A New Yeast Metabolon Involving at Least the Two First Enzymes of Arginine Biosynthesis

ACETYLGLUTAMATE SYNTHASE ACTIVITY REQUIRES COMPLEX FORMATION WITH ACETYLGLUTAMATE KINASE*

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Open reading frame YJL071W of Saccharomyces cerevisiae was shown to be ARG2 and identified as the structural gene for acetylglutamate synthase, first step in arginine biosynthesis. The three Ascomycete acetylglutamate synthases characterized to date appear homologous, but unlike the other enzymes of the yeast arginine biosynthesis pathway, they showed no significant similarity to their prokaryotic equivalents. The measured synthase activity did not increase with the number of ARG2 gene copies unless the number of ARG5/6 gene copies was increased similarly. ARG5/6 encodes a precursor that is matured in the mitochondria into acetylglutamate kinase and acetylglutamyl-phosphate reductase, catalyzing the second and third steps in the pathway. The results imply that the synthase must interact stoichiometrically in vivo with the kinase, the reductase, or both to be active. Results obtained with synthetic ARG5 and ARG6 genes suggested that both the kinase and the reductase could be needed. This situation, which has completely escaped notice in yeast until now, is reminiscent of the observation in Neurospora crassa that nonsense arg-6 kinase/reductase mutants lack synthase activity (Hinde, R. W., Jacobson, J. A., Weiss, R. L., and Davis, R. H. (1986) J. Biol. Chem. 261, 5848–5852). In immunoprecipitation experiments, hemagglutinin-tagged synthase coprecipitated with a protein proven by microsequencing to be the kinase. Western blot analyses showed that the synthase has reduced stability in the absence of the kinase/reductase. Our data demonstrate the existence of a new yeast arginine metabolon involving at least the first two, and possibly the first three, enzymes of the pathway. Hypotheses regarding the biological significance of this interaction are discussed.

Arginine biosynthesis in microorganisms follows a common general pathway in which prior N-acetylation of glutamate is essential to allowing specific synthesis of ornithine via the acetylated derivatives cycle. This makes arginine biosynthesis independent of biochemically analogous proline biosynthesis (Fig. 1) (1, 2). The initial acetylation reaction, catalyzed by N-acetylglutamate synthase (EC 2.3.1.1), occurs at the expense of an acetyl-CoA molecule. In most microbes, the high energy cost of this reaction is limited by the fact that the acetyl group of acetylornithine is recycled to a glutamate molecule in a step catalyzed by an ornithine N-acetyltransferase (EC 2.3.1.35). This cyclic metabolic organization confers an anaplerotic character to the first step in biosynthesis, which merely feeds the cycle to compensate for dilution by growth. Avoidance of futile metabolism of acetylglutamate, independently of its origin, requires a double control of activity on the first two enzymes at the pathway entrance.

In humans there is no acetylated derivatives pathway specialized in ornithine biosynthesis. Some ornithine is made from glutamyl-phosphate semialdehyde, an intermediate in the pathway to proline, but most of the arginine produced is hydrolyzed in the urea cycle, which ensures detoxification of excess ammonium. Arginine thus remains an “essential” amino acid, required in food. Although no acetylated derivatives cycle has been identified, acetylglutamate synthase is present. It is viewed as the first committed step in the urea cycle because carbamoyl-phosphate synthase I (EC 6.3.4.16), forming the carbamoyl phosphate needed together with ornithine to produce citrulline, must be activated specifically by acetylglutamate to function (3). Thus, factors regulating the activity of acetylglutamate synthase are relevant to the control of the urea cycle. It has been shown, for example, that arginine stimulates the activity of rat acetylglutamate synthase (4).

