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Relationship between Two Major Immunoreactive Forms of Arginase in *Neurospora crassa*

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Two major immunoreactive proteins of M_r 41,700 and 36,100 have been detected in crude mycelial extracts with polyclonal antibodies raised against arginase purified from *Neurospora crassa*. The latter corresponded to the protein used to obtain the antibodies. Both polypeptides were either missing or present in very low amounts in mutant strains having little or no detectable arginase activity. The relative proportion of the two species was altered in strains containing the nitrogen catabolite regulatory mutation *nit-2*. Peptide mapping indicated that the two species are very closely related, but several of the peptides which appeared to be identical by staining reacted differently with the antibodies. Both species were produced by in vitro translation of poly(A)$^+$ mRNA, although the larger species was produced to a much smaller extent than was expected from its abundance in vivo. The results suggest the existence of multiple forms of arginase in *N. crassa* which differ in their response to nitrogen catabolite regulation.

Arginase (EC 3.5.3.1) from *Neurospora crassa* is a catabolic enzyme which is maximally active under conditions of nitrogen starvation (24) or arginine excess (40). The specific activity of arginase increases threefold when mycelia are grown with arginine as the sole nitrogen source. This induction is accompanied by a 75-fold increase in the cytoplasmic pool of arginine (13). However, it is not known whether this increase in enzyme activity results from de novo protein synthesis or from activation of preexisting arginase molecules.

When a wild-type strain is grown in medium containing glutamine, glutamate, or ammonium, the addition of arginine causes only a small increase in arginase specific activity (37). This effect has been termed "nitrogen catabolite control." Arginase is present at fully induced levels in mutant strains having the *am* mutation (lacking NADP:glutamate dehydrogenase, and hence having low glutamate and glutamine pools) whether the strain is grown in arginine alone or in arginine-ammonium medium (37). This suggests that nitrogen catabolite control is mediated by glutamine or glutamate or by a secondary effect directly influenced by these two species.

One known nitrogen catabolite repression mechanism is mediated by the *nit-2* locus in *N. crassa*. The *nit-2* gene product is a positive, *trans*-acting, pleiotropic regulatory effector necessary for expression of genes whose products are required for utilization of several alternative nitrogen sources (31). Glutamine appears to cause inactivation of the *nit-2* gene function, possibly through maintenance of an octameric glutamine synthetase, which may itself be the negative effector (14). Arginase levels are unaffected by the *nit-2* mutation (15), indicating that the *nit-2* regulatory mechanism is not responsible for nitrogen control of arginase levels. The *nmr* locus, which is responsible for nitrogen catabolite repression of nitrate assimilatory genes, also does not appear to regulate arginase levels in *N. crassa* (15, 36).

We recently reported preliminary evidence for two and possibly three forms of arginase in *N. crassa*; two major immunoreactive proteins were detected by immunoblot analysis of crude extracts with antiserum directed against the purified enzyme (5a). The latter migrates as a single species during sodium dodecyl sulfate-gel electrophoresis. We have attempted to determine the structural relatedness of the two major immunoreactive proteins and to determine whether they arise from the same mRNA molecule. Also, because the mechanism of induction and its modulation by nitrogen source are unknown, the antiserum raised to the purified protein was used as a probe of protein levels in various nitrogen regulatory mutants.

Here we report that the two major protein species are structurally similar but appear to be encoded by separate poly(A)$^+$ mRNA molecules. We found that the induction of arginase by arginine is due to de novo protein synthesis, resulting in an increased amount of both proteins. We failed to detect induction of arginase in log-phase mycelia of mutants containing the *nit-2* mutation and observed a difference in the relative amounts of the two proteins.

MATERIALS AND METHODS

Strains and growth conditions. The *N. crassa* wild-type strain (LA1) and strains with four different *aga* mutations (alleles UM-903, UM-906, UM-908, and UM-913) were provided by R. H. Davis. The nitrogen regulatory mutant LA116 (*nit-2*, allele nr37) was obtained from the Fungal Genetics Stock Center, Kansas City, Mo. (FGSC983). The nitrogen regulatory mutant LA134 (*nmr*, allele 304) was provided by R. H. Garrett. All strains were grown at 30°C in Vogel minimal medium (38) with 1.5% sucrose as the carbon source. Where indicated, mycelia were grown in minimal medium with or without NH_4NO_3 and with 0.1% arginine. Strains having an *aga* mutation were supplemented with 0.02% putrescine when grown in arginine-supplemented medium. Mycelia were collected by filtration in the early- to mid-log phase unless noted otherwise. The mycelia to be used as a source of arginase for immunoblot analysis or peptide mapping could be stored at −20°C until needed. Mycelia to be used for RNA purification were processed immediately.

For preparation of [^{35}S]sulfate-labeled extract, strain LA1 was grown in minimal medium without sulfate or NH_4NO_3

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but supplemented with 0.1% arginine and 80 μM $MgSO_4$. $Na^{35}SO_4$ (ICN Pharmaceuticals Inc.) was added to a final level of 4 μCi/ml, and the culture was grown to the stationary phase. For preparation of poly(A)$^+$ mRNA, strain LA1 was grown in minimal medium without NH_4NO_3 supplemented with 0.1% arginine. Strain LA116 was grown in the above medium plus NH_4NO_3.

