosphate reductase.

Strain	Allele	Medium	Enzyme activities	
			Kinase microunits/mg	Reductase ΔF/Δt/mg
Wild type				
LA1		MM	176.9	1.85
<i>arg-6</i> : complementation group A				
LA54	CD29	MM + arginine	4.0	2.83
LA322	CD136	MM + arginine	3.2	2.75
LA323	CD174	MM + arginine	3.5	2.36
<i>arg-6</i> : complementation group B				
LA302	CD34	MM + arginine	84.1	0.01
LA319	CD116	MM + arginine	36.4	0.01
LA310	CD65	MM + arginine	18.3	0.03
LA317	CD92	MM + arginine	12.4	0.00
LA304	CD44	MM + arginine	10.2	0.00
LA318	CD111	MM + arginine	6.3	0.03
LA59	CD25	MM + arginine	4.3	0.01
LA305	CD46	MM + arginine	3.1	0.02
<i>arg-6</i> : noncomplementing group				
LA55	CD63	MM + arginine	0.1	0.00
LA56	CD68	MM + arginine	4.6	0.00
LA320	CD121	MM + arginine	0.1	0.00

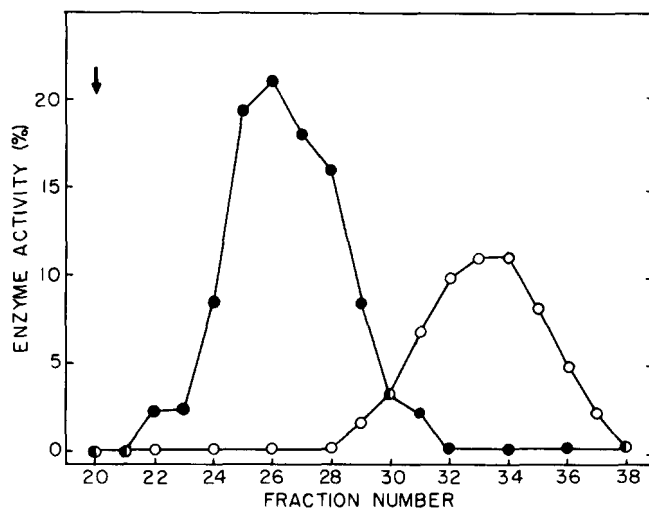


FIG. 1. Separation of acetylglutamate kinase and acetylglutamyl-phosphate reductase activities by gel filtration on Sephadex G-200. Mitochondria were purified from mycelia of strain LA16, and 3.0 ml of a matrix fraction was applied to the column. Ten-ml fractions were collected. The arrow indicates the void volume. Per cent enzyme activity recovered: ●—●, acetylglutamate kinase; ○—○, acetylglutamyl-phosphate reductase.

adex G-200 yielded two well-separated peaks of activity, representing 100% of the acetylglutamate kinase activity and 54% (unstable in dilute form) of the acetylglutamyl-phosphate reductase activity applied to the column. Variations in the extraction technique including digitonin lysis of the mitochondria or sonication under conditions minimizing proteolytic damage failed to yield any evidence of a larger species having both activities.

Two additional approaches were used in order to further substantiate the conclusion that the two enzyme activities reside on distinct polypeptides. First, the kinase and reductase

TABLE III

Separation of acetylglutamate kinase and acetylglutamyl-phosphate reductase activities by Blue A Dyematrix affinity chromatography

Mitochondria were purified from strain LA16 mycelia, and 400  $\mu$ l of a matrix fraction was applied to the column. The column was eluted as described under "Experimental Procedures."

Elution conditions	Enzyme activities	
	Kinase	Reductase
	% recovered	
Blue A start buffer	0	0
NADP	28	92
EDTA	126	0

activities were found to precipitate in distinct but overlapping fractions upon ammonium sulfate fractionation (data not shown). In the second approach, a crude extract was concentrated by ammonium sulfate precipitation and desalted on Penefsky columns, and the enzymes were then separated by specific stepwise elution from a Blue A Dyematrix column as shown in Table III. Elution with 1 mM NADP<sup>+</sup> resulted in recovery of 92% of the reductase activity. Subsequent elution with 10 mM EDTA yielded greater than 100% of the kinase activity applied to the column. This is probably due to inhibition of the kinase activity in the crude extract by residual sulfate, which is subsequently removed during chromatography on the Dyematrix column.