In the yeast Saccharomyces cerevisiae and the other Ascomycetes studied to date, acetylglutamate synthase and the next four enzymes forming the acetylated derivatives cycle are located in the mitochondria (5). In S. cerevisiae, ornithine is exported to the cytosol for further processing to arginine, whereas in a number of other yeasts (mostly obligate aerobes) and Neurospora crassa, ornithine is transcarbamylated to citrulline in the mitochondria, and it is citrulline that is exported to the cytosol. In the latter cases the carbamoyl phosphate required to make citrulline is produced in the mitochondria as well. As expected from the pathway organization, arginine feedback regulation in S. cerevisiae and N. crassa controls the activity of the enzymes catalyzing the first two steps: N-acetylglutamate synthase (6, 7) and N-acetylglutamate kinase (EC 2.7.2.8) (8, 9).

N-Acetylglutamate kinase (EC 2.7.2.8) and N-acetylglutamyl-phosphate reductase (EC 1.2.1.38) are encoded by a single gene in all Ascomycetes studied to date: ARG5/6 in S. cerevisiae (10) and Candida albicans (11), arg-6 in N. crassa (12), and arg11 in Schizosaccharomyces pombe (13). Each of these genes encodes a polyprotein precursor with an N-terminal kinase domain and a C-terminal reductase domain. The precursor is cleaved into two distinct enzymes in the mitochondria. In S. cerevisiae for example, deletion of the N-terminal
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**Fig. 1.** Arginine biosynthetic pathways in *S. cerevisiae* and *E. coli*. The yeast genes are designated by their historical nonsequential numbers. The nomenclature of the bacterial genes goes by sequential lettering. The acetylated derivatives cycle is proper to yeast, where it occurs in the mitochondria. *E. coli* uses the linear pathway, which includes an acetylornithinase (product of *argE*) and proceeds in the cytosol.

mitochondrial targeting peptide results in the accumulation of an uncleaved but enzymatically active polyprotein in the cytosol (10). In prokaryotes (both bacteria and archaea), acetylglutamate kinase and acetylglutamyl-phosphate reductase are encoded by separate genes (1). An *Arabidopsis thaliana* gene encoding a single acetylglutamate kinase-like protein has also been identified (data base CAB66113).

When the sequences of the complex genetic loci of the Ascomycetes are aligned with those of the homologous prokaryotic genes encoding the kinase (*argB*) and the reductase (*argC*) separately, there appear two extra domains in the Ascomycete loci: one encoding the expected N-terminal mitochondrial targeting peptide and another encoding an intriguing 200-amino acid domain which, after maturation in front of the reductase region, presumably forms the C-terminal domain of the kinase (10, 12). No function has been assigned to this region so far. It is absent from the putative *A. thaliana* kinase gene.

Much attention has focused on the regulation of acetylglutamate synthase activity in many organisms, including ureotelic mammals. This is not surprising for a key enzyme in the arginine biosynthetic pathway or urea cycle. In contrast, the synthase gene has been much less studied and characterized. So far five bacterial acetylglutamate synthase genes are known, and only two eukaryotic genes, *arg-14* in *N. crassa* (14) and the *S. pombe* gene (locus CAA22186, EMBL SPBC725, accession no. AL034352.1, direct submission by Lyne, M., Rajandream, M. A., Barrell, B. G., and Rieger, M.). An unexpected feature of *N. crassa* acetylglutamate synthase is the absence of a discernible evolutionary relationship with its bacterial counterparts (14).

In *S. cerevisiae*, no formal characterization of the synthase gene has been published yet, except that our own data were reported, with permission, by Vandenbol and Portetelle in the framework of the yeast open reading frame (ORF) characterization project (15).

Until now, the only *S. cerevisiae* mutants known to lack synthase activity have been those of the *arg2* complementation group, so this locus is presumed to encode the structural gene of the synthase. Yet in the filamentous fungus *N. crassa*, acetylglutamate synthase activity is absent not only in *arg-14* mutants, deficient in the structural gene, but also in some *arg-6* mutants (7, 16). This suggests that acetylglutamate kinase and/or acetylglutamyl-phosphate reductase might play a role in the proper functioning of acetylglutamate synthase, probably at a post-translational level (14).