Arginase assay. Arginase activity was measured by the procedure of Davis and Mora (12). Urea was quantitated by the method of Archibald (3). One unit of activity is the production of 1 μmol of urea per min.

Protein assay. Protein was quantitated by the method of Bradford (6) with the Bio-Rad protein reagent concentrate microassay technique. Bovine serum albumin was used as a standard.

Antiserum generation. The antiarginase polyclonal serum used for these studies was described elsewhere (5a). Polyclonal antiserum raised against purified *N. crassa* ornithine carbamoyltransferase was the gift of Marjorie Bates.

Immunoglobulin purification. The immunoglobulin fraction from antiserum raised against arginase was purified by a modification of the technique of Levy and Sober (25). Serum (5 ml) was brought to 33% saturation with solid $(NH_4)_2SO_4$ and stirred for 30 min at 4°C. The solution was then centrifuged at 27,000 × *g* for 15 min at 2°C. The pellet was suspended in a small volume of 17.5 mM NaH_2PO_4 (pH 6.3) and dialyzed three times for 4 h against 1 liter of the same buffer at 4°C. The dialyzed solution was applied to a 5- by 2.5-cm DE-52 (Whatman) column equilibrated with the dialysis buffer at 4°C. Proteins were eluted with the dialysis buffer. Fractions having absorbance at 280 nm were pooled and concentrated (Amicon Ultrafiltration Cell, YM10 membrane) at 4°C. The purity of the immunoglobulin fraction was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with slab gels with a 10% resolving gel (5a). Activity depletion assays (data not shown) indicated that 20 μl of immune serum or 10 μl of purified immune immunoglobulin was sufficient to precipitate all of the arginase activity and protein from 1 mg of crude extract protein (induced wild-type culture). Twice the volume of the corresponding preimmune serum or purified preimmune immunoglobulin contained an equivalent amount of immunoglobulin and was used as a control.

Immunoblot analysis. Mycelia were extracted with a Beadbeater (Biospec Products) apparatus or by sand grinding as described elsewhere (5a). The extraction buffer contained 50 mM Tris chloride, 20 mM arginine, 1 mM EDTA, 1 mM 2-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride (pH 7.5). The buffer used for preparation of extracts from strains having the *aga* mutation contained the above components plus 150 mM NaCl. Extracts were centrifuged at 27,000 × *g* for 15 min at 2°C. Crude extracts of strains with *aga* mutations were immunoprecipitated to concentrate the arginase before immunoblot analysis. Extracts were centrifuged in a microcentrifuge for 10 min at 4°C to remove any residual aggregated material. A sample containing 1 mg of crude extract protein was treated with 30 μl of a 10% suspension of *Staphylococcus aureus* cells for 15 min at 0°C followed by centrifugation for 2 min at 4°C in a microcentifuge. The clarified supernatant was removed to a new tube, 20 μl of antiserum was added, and the solution was incubated at 0°C for 45 min. Then 40 μl of the 10% *S. aureus* cell suspension was added, and incubation was continued at 0°C for 60 min. The mixture was centrifuged in a microcentrifuge at 4°C for 2 min, and the supernatant was discarded. The pellet was washed by vigorous mixing in 500 μl of 50 mM Tris chloride–150 mM NaCl–5 mM EDTA–1 mM 2-mercaptoethanol–1 mM phenylmethylsulfonyl fluoride–0.05% Triton X-100 (pH 7.5) and centrifuged for 1 min at room temperature, the supernatant was discarded. This washing step was repeated twice. Arginase-antibody complexes were solubilized by adding 40 μl of 3% sodium dodecyl sulfate and heating at 100°C for 5 min. The suspension was centrifuged at room temperature in a microcentrifuge, and the supernatant was retained for immunoblot analysis.

Immunoblot analysis of samples derived from 50 μg of crude extract protein was as described elsewhere (5a). Purified arginase (50 to 250 ng) was used as a standard for quantitation of the amount of arginase.

Peptide mapping. Cell extracts of strain LA116 grown in arginine-supplemented medium were prepared by sand grinding. The extraction buffer was 50 mM Tris chloride–20 mM arginine–150 mM NaCl–1 mM 2-mercaptoethanol–5 mM EDTA–1 mM phenylmethylsulfonyl fluoride (pH 7.5). The extract was centrifuged at 27,000 × *g* for 15 min at 2°C to remove cell debris. The supernatant was centrifuged again in the microcentrifuge for 10 min at 4°C.