**Determination of Molecular Weight**—In order to get additional information on the structure of the *arg-6*-encoded protein(s), we determined the native and subunit molecular weights. Native molecular weights were determined by gel filtration chromatography on TSK-3000. Acetylglutamyl-phosphate reductase and acetylglutamate kinase activities migrated with molecular weights corresponding to 93,000 and 400,000, respectively (Fig. 2). Partially purified preparations of acetylglutamate kinase and acetylglutamyl-phosphate reductase were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and several bands were evident in both sample lanes (data not shown). Based on several trials using both 10 and 12.5% polyacrylamide gels, the subunit molecular weight of acetylglutamate kinase was determined to be between 49,000 and 52,000. This is consistent with an octameric structure. Acetylglutamyl-phosphate reductase had an apparent subunit molecular weight between 38,000 and 46,000, consistent with a dimeric structure. The purification, further characterization of these enzymes, and determination of the subunit molecular weights of the purified proteins will be presented elsewhere.<sup>2</sup>

## DISCUSSION

The *arg-6* locus had previously been shown to be genetically complex (1). The absence of kinase and reductase activities in several strains carrying mutations at the *arg-6* locus indicates that it codes for both enzymes (Table II). The results of the biochemical characterization of 40 mutations at the *arg-6* locus are consistent with the expectations based on the genetic data. All the mutants in complementation group A lack acetylglutamate kinase activity, those in group B lack acetylglutamyl-phosphate reductase activity, and those in the noncomplementing group lack both activities.

The *arg-6* alleles were also screened for suppressibility by a nonsense suppressor (3). The results, summarized in Table IV, revealed a nonrandom distribution of suppressible alleles among the three complementation groups. Furthermore, a

<sup>2</sup> A. U. Wandinger-Ness and R. L. Weiss, manuscript in preparation.

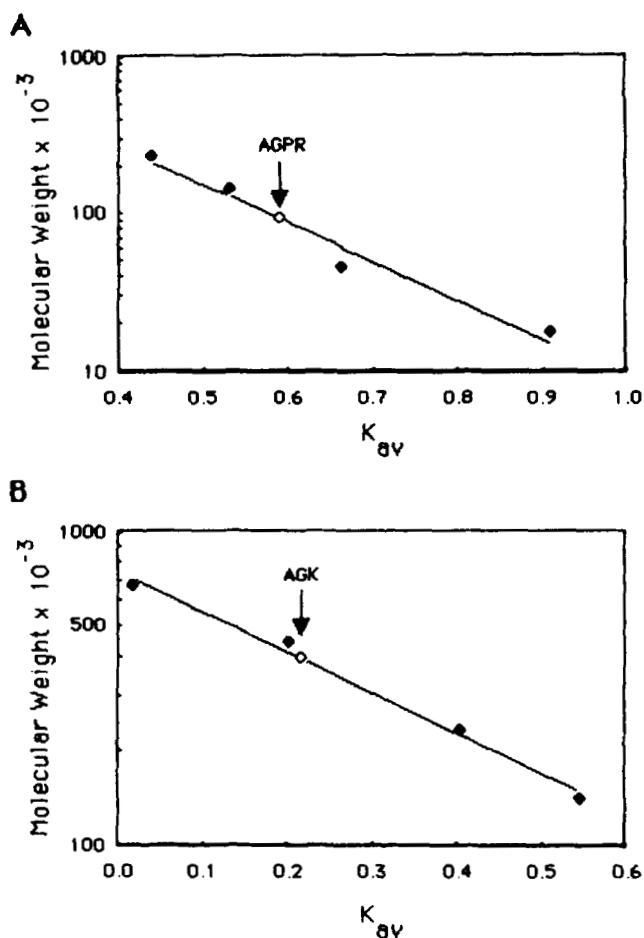


FIG. 2. Molecular weight versus  $K_{av}$  plots as determined for acetylglutamyl-phosphate reductase (AGPR) (A) and acetylglutamate kinase (AGK) (B) on a TSK-3000 column as described under "Experimental Procedures." Closed symbols represent molecular weight standards listed under "Experimental Procedures." Open symbols represent peak enzyme activities of acetylglutamyl-phosphate reductase and acetylglutamate kinase, respectively.

TABLE IV

Genetic analysis of *arg-6* mutants

Data are from Ref. 3.		
Complementation group	No. of mutants	Suppressible mutants
A	4	0
B	27	12
Noncomplementing	12	1

strong translational polarity was observed in the case of the noncomplementing class mutants, where both of the activities are absent as a result of a single mutation (both are restored in the presence of a nonsense suppressor). These results suggested that the *arg-6* locus encodes a bifunctional protein, with acetylglutamyl-phosphate reductase being translation-distal.