In this paper, we demonstrate conclusively that the *ARG2* gene is the structural gene for acetylglutamate synthase in *S. cerevisiae*, but we also show that acetylglutamate synthase activity does not increase in proportion to the number of *ARG2* gene copies unless the number of *ARG5,6* gene copies is increased similarly. We provide evidence suggesting that the synthase is not active in vivo unless it associates stoichiometrically at least with the *ARG5,6*-encoded kinase and possibly also with the *ARG5,6*-encoded reductase. We show that the kinase communoprecipitates in vitro with hemagglutinin (HA)-tagged acetylglutamate synthase and that the uncomplexed synthase is unstable. We discuss the possible functional relevance of this new yeast arginine metabolon in relation to the cyclic character of the arginine pathway and the resulting requirement for arginine feedback control on the first two steps of the pathway.

**Experimental Procedures**

*Strains and Growth Conditions*

*Escherichia coli*—XA4 (F’ *argA, nalA, λ−, λ+* hsdR) was obtained from A. Mountain et al. (17).

*S. cerevisiae*—The wild-type strain in this laboratory is YM1278b (Mat a). MG471 (Mat a ura3–471) and MG535 (Mat a, arg2–535) are genetically and enzymatically characterized strains of our laboratory derived from YM1278b by M. Grenson. We obtained 13S826b (ura3–3, arg2–535) as a recombinant issued from a cross between MG535 and 10R33b (Mat, ura3–), a strain provided by E. Dubois. Strain AB1 (Mat a, ura3–arg2–gen5), derived from 10R33b, and strain YeBR6 (Mat a ura3–4, arg5–gen5) and YeBR6 (Mat a ura3–471, arg5–gen5, arg5–) were constructed for this work (see below). Strain 14S31b (Mat a ura3–his3–) was obtained from a cross between 13S31a (18) and YM1427 (a gift from M. Johnston).

All yeast strains were grown at 30°C on a minimal medium containing 0.6% (NH₄)₂SO₄, 3% glucose, vitamins, and trace minerals (19). Where required, uracil, l-histidine, or l-arginine were added to a concentration of 25 μg/ml.

When genes under the transcriptional control of a GAL promoter had to be induced, cells were grown on minimal galactose medium containing 3% galactose as carbon source instead of the usual minimal medium (containing 3% glucose).

*Oligonucleotides Used in This Work*

AA5: GCCGGGATCCTTATGTTATTGAAAAATATACGTCAGAGGAGG

AA6: GCCGGTGCGACAAGTGTCATCATCTAGTGCTTAGAAAGGGTGGG

1 The abbreviations used are: ORF, open reading frame; HA, hemagglutinin; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; IPTG, isopropyl-1-thio-β-D-galactopyranoside.
ARG5,6 encoding the first 38 residues of the mitochondrial leader peptide of mid pYB8 was obtained by recombinant PCR. Two first step PCR-amplified fragments were made, corresponding to the DNA sequences encoding the first 38 residues of the mitochondrial leader peptide of ARG5,6 (known to be sufficient for targeting (10)), and to residues 494–863 comprising the acetylglutamyl-phosphate reductase domain and the maturation site in front of it. Because primers BY9 and BY10 were designed with a mutual overlap, the two first step fragments can self-anneal and be elongated and amplified with the external primers BY4 and BY5. This recombinant fragment was then digested with EcoRI and BamHI and cloned in a similarly cut vector. Plasmid pH16 was constructed similarly by recombinant PCR, using primers BY4/AA71 and AA73/BY5 to generate two overlapping PCR fragments whose self-annealing and PCR amplification with oligonucleotides BY4 and BY5 generates an ARG5,6 ORF with a row of 10 histidines inserted in-frame between amino acids 83 and 84 of the precursor protein.

**DNA Sequencing**

The DNA sequences of the ARG2 ORFs cloned in plasmids pYB1, pYB2, pAA8a, and pAA8c were determined. The sequence of ARG2 in pAA8c, amplified using S288c genomic template DNA, was 100% identical to the data base sequence. The sequence of the ARG2 gene in pYB1, pYB2, and pAA8a displays 11 differences at nucleotide level with respect to the data base sequence. These differences, starting from the initiator ATG, are A26G, A195T, C349T, A486G, C492T, G501A, A822G, A1156G, T1161C, C1168T, and T1239G. Four of these differences result in modifications at the deduced amino acid level, i.e., E9G, L117F, K386E, and P905S. Because the three sequenced clones were issued from independent PCR amplifications, these differences must be proper to the ARG2 sequence and not PCR-linked errors.