Arginase was precipitated as described above, with the following exceptions: (i) a volume of extract containing 3 mg of protein was used; (ii) the sources of antibody were 10 μl of purified immune immunoglobulin or 40 μl of decomplemented preimmune serum; (iii) the washing buffer contained 50 mM Tris chloride–150 mM NaCl–5 mM EDTA–1 mM 2-mercaptoethanol–1 mM phenylmethylsulfonyl fluoride (pH 7.5); (iv) the wash steps with the Tris chloride buffer were followed by two additional washes with water; and (v) the addition of 40 μl of 25 mM NaOH, followed by incubation at room temperature for 15 min, was used to solubilize the arginase-antibody complexes. Samples were prepared for electrophoresis as described elsewhere (5a).

The procedure of Josefsson and Randall (19) was used for peptide mapping. Immunoprecipitated samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis with a slab gel and a 10% resolving gel. The gel was stained with Coomassie brilliant blue (39) and then washed in water. Gel slices containing the individual proteins were incubated for 5 min in 1 ml of 1× Laemmli stacking gel buffer (22). Proteolysis and second-dimension electrophoresis were performed with a slab gel with a 5-cm-tall 2.5% stacking gel and a 15% resolving gel. Gel slices were diluted in 1× Laemmli stacking gel buffer containing 0, 50, or 250 (or 200) ng of *Staphylococcus aureus* V8 protease. Molecular weight standards were 500 ng each of egg albumin (45,000), glyceraldehyde-3-phosphate dehydrogenase (36,000), β-lactoglobulin (18,400), and α-lactalbumin (14,200). Before samples were loaded on the gel, 10 μl of a solution containing 0.05% bromphenol blue and 80% glycerol was added to all protease and molecular weight standard samples.

Silver staining. Gels to be silver stained were fixed in 25% isopropanol and 10% acetic acid overnight. The gels were washed in water, incubated in 50% methanol for at least 2 h, and then washed in water again. The positions of peptide bands were determined by silver staining by the Oakley et al. protocol (28) with the following modifications. A solution of 5% Norit-EX-purified (2), unbuffered glutaraldehyde was used as the source of glutaraldehyde. The final volume of the silver solution in ammonia was 200 ml instead of 100 ml, and staining was for 1 h instead of 15 min. The color development was terminated by incubation in 50% methanol for 5 min, followed by incubation in water.

Immunoblot analysis. Gels were subjected to immunoblot analysis as outlined elsewhere (5a), except that the blotting step was overnight instead of for 2 h. Exposure was at 70°C with preflashed (23) film for 1 week.

Preparation of ^{35}S-labeled arginase. Mycelia were extracted by sand grinding as described above with 50 mM Tris chloride–20 mM arginine–1 mM 2-mercaptoethanol–1 mM EDTA–1 mM phenylmethylsulfonyl fluoride (pH 7.5). The extract was centrifuged in the microcentrifuge for 5 min at 4°C, and the supernatant was retained. This was repeated once. The supernatant was assayed for protein amount and for incorporation of ^{35}S into protein (alkali-stable, trichloroacetic acid-precipitable radioactivity).

A volume of the extract containing 7.2×10^6 cpm of ^{35}S-labeled protein was treated with 10 μl of purified immunoglobulin or 17 μl of purified preimmune immunoglobulin at 0°C for 90 min. Then 20 μl of the 10% *S. aureus* cell suspension was added, and incubation was continued for an additional 30 min. The insoluble material was collected by centrifugation in a microcentrifuge for 2 min at 4°C, washed by suspension in 500 μl of 50 mM Tris chloride–150 mM NaCl–0.1% sodium dodecyl sulfate–0.05% Triton X-100–5 mM EDTA–1 mM phenylmethylsulfonyl fluoride (pH 7.5), and collected by centrifugation in the microcentrifuge for 1 min at room temperature (1). This was repeated three times. The insoluble material was then washed once more with 500 μl of 50 mM Tris chloride–1 mM 2-mercaptoethanol–0.1 mM phenylmethylsulfonyl fluoride (pH 7.5). The antibody-antigen complexes were solubilized by treating with 35 μl of 3% sodium dodecyl sulfate at 100°C for 5 min. The suspension was centrifuged for 2 min at room temperature in the microcentrifuge, and the supernatant was withdrawn to a new tube. A volume of immune immunoglobulin-precipitated extract containing 4,000 cpm and a volume of preimmune immunoglobulin-precipitated extract containing an equivalent amount of immunoglobulin protein were subjected to electrophoresis.

Poly(A)$^+$ mRNA purification. The procedure used to purify poly(A)$^+$ mRNA was previously described (27). The average yield of poly(A)$^+$ mRNA from 16 g (wet weight) of *N. crassa* mycelia was 120 μg.

In vitro translation of poly(A)$^+$ RNA. Poly(A)$^+$ mRNA was translated in vitro with a nuclease-treated rabbit reticulocyte lysate system according to the instructions of the manufacturer (Promega Biotec). A volume of 12.5 μl (12.5 μg) of poly(A)$^+$ mRNA was treated with 1.3 μl of 50 mM methylmercury hydroxide (5 mM, final concentration) at 0°C for 5 min to disrupt any secondary structure (9, 29). Then, in the following order, were added 3 μl of 50× amino acid mixture minus methionine, 450 μCi of [^{35}S]methionine (ICN) in 40 μl, and 100 μl of rabbit reticulocyte lysate. The mixture was incubated at 28°C for 90 min. The reaction was stopped by lowering the temperature to 0°C. Incorporation of the [^{35}S]methionine into protein was determined as alkali-stable, trichloroacetic acid-precipitable radioactivity.