Multifunctional loci in which several enzymatic activities are associated with a single polypeptide chain have been reported in several eukaryotic organisms (17, 18). One advantage of a bifunctional protein would be that the activities which synthesize and utilize the labile intermediate, acetylglutamyl phosphate, are on the same protein. However, the kinase and reductase activities were readily separable as judged by several criteria (Figs. 1 and 2 and Table III). This is also true in *S. cerevisiae* (6). Even the addition to all buffers of a protease inhibitor, phenylmethylsulfonyl fluoride (which

established the existence of a pentafunctional protein of the *arom* complex in *N. crassa* (14)), failed to provide any evidence for a large bifunctional protein in the mitochondrial matrix.

Three models of the gene-protein relationship can be proposed based on these results. First, the *arg-6* locus in fact comprises two loci with corresponding mRNAs and polypeptides. If this were the case, the noncomplementing group, lacking both activities, would be rare, and none would be expected to be a nonsense mutation. Neither expectation is borne out. Second, the locus is transcribed into a single mRNA transcript which is processed into two, each encoding a separate enzyme. Alternatively, a single mRNA could have two independent initiation sites for protein synthesis, one for each enzyme. These models would not be compatible with the large number of noncomplementing mutants (a nonsense mutant would not be expected to have a polar effect), and polycistronic mRNAs have thus far been found only in prokaryotic organisms. Third, the locus encodes a single mRNA which is translated into a single polypeptide. The latter is then cleaved to yield the two physically separable enzymes. In this model, many mutations of the noncomplementing class would be expected and some of these would be suppressible nonsense mutations. No complementing nonsense mutations would be expected in the translation-proximal region because their polarity would preclude complementation activity for the distal function.

Both of the expectations of the one mRNA/one protein model are realized: there are many noncomplementing mutants, one a nonsense type, and there are no nonsense mutants in the admittedly small sample of group A mutants. In this model, the group B (reductase<sup>-</sup>) mutations (among which are many nonsense mutants) are presumed to be translation-distal. The failure to detect a larger molecular weight precursor form carrying both activities is not inconsistent with this model for several reasons. Such a precursor might be inactive and cleavage may be concomitant with import into mitochondria or it might be present in small quantities and/or unstable. There is accumulating evidence that proteins destined for the mitochondrion are synthesized in the cytoplasm usually as larger molecular weight precursors (19, 20) and/or in an inactive form (21). These precursors are post-translationally processed, and subsequently imported following binding to a putative mitochondrial receptor (reviewed in Ref. 22).

The observation that mutants lacking acetylglutamyl-phosphate reductase (complementation group B) had decreased acetylglutamate kinase activity can also be explained by the proposed model. The mutation might interfere with normal processing and/or transport or an incorrectly processed form may be less active. Alternatively, even though the acetylglutamate kinase and acetylglutamyl-phosphate reductase reside on different polypeptides, they are likely to be associated *in vivo* to ensure efficient utilization of the labile intermediate, acetylglutamyl phosphate. Therefore, in the absence of functional acetylglutamyl-phosphate reductase, acetylglutamate kinase may be less stable or less active.

The determination of native and subunit molecular weights of acetylglutamate kinase and acetylglutamyl-phosphate re-

ductase further supports the hypothesis that these activities reside on distinct polypeptides. Acetylglutamate kinase appears to be an octameric protein, while acetylglutamyl-phosphate reductase is a dimer. The proposed model that these proteins arise from a single precursor implies that they are synthesized stoichiometrically. If the kinase and the reductase are associated *in vivo*, these data suggest that four acetylglutamyl-phosphate reductase dimers are associated with every acetylglutamate kinase octamer. This association, if it exists, is not conserved *in vitro* in cell extracts, since the activities were never found to co-migrate during chromatography on a variety of resins. Further work is necessary before definitive conclusions about the structure and/or assembly of these proteins *in vivo* can be made.

A direct test of the proposed model requires that a single precursor polypeptide containing acetylglutamate kinase and acetylglutamyl-phosphate reductase be detected. If this is not found to be the case, then translation of the two enzymes may reveal a novel mechanism in eukaryotic protein synthesis, mRNA metabolism, or protein-protein interaction. The more extensive analysis of the yeast *argBC* locus has yielded the same picture of the two enzymes' genetic determination and the same hypotheses of gene-enzyme relationship. Whatever mechanism of gene action prevails, it is probably common to the two organisms.

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