### Construction of Strains AB1, YeBR5, and YeBR6

We used the method of A. Wach (21) to construct strain AB1, where the genomic ARG2 ORF is deleted between the ATG and the stop codon and transplanted by a heterologous cassette, kanMX4, selected on the basis of the Geneticin (G418) resistance it confers to yeast. In this method, two homologous regions are amplified, first by PCR using two overlapping oligonucleotides. The PCR fragments obtained in the first step can be used as primers to amplify the selectable marker while linking it to the long flanking homology regions, ensuring efficient recombination with the homologous chromosome in strains transformed with this recombinant DNA. The two oligonucleotide pairs used in this construction were AA5/AA7 and AA8/AA6.

### Plasmid Constructs

Most constructions were straightforward clonings of PCR-amplified fragments, using the oligonucleotide primers and vectors indicated in Table I. At their 5'-ends, the primers included "add-on" sequences corresponding to the wanted restriction sites (in bold in the oligonucleotide list), allowing classical sticky end cloning into the corresponding sites of the vectors. In vectors pYX213 and pXY223 (from R&D Systems), the cloned ORFs are expressed from a GAL promoter. Plasmid pYB8 was obtained by recombinant PCR. Two first step PCR-amplified fragments were made, corresponding to the DNA sequences encoding the first 38 residues of the mitochondrial leader peptide of ARG5,6 (known to be sufficient for targeting (10)), and to residues 494–863 comprising the acetylglutamyl-phosphate reductase domain and the maturation site in front of it. Because primers BY9 and BY10 were designed with a mutual overlap, the two first step fragments can self-anneal and be elongated and amplified with the external primers BY4 and BY5. This recombinant fragment was then digested with EcoRI and BamHI and cloned in a similarly cut vector. Plasmid pH16 was constructed similarly by recombinant PCR, using primers BY4/AA71 and AA73/BY5 to generate two overlapping PCR fragments whose self-annealing and PCR amplification with oligonucleotides BY4 and BY5 generates an ARG5,6 ORF with a row of 10 histidines inserted in-frame between amino acids 83 and 84 of the precursor protein.

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**TABLE I**

| Plasmids | Cloning vector | Origin and nature of insert |
|----------|----------------|-----------------------------|
| pAA8a    | pFL1 (2 μm URA3) | Sigma128b Genomic fragment bearing the ARG2 gene: PCR-amplified with AA16/AA6 |
| pAA8c    | S288c          | PCR-amplified ARG2 ORF (primers BY1 and BY2) |
| pAA9a    | pFL38 (ARS/SCN, URA3) | Sigma128b PCR-amplified ARG2 ORF inserted with its 3'-end in fusion with the sequence encoding pAA9c |
| pAA9b    | S288c          | HA epitope (primers BY1/BY3) |
| pYB1     | pYX213 (2 μm URA3, pGAL) | Sigma128b PCR-amplified ARG2 ORF (primers BY1 and BY2) |
| pYB2     |                 | HA epitope (primers BY1/BY3) |
| pYB3     | pYX223 (2 μm HIS3, pGAL) | PCR-amplified ARG5,6 ORF (primers BY4 and BY5) |
| pYB4     |                 | PCR-amplified ARG7 ORF (primers AA37 and AA40) |
| pYB5     |                 | PCR-amplified ARG8 ORF (primers BY6 and BY7) |
| pYB6     |                 | PCR-amplified mitochondrial leader and ARG6 domain of ARG5,6 (primers BY4 and BY8) |
| pYB7     |                 | Recombinant PCR with ARG5,6 ORF (primers BY4 and BY9) and ARG5 domain of ARG5,6 (primers BY10 and BY5) |
| pYB8     |                 | Recombinant PCR with ARG5,6 ORF (primers BY4/AA71) and ARG5,6 domain (primers AA73/BY5), with insertion of 10 His residues at the N terminus of the mature acetylglutamate kinase |
| pOS16    |                 | FL100 ARG3 ORF inserted in frame upstream of the HA epitope sequence (20) |
| pAA6     | pTrc99A        | Sigma128b PCR-amplified ARG2 ORF (primers AA29 and AA30) inserted downstream of IPTG-inducible tryp-lac promoter |
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**TABLE II**