The reaction mixture was brought to 3% sodium dodecyl sulfate and heated at 100°C for 5 min. The solution was then diluted 10-fold with 50 mM Tris chloride–150 mM NaCl–5 mM EDTA–1 mM phenylmethylsulfonyl fluoride–1% Triton X-100 (pH 7.5). This solution was then centrifuged in the microcentrifuge for 2 min at room temperature to remove insoluble aggregates. The supernatant was transferred to new tubes so that each tube contained approximately 5×10^6 cpm of radioactivity. Each sample was treated with either 2 μl of purified immune antiarginase immunoglobulin, 3.4 μl of purified preimmune immunoglobulin, or 5 μl of immune or control preimmune anti-ornithine carbamoyltransferase serum. For competition experiments, 71 or 710 ng of purified arginase was added to some of the reactions. After incubation at 4°C overnight, 5 μl of the 10% *S. aureus* cell suspension was added to each tube, and incubation was continued for an additional 30 min. The insoluble material was collected by centrifugation in the microcentrifuge for 2 min at 4°C and washed as described above for the ^{35}S-labeled extract immunoprecipitates. The antigen-antibody complexes were solubilized in 20 μl of 3% sodium dodecyl sulfate by heating at 100°C for 5 min and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Fluorography. Immunoprecipitated ^{35}S-labeled proteins from extracts or in vitro translation reaction mixtures were visualized by fluorography with sodium salicylate (8) and preflashed film (23).

RESULTS

Immunoblot analysis of the arginaseless mutants. Whole cell extracts of the four arginaseless mutants and the wild-type strain were prepared as indicated in Materials and Methods. Immunoblot analysis was used to detect proteins which cross-reacted with antiserum directed against purified arginase (Fig. 1). The wild-type extract (74A) contained both the M_r 41,700 and M_r 36,100 proteins observed elsewhere (5a). The M_r 36,100 form corresponds to the protein possessing arginase activity which was purified. Of the four strains having different alleles of the *aga* mutation, only strain LA166 (allele UM908) contained detectable amounts of the two immunoreactive proteins. This strain and strain LA154 (allele UM903) possessed measurable arginase activity, although the two protein species could not be detected in the extract from the latter. The faint background in the other lanes resulted from the large amount of immunoglobulin used for immunoprecipitation.

Immunoblot analysis of the enzyme from the wild type and

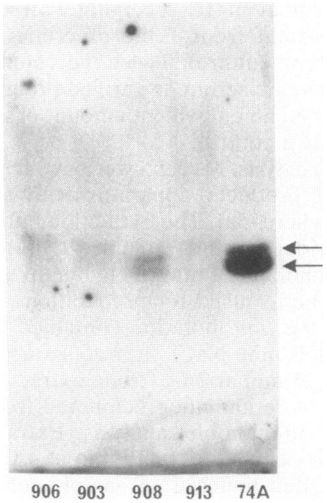

FIG. 1. Immunoblot analysis of the wild type and four arginaseless mutants. Immune replica analysis was performed as described in Materials and Methods. The lanes marked 906, 913, 908 and 903 contain extracts from the four arginaseless mutants (numbers represent alleles of the *aga* mutation), and 74A is the wild-type strain. The four mutants were grown in medium supplemented with NH_4^+, arginine, and putrescine, whereas strain LA1 was grown in the presence of arginine. The positions of the two immunoreactive species are marked with arrows.

two nitrogen regulatory mutants grown on various nitrogen sources. Whole cell extracts of strains LA1 (wild type), LA116 (*nit-2*), and LA134 (*nmr-1*) grown on various nitrogen sources were prepared as described in Materials and Methods. Immunoblot analysis with antiserum directed against arginase was performed (Fig. 2). Two major immunoreactive species (M_r 41,700 and 36,100) were detected in the extract from strain LA1 grown on ammonium-arginine (Fig. 2, lane 2) or arginine (lane 3) as the nitrogen source. The amounts of both major species and the arginase specific activity were greatest when arginine was the sole nitrogen source (Table 1).

Strain LA116 (*nit-2*) had three major immunoreactive species (Fig. 2, lanes 4 through 6). The higher-molecular-weight species may be aggregates of the major immunoreactive species. The ratio of the 41,700-M_r protein to the 36,100-M_r protein differed from that of the wild type; this mutant also failed to achieve maximum induction of arginase activity (Table 1). The differences in ratios and induction were exhibited only by log-phase mycelia; stationary-phase mycelia were identical to the wild type (data not shown). Strain LA134 (*nmr-1*) behaved similarly to the wild-type strain (Fig. 2, lanes 10 through 12): levels of the two major immunoreactive species and the specific activity of arginase (data not shown) increased upon the addition of arginine to minimal medium and were greatest with arginine as the sole nitrogen source. Controls with preimmune sera were all blank (data not shown).