| Strain | Genotype | Plasmid | Relevant traits of plasmids | Growth media | Specific activity nmol/min mg⁻¹ protein |
|--------|----------|---------|-----------------------------|--------------|--------------------------------------|
| 1      | 1278b    | Wild-type | None                        | Mam          |                                      |
| 2      | 13S62b   | ura3⁻, arg2-535 | pAA8                      | 2 µm URA3-ARG2 | 7.1                                  |
| 3      |          |         | pAA9                        | ARS/URA3-ARG2 | 7.7                                  |
| 4      | 14S31b   | ura3⁻, his3⁻ | pYB1 + pYX223              | 2 µm URA3-pGALAR2 + 2 µm HIS3 | 10.2                                |
| 5      |          |         | pYB1 + pYB3                | URA3-pGALAR2 + 2 µm HIS3-pGALAR5,6 | 140                                 |
| 6      |          |         | pYB1 + pYB4                | URA3-pGALAR2 + µm HIS3-pGALAR8 | 10.5                                 |
| 7      |          |         | pYB1 + pYB5                | URA3-pGALAR2 + µm HIS3-pGALAR7 | 12.3                                 |
| 8      |          |         | pYB1 + pYB6                | URA3-pGALAR2 + µm HIS3-pGALAR11 | 7.3                                  |
| 9      |          |         | pYB1 + pYB7                | URA3-pGALAR2 + µm HIS3-pGALAR6 | 14.5                                 |
| 10     |          |         | pYB1 + pYB8                | URA3-pGALAR2 + µm HIS3-pGALAR6 | 4.3                                   |
| 11     |          |         | pYB2 + pYX223              | 2 µm URA3-pGALAR2-HA + µm HIS3 | 3.9                                   |
| 12     |          |         | pYB2 + pYB3                | URA3-pGALAR2-HA + 2 µm HIS2-pGALAR5,6 | 138                                 |
| 13     |          |         | pYB1 + pHP16               | 2 µm URA3-pGALAR2 + 2 µm HIS3-pGAL(HIS)10ARG5,6 | 8.2                                  |
| 14     |          |         | pYB2 + pHP16               | 2 µm URA3-pGALAR2-HA | 3.1                                  |
| 15     |          |         | pYX213 + pHP16             | 2 µm URA3    | 3.0                                   |
| 16     | YeBR6    | ura3⁻, arg6::gen′ arg5    | pYB2 + pYB3               | 2 µm URA3-pGALAR2-HA + 2 µm HIS2-pGALAR6 | 120                                 |
| 17     |          |         | pYB2 + pHP16               | URA3-pGALAR2-HA + 2 µm HIS2-pGAL(HIS)10ARG5,6 | 2.5                                  |
| 18     |          |         | pYX213 + pHP16             | 2 µm URA3    | NM*                                  |

*Not measurable.

**Enzyme Activity Assays**

**Acetylglutamate Synthase**—This enzyme activity was measured by a radioassay using l-[¹⁴C]glutamate and acetyl-CoA as substrates. French press cell extracts (from 2 liters cultures at OD 0.4) were desalted on Sephadex G-25 against 20 mM Tris-HCl, pH 7.5, containing French press cell extracts (from 2 liters cultures at OD 0.4) were decreased by half if the reaction mixture was incubated for 30 min.

**Activities** and under 20%.

**Long Term in Vivo Labeling of Cells with ³⁵S-Amino Acids and Immunoprecipitation of HA-tagged Proteins**

A 400-ml overnight culture of the tested strain grown on minimal galactose medium supplemented with the required amino acids was grown to OD 0.25–0.3. After centrifugation (4500 rpm, SSS4 rotor) for 10 min at room temperature, the cells were resuspended in 1 ml of prewarmed medium and incubated at 30 °C for another 10 min. Then 250 µCi/ml [³²S]methionine (>37 TBq/mmol, >1,000 Ci/mmol, in vivo cell labeling grade), 250 µCi/ml [³²S]cysteine (same specific activity) (both labeled compounds were from Amersham Pharmacia Biotech), and a 20-fold excess of cold amino acids were added. The culture was then incubated for 2 more h at 30 °C. After a short centrifugation in a microcentrifuge to remove the supernatant, the cells were washed with 1 ml of phosphate-buffered saline, and the pellet was resuspended in 1 ml of lysis buffer (50 mM Tris-Cl, 150 mM NaCl, pH 7.5). The cells were then twice in (50 mM Tris-HCl, pH 7.5) and once in (10 mM Tris-HCl, pH 7.5). The immunoprecipitated proteins were separated by SDS-PAGE (PAGE) in 12% gels, the proteins were transferred to an ECL Hybond nitrocellulose membrane (Amersham Pharmacia Biotech) in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol) in a Mini PROTEAN 3 blotting cell (Bio-Rad). Specific primary mouse anti-HA antibody and mouse anti-His antibody (Roche) (4 µl/mg each) and 40 units/ml horseradish peroxidase-labeled secondary antibody (Roche) were used to detect the tagged proteins.

**Western Blots**

Further analysis of the proteins was performed by a standard chemiluminescence Western blotting protocol (Roche). After SDS-PAGE in 12% gels, the proteins were transferred to an ECL Hybond nitrocellulose membrane (Amersham Pharmacia Biotech) in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol) in a Mini PROTEAN 3 blotting cell (Bio-Rad). Specific primary mouse anti-HA antibody and mouse anti-His antibody (Roche) (4 µl/ml each) and 40 units/ml horseradish peroxidase-labeled secondary antibody (Roche) were used to detect the tagged proteins.

**N-terminal Amino Acid Microsequence Analysis**

Sequencing was done by R. Wattiez in the Laboratory of Biochemistry of the University of Mons-Hainaut (22). Proteins were electroblotted onto PVDF membranes (Sequi-Blot PVDF membrane; Bio-Rad) using 25 mM Tris, 192 mM glycine, 0.1% SDS as the cathode buffer and 25 mM Tris, 192 mM glycine, 2% methanol as the anode buffer. Before electroblotting, the membranes were soaked in methanol for 30 s and in anode buffer for at least 10 min. Protein transfer was carried out for 1 h at 24 V in a semidyblotting apparatus (Biolyon, France). Just before staining, the membranes were washed four times with MilliQ water. The PVDF membrane-bound proteins were visualized by staining with Coomassie Brilliant Blue R-250. The bands on the PVDF membrane were excised, and the N-terminal amino acid sequences of the corresponding proteins were determined.
RESULTS

**YJL071W Is Allelic to ARG2 and Is the Structural Gene for Acetylglutamate Synthase—**FastA and BLAST searches of the yeast data base with the amino acid sequence of the *E. coli* acetylglutamate synthase identified no ORF showing significant similarity to the prokaryotic enzyme. In contrast, a query with the sequence of the *N. crassa* acetylglutamate synthase encoded by the *arg-14* gene identified ORF YJL071W, displaying 30% identity over a length of about 330 residues. We amplified the YJL071W ORF region by PCR, from base pair –389 upstream from the initiator codon to base pair 361 downstream from the stop codon, using as template genomic DNA from our wild-type strain Y1278b or from the S288c data base strain. The amplified fragments were cloned in a multicopy vector (pAA8a or pAA8c, respectively) and in a single copy vector (pAA9a or pAA9c, respectively). All of the clones were able to complement fully the *arg2* mutation of strain 13S62b, which displays no detectable acetylglutamate synthase activity. In 13S62b, moreover, the presence of the plasmids resulted in a synthase activity about twice as high as in the wild-type strain Σ1278b, i.e. about 7.5 nmol of acetylglutamate produced per min and per mg of total protein. Although it was unclear at the time why an expected 10–15-fold increase in gene copy number (resulting from the presence of a 2 μm-based pAA8 plasmid) did not lead to a similar enzyme activity increase, the assays nevertheless showed a clear correlation between the appearance of acetylglutamate synthase activity and the presence of ORF YJL071W. This correlation was confirmed further by the phenotype of strain AB1, lacking the entire ORF YJL071W; this strain displayed no acetylglutamate synthase activity, and its growth was arginine-dependent.