Densitometric tracings of purified arginase standards (Fig. 2, lanes 7 through 9) were used to generate a standard curve, which was used to determine the amount of the immunoreactive species in the extracts of LA1 and LA116 (Table 1). The specific activity of arginase correlated most closely with the amount of the 36,100-M_r species. The amount of the 41,700-M_r form was approximately the same for both strains grown in the same medium. This result was obtained in three separate experiments with log-phase mycelia. The appearance and abundance of the 26,800-M_r protein were more variable, suggesting that it may be a degradation product of one of the other proteins.

Peptide map analysis of the immunoreactive M_r 41,700 and 36,100 proteins. The in gel proteolysis technique of Cleveland et al. (10) as modified by Josefsson and Randall (19) was used for peptide map analysis of the 41,700- and 36,100-M_r immunoreactive species. The *S. aureus* V8 protease, which cleaves on the carboxyl side of peptide bonds involving glutamate and aspartate (18), was used for the proteolysis reactions.

TABLE 1. Activity of arginase and level of immunoreactive protein in wild-type and regulatory mutant strains grown on various media

Strain	Medium	Sp act (U/mg)	ng of protein of M_r:			Ratio[a]
			41,700	36,100	26,800	
LA1	NH_4NO_3	1.24	2	17	0	8.5
	NH_4NO_3 + arginine	1.88	15	51	0	3.4
	Arginine	4.04	22	91	0	4.1
LA116	NH_4NO_3	1.11	4	16	2	4.0
	NH_4NO_3 + arginine	1.95	18	30	3	1.7
	Arginine	2.47	23	46	5	2.0

[a] Ratio of 36,100-M_r protein to the 41,700-M_r protein.

The 41,700- and 36,100-M_r proteins without added protease (Fig. 3, lanes 1 and 2) migrated as doublets when visualized by silver staining. The presence of doublets in the lanes corresponding to undigested protein is probably due to mechanical shearing or acid-catalyzed cleavage of the proteins during fixing and staining of the first-dimension gel. The addition of 50 ng of protease (Fig. 3, lanes 4 and 5) showed identical patterns of digestion products for the two proteins. The results of cleavage reactions with 250 ng of protease (Fig. 3, lanes 6 and 7) showed more complete digestion and patterns identical to those obtained with 50 ng of protease.

When analyzed by immunoblot analysis, reactions with 50 ng of *S. aureus* V8 protease with the 41,700- and 36,100-M_r proteins (Fig. 4, lanes 3 and 4, respectively) yielded several peptides which appeared to be common to both proteins and other peptides which differed in intensity or were unique to one of the two immunoreactive proteins. Treatment of the two proteins with 200 ng of protease (Fig. 4, lanes 5 and 6) yielded several common peptides, but some of these differed in apparent amount between the proteins. The 36,100-M_r species (Fig. 4, lane 6) also had some unique peptides. Overall, the peptides generated ranged in size from M_r 8,700 to 27,100. The digestion products visualized in the immunoblot (Fig. 4) were of the same molecular weight as those seen in the silver-stained gels (Fig. 3). However, the

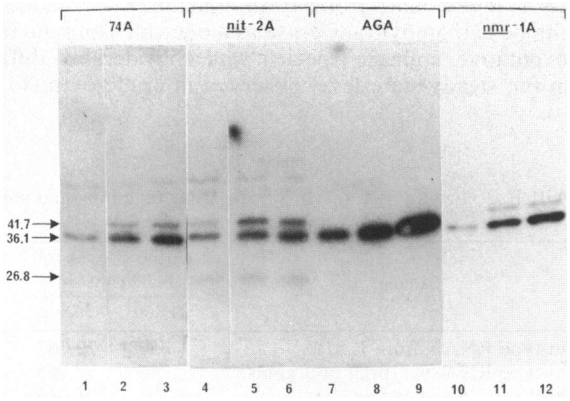

FIG. 2. Immunoblot analysis of wild-type and mutant strains grown on various nitrogen sources. Lanes: 1 through 3, wild-type strain LA1 grown on NH_4NO_3, NH_4NO_3 plus arginine, and arginine alone, respectively, as the nitrogen source; 4 through 6, same except strain LA116 (*nit-2*); 7 through 9, 200, 100, and 50 ng, respectively, of purified arginase; 10 through 12, as lanes 1 through 3, except strain LA134 (*nmr-1*).

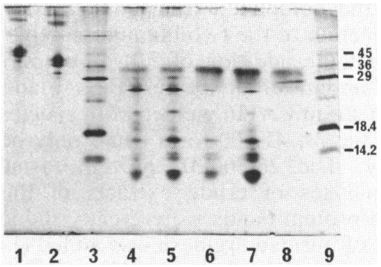

FIG. 3. Silver-stained peptide maps of the two immunoreactive proteins. Peptide mapping followed by silver staining was performed as outlined in Materials and Methods. Lanes: 3 and 9, molecular weight standards; 1 and 2, undigested 41,700- and 36,100-M_r proteins, respectively; 4 and 5, 41,700- and 36,100-M_r proteins, respectively, and 50 ng of protease; 6 and 7, 41,700- and 36,100-M_r proteins, respectively, and 250 ng of protease; 8, 250 ng of protease alone.