Allelism of the YJL071W and ARG2 genetic loci was established in two ways: first, by showing that not a single arginine-prototrophic recombinant arose from a cross between strain AB1 (*Mata, ura3*, YJL071::gen*) and strain MG535 of 19 tetrads analyzed; and second, by demonstrating noncomplementation between the recessive arginine deficiencies of strains MG535 and AB1 (*Mata, ura3*, YJL071::gen*). The diploids obtained by crossing these strains (and induced by their sporulation) were unable to grow on minimal medium.

No Evolutionary Relationship Is Detectable between Prokaryotic and Fungal Acetylglutamate Synthases; the Yeast Enzyme Cannot Complement an *argA* Mutation in *E. coli*—**Fig. 2A shows the Clustal X alignment of the five bacterial acetylglutamate synthase sequences available to date. A few highly conserved regions appear. An equivalent alignment of the three available Ascomycete sequences also highlights a series of important regions (Fig. 2B), but not one of the domains conserved in Ascomycetes overlaps with a bacterial conserved domain (Fig. 2C). This strengthens the conclusion of Yu et al. (14), based on a comparison of *N. crassa* enzyme with those of *E. coli* and *Pseudomonas aeruginosa*, that fungal and bacterial acetylglutamate synthases show little similarity. Although no archeal acetylglutamate synthase has been characterized to date, BLAST searches of the fully and partially sequenced archeal genomes produced three significant alignments when using the *E. coli* synthase sequence as a query (*Archeoglobus fulgidus, Methanococcus jannaschii*, and *Methanobacterium thermautotrophicum*) and none with the yeast synthase, suggesting a commun origin for all prokaryotic acetylglutamate synthases.

We tried to restore arginine-dependent growth of an *argA*–*E. coli* mutant by transforming it with plasmid pAA6, where the yeast ARG2 ORF is expressed under the transcriptional control of the bacterial *try-lac* promoter and the translational control of an appropriate Shine-Dalgarno sequence. Even after IPTG induction, not the slightest growth improvement was detectable in the absence of added arginine; the results were the same whether the bacterial strain was transformed with pAA6 or with the empty pTrc99 cloning vector. Similarly, no increase in synthase activity was detected in extracts of IPTG-induced XA4 (pAA6) compared with XA4 (pTrc99A). Thus for some reason, the eukaryotic enzyme is either not functional or not stable in the prokaryotic environment.

**Acetylglutamate Synthase Activity Requires Coexpression of Equivalent Gene Copy Numbers of ARG2 and ARG5,6—**As shown above, we observed no increase in acetylglutamate synthase activity upon increasing the number of ARG2 gene copies. A possible explanation for this intriguing fact is that a required further partner protein was lacking. One or more of the other mitochondrial enzymes involved in arginine biosynthesis seemed, physiologically, the most likely candidate(s).