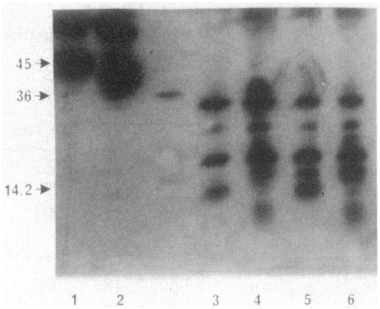

FIG. 4. Immunoblot analysis of peptide maps from the two immunoreactive proteins. Peptide mapping followed by immune replica analysis was performed as described in Materials and Methods. The positions of molecular weight markers were determined by amido black staining of one lane of the blot as described in Materials and Methods. The numbers by the arrows correspond to the molecular sizes in kilodaltons of proteins that migrate to those positions. Lanes: 1 and 2, undigested 41,700- and 36,100-M_r proteins, respectively; 3 and 4, 41,700- and 36,100-M_r proteins, respectively, and 50 ng of protease; 5 and 6, 41,700- and 36,100-M_r proteins, respectively, and 200 ng of protease.

relative intensity of specific peptide bands differed for the two proteins in Fig. 4, whereas it was invariant in Fig. 3.

Translation of poly(A)$^+$ mRNA from the wild type and the *nit-2* mutant. To further investigate the relationship between the two major immunoreactive species, poly(A)$^+$ mRNA was purified from strains LA1 and LA116 and translated in vitro by using a rabbit reticulocyte lysate. An amount of purified antibody adequate to precipitate all reactive species was used for immunoprecipitation of translated proteins from samples containing equal amounts of incorporated ^{35}S label after the in vitro translation reaction. Antibodies directed against ornithine carbamoyltransferase were used to standardize the amounts of translated material, since the level of this enzyme is not regulated by the nitrogen source in *N. crassa* (11).

A crude extract of strain LA1 was uniformly labeled with $^{35}SO_4$ and immunoprecipitated with immune immunoglobulins to provide a control for steady-state amounts of the two proteins (Fig. 5, lane 7). Preimmune immunoglobulin yielded no proteins visible in crude extracts (data not shown) or in in vitro translation reactions (Fig. 5, lanes 2, 5, 9, and 13).

The 41,700- and 36,100-M_r species, plus some fainter bands, were produced by translation of RNA from strain LA1 (Fig. 5, lane 6). The addition of 71 ng of unlabeled arginase to the completed translation mixture caused no apparent depletion of the two immunoreactive species (Fig. 5, lane 3), but the addition of 710 ng of purified arginase reduced the amount of all species (Fig. 5, lane 4).

RNA from strain LA116 yielded both species (Fig. 5, lane 8), although the M_r 41,700 form could only be detected by densitometry. The 26,800-M_r protein visualized in immunoblot analyses of crude extracts of this strain was absent. Both protein bands were greatly reduced or absent when 710 ng of purified arginase was added (Fig. 5, lane 9).

RNA from strains LA1 and LA116 yielded a dark band corresponding to a species of M_r 41,400 when treated with anti-ornithine carbamoyltransferase serum (Fig. 5, lanes 10 and 11). Neither this band nor any of the background bands was affected by the addition of 710 ng of purified arginase (Fig. 5, lane 12).

Table 2 is a summary of the relative band intensities determined by densitometric tracing of the fluorograph in Fig. 5. The numbers have been corrected for differences in mRNA levels and translation efficiency by using the ornithine carbamoyltransferase as a control. The ratio of the two putative arginase species was considerably different than the steady-state level observed in crude extracts. Im-

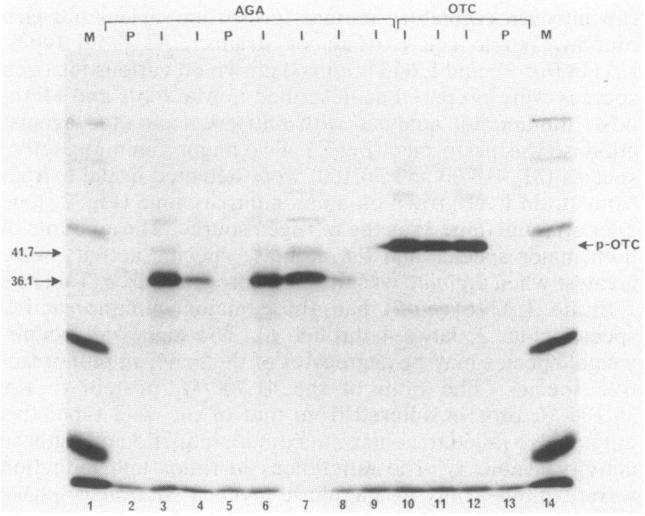

FIG. 5. Immunoprecipitation of crude extract and in vitro-translated, ^{35}S-labeled proteins. Immunoprecipitation was as outlined in Materials and Methods. Lanes; 1 and 14, ^{14}C-labeled molecular weight standards (the arrows mark the positions of 41,700- and 36,100-M_r immunoreactive proteins); 2 through 6, immunoprecipitated proteins from in vitro translation reactions with poly(A)$^+$ mRNA from strain LA1 and anti-arginase (AGA) antibody; 2, preimmune-precipitated protein and 710 ng of purified arginase; 5 and 6, preimmune immunoglobulin- and immune immunoglobulin-precipitated reactions, respectively; 3 and 4, immune immunoglobulin-precipitated material and 71 and 710 ng of purified arginase, respectively; 7, material precipitated with anti-arginase immunoglobulin from an ^{35}S-labeled crude extract of strain LA1, 8 and 9, antiarginase-precipitated proteins from in vitro translation reactions with poly(A)$^+$ mRNA from strain LA116, and 0 and 710 ng of purified arginase, respectively; 10 through 13, in vitro translation products immunoprecipitated with anti-ornithine carbamoyltransferase antibody; 13, preimmune serum-precipitated material from a strain LA1 reaction; 10 and 11, immune serum-precipitated proteins from LA1 and LA116 reactions, respectively; 12, immune serum-precipitated material from a strain LA1 reaction and 710 ng of AGA. Abbreviations: I, immune antibody or serum used for precipitation; P, preimmune antibody or serum used for precipitation; M, molecular weight standards; OTC, ornithine carbamoyltransferase.

TABLE 2. Products of in vitro translation reactions and steady-state labeling

Sample	Arbitrary units of proteins of M_r:		Ratio
	41,100	36,500	
Wild-type RNA (strain LA1)[a]	754	22,582	30.0
Wild-type RNA + 710 ng of arginase[b]	246	7,955	32.0
Wild-type, ^{35}S-labeled extract[c]	4,796	21,043	4.4
Mutant RNA (strain LA116)	545	5,761	11.0

[a] The indicated source of RNA was translated in vitro, and antigenic proteins were precipitated by using antiarginase antibody.

[b] The indicated amount of purified arginase was added during the immunoprecipitation.

[c] An ^{35}S-labeled extract was immunoprecipitated by using antiarginase antibody.

munoprecipitation of both species was affected equally by the addition of purified arginase.

DISCUSSION

The 36,100- and 41,700-M_r proteins detected by immunoblot analyses with antiarginase immunoglobulin were present in the wild type and the two nitrogen regulatory mutants under every growth condition tested. The 26,800-M_r species was often absent. Immunoblot analysis of four arginaseless strains revealed that the 41,700- and 36,100-M_r proteins were present or absent in tandem. These data suggest that the 41,700- and 36,000-M_r proteins are encoded or regulated by the *aga* locus in *N. crassa*.

The amounts of the immunoreactive proteins in crude extracts varied as a function of the strain and the growth medium. Strain LA134 (*nmr*) and LA1 (wild type) exhibited the same increase in activity and immunoreactive proteins in response to arginine in the growth medium. Thus, the *nmr* locus appears to have no effect on the induction of arginase. However, strain LA116 (*nit-2*) differed from strains LA1 and LA134 in several ways. The 26,800-M_r protein was present under all growth conditions tested, and this strain did not achieve the same levels of arginase activity as did strain LA1 when grown on arginine as the sole nitrogen source. The ratio of the 36,100-M_r protein to the 41,700-M_r protein was lower than that of strain LA1 under the same growth conditions due to a decrease in the amount of the 36,100-M_r form. The above observations are consistent with the 36,100-M_r species possessing all or most of the enzymatic activity of arginase. This is also supported by the results of purification of arginase from *N. crassa*: the 36,100-M_r protein was purified and possessed substantial activity (5a); chromatographic fractions containing predominantly the 41,700-M_r species had little or no enzymatic activity (Borkovich and Weiss, unpublished observations).

A defective arginine transport system in strain LA116 (15) may prevent entry of sufficient arginine into mycelia to achieve full induction of arginase. This effect would have to be seen in log-phase cultures grown on ammonium nitrate (results presented here) but not in stationary-phase mycelia cultured with ammonium tartrate and hypoxanthine (14), possibly due to the existence of a higher arginine pool in stationary-phase mycelia than in rapidly dividing log-phase cultures. However, because the M_r 41,700 species is present at the same levels in strain LA116 as in strain LA1, its regulation must be more sensitive to arginine than that of the 36,100-M_r species or influenced by a secondary metabolite or locus.

The 41,700- and 36,100-M_r proteins yielded identical silver-stained peptide maps after cleavage by *S. aureus* V8 protease, whereas immunoblot analysis yielded a slightly different pattern of digestion products for the two proteins. The differences seen by immunoblot analysis may be due to peptides of differing antigenicity but the same molecular weight. This suggests that the 41,700- and 36,100-M_r proteins may be distinct proteins with differing primary amino acid sequences. The overall difference in molecular weight is not apparent in the silver-stained maps: the peptides appear to be the same size. This may be due to (i) nonspecific protease digestion; (ii) differences in peptide sizes too small to be detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis; or (iii) differences at the amino or carboxyl terminus whose peptide product(s) from the 41,700-M_r protein run(s) at the dye front of the gel.

Translation of poly(A)$^+$ mRNA from strain LA1 grown with arginine as the nitrogen source resulted in the production of both the 41,700- and 36,100-M_r immunoreactive proteins. Poly(A)$^+$ mRNA from strain LA116 also yielded both species. The appearance of both proteins as in vitro translation products suggests that the two proteins are not posttranslational variants of one another, whether by covalent modification or proteolysis. These observations suggest that the arginine-mediated control of arginase levels occurs before final production of poly(A)$^+$ mRNA, either at transcription, during splicing of the heterogeneous nuclear RNA, or in the stability of the RNA produced, and that induction of arginase is due to an increase in the level of the 36,100-M_r protein.

The incorporation of $^{35}SO_4$ into the two protein species was equal to the protein ratio found after immune replica analysis under the same conditions, indicating that sulfur-containing amino acids are distributed proportionately between the two species. However, the ratio of the two proteins after in vitro translation was 10 times higher than the corresponding ratio of protein in crude extracts. This could be due to a greater amount of methionine present in the 36,100-M_r protein than the 41,700-M_r protein. It could also be explained by differential translational control of the two mRNAs or preferential proteolysis of the 36,100-M_r protein species in vivo or both.

The 26,800-M_r protein was not produced during in vitro translation reactions with mRNA from strain LA116. A protein of this approximate molecular weight was present in translation reactions with mRNA from strain LA1, but it varied in intensity with other background bands present in the fluorograph. In other in vitro translation experiments, this species was present in overexposed fluorographs of preimmune-treated reactions. Therefore, it is likely that the 26,800-M_r form is not translated from a unique poly(A)$^+$ mRNA but results from proteolysis of either the 41,700- or 36,100-M_r protein in vivo.

Several models could explain the relationship between the M_r41,700 and 36,100 proteins. Because both proteins are present or absent in tandem in the arginaseless strains, the *aga* mutation may represent a regulatory locus encoding a positive regulator of the structural genes for the 41,700- and 36,100-M_r proteins. Both the 41,700- and 36,100-M_r proteins would have to possess enzymatic activity in this model, and an arginaseless strain could be generated only if the regulatory locus or both structural genes were mutated. An alternative to this model is that the 41,700-M_r protein is related in structure and regulation, but not in function, to the enzymatically active 36,100-M_r protein. However, because all mutant strains selected for arginase deficiency contain both or neither of the two proteins, it is unlikely that their structural and regulatory similarity is coincidental.

The translation results could also be explained by differential splicing of a single heterogeneous nuclear RNA or preferential stabilization of separate poly(A)$^+$ mRNA molecules. Regulation of splicing would have to yield more of the poly(A)$^+$ mRNA coding for the 36,100-M_r protein. However, since the amount of poly(A)$^+$ mRNA encoding the 41,700-M_r species is relatively more abundant at lower arginine concentrations, the splicing reaction would itself have to be regulated by arginine. If a model involving differential stabilization of poly(A)$^+$ mRNA molecules is invoked, then an increase in arginase level by arginine could be effected by increased stabilization of poly(A)$^+$ mRNA molecules with or without an increase in transcription. This mechanism requires the preferential stabilization of poly(A)$^+$ mRNA encoding the 36,100-M_r protein or instability of the 41,700-M_r poly(A)$^+$ mRNA. Evidence for stabilization and destabiliza-

tion of RNA as a control of gene expression has been reported in *Saccharomyces cerevisiae* for arginase and ornithine carbamoyltransferase RNAs (26).

A final possibility requires the existence of alternate promoters for transcription of separate heterogeneous nuclear RNA molecules encoding the 41,700- and 36,100-M_r proteins. The promoter for the 41,700-M_r species mRNA would have to be more sensitive to the arginine pool and result in greater increases in the amount of mRNA for the 41,700-M_r protein. However, the promoter for the 36,100-M_r protein mRNA would have to be transcribed more efficiently generally. Evidence for alternate promoters for a given gene has been reported for the yeast invertase gene (7). Under various growth conditions, synthesis of the two mRNAs coding for the cytoplasmic and secreted forms of invertase can be coordinately or independently regulated (7, 30). Another case of a single structural gene with multiple promoters is the glutamine synthetase gene in *Escherichia coli* (32). This gene gives rise to two different mRNA molecules, again with some coordinate and some independent means of regulation.

Determination of the exact relationship between the 41,700- and 36,100-M_r proteins obviously requires further investigation. However, it seems clear that the structure and regulation of arginase in *N. crassa* is fairly complex. The existence of multiple forms of arginase in different mammalian tissues and their differential regulation is well documented (4, 5, 16, 17, 20, 21, 33–35). If the 41,700- and 36,100-M_r proteins both possessed arginase activity, then *N. crassa* would be the first nonvertebrate known to contain more than one form of arginase.

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