To test this hypothesis, we cloned the ARG2 ORF under the control of a GAL promoter in a 2 μm-based URA3 vector, obtaining plasmid pYB1. On the other hand, we cloned the ARG5,6, ARG8, ARG7, and ARG11 genes, encoding the acetyl cycle enzymes and the ornithine transporter. The vector used to clone these genes was identical to that used for the ARG2 ORF except that it carried a HIS3 selection marker. The resulting plasmids were, respectively, pYB3, pYB4, pYB5, and pYB6. The functional character of all of the cloned genes was tested by complementation of the appropriate yeast mutations. We then cotransformed strain 14S31b (ura3*, his3*) with pYB1 and either pYB3, pYB4, pYB5, or pYB6, or the empty vector pXY223 used as a negative control. Transformants were grown on galactose, and acetylglutamate synthase activity was measured. Table II (rows 4–8) shows that all transformants displayed a similar low activity of about 7–12 nmol⋅min⁻¹⋅mg protein⁻¹, except the one harboring both pYB1 and the plasmid bearing the ARG5,6 gene (pYB3). In this case, the activity was 14 times higher than in the transformant harboring pYB1 and the empty vector. Thus, an activity increase proportional to the ARG2 gene copy number increase was obtained only if the ARG5,6 gene copy number was increased accordingly. This suggests that acetylglutamate synthase is active *in vivo* only if it associates stoichiometrically with acetylglutamate kinase and/or acetylglutamyl-phosphate reductase. In *vivo*, however, we observed no synthase activity increase upon mixing extracts of strains overexpressing ARG2 and ARG5,6 separately. This suggests that the synthase either does not fold properly or is unstable in the absence of the kinase and/or reductase.

**Coexpression from High Copy Number Plasmids of the ARG2 Gene and a Synthetic Gene Encoding Either the Kinase or the Reductase Is Not Sufficient to Promote High Synthase Activity—**As ARG5,6 encodes two proteins, either one or both of them might be required to render the synthase functional. To investigate this, we constructed two artificial genes: ARG6, corresponding to the kinase domain of ARG5,6, and ARG5, corresponding to the reductase domain. Using the same 2 μm-based HIS3 vector as above, we constructed pYB7 and pYB8, expressing respectively ARG6 or ARG5 under the control of the GAL promoter (Fig. 3).

That each of the artificial genes is functional was shown by phenotypic complementation. Plasmid pYB8, on the one hand,
FIG. 2. Clustal X multiple alignments of acetylglutamate synthases. The synthases of prokaryotes E. coli, Buchnera aphidicola, P. aeruginosa, Neisseria meningitidis, and Pseudomonas putida are compared in panel A. The gray boxes with black bold letters are the residues conserved in the five bacterial enzymes. The synthases of Ascomycetes N. crassa, S. pombe, and S. cerevisiae are compared in panel B. The black boxes with white letters are the residues conserved in the Ascomycete synthases. A potential membrane anchor sequence is boxed. Panel C aligns the synthases of S. cerevisiae and E. coli. Four open circles indicate the only residues that show up as conserved in this sequence comparison while corresponding to positions also conserved in the bacterial and Ascomycete synthases families.
could transform to arginine prototrophy strain YeBR5 (ura3, Δarg5::genR), which lacks the promoter-distal reductase-encoding ARG5 region of the ARG5,6 gene. On the other hand, cotransformation with pYB7 and pYB8 was necessary to render arginine-independent strain YeBR6 (ura3, Δarg6::genR/Δarg5), which expresses neither the kinase (deletion) nor the reductase (polar effect of Δarg6::genR on the distal ARG5 domain). This shows that pYB7 expresses the kinase gene.

Acetylglutamate synthase activity was then assayed in 14S31b (pYB1+pYB3) (used as a control) and 14S31b (pYB1+pYB7) and 14S31b (pYB1+pYB8) (Table II, rows 9 and 10). Neither pYB7 nor pYB8 alone conferred increased synthase activity in the presence of pYB1. This contrasts with the 14-fold increase conferred by pYB3 under the same conditions. It suggests that both the kinase and the reductase are required to render the synthase functional, at least if it can be demonstrated that the amounts of kinase and reductase produced separately from pYB7 and pYB8 are similar to those produced from pYB3 bearing the natural ARG5,6 locus.

To test this, we analyzed relevant total protein extracts by SDS-PAGE. First we compared extracts of cells harboring pYX213+pYX223 (empty vectors) with extracts of cells harboring pYX213+pYB3 (Fig. 4, lanes 2 and 3). Only one clear extra band was observed in the latter case. It was located at 38 kDa, the expected molecular mass of the reductase. This shows that pYB7 expresses the kinase gene.

Acetylglutamate synthase activity levels in transformants bearing pYB1+pYB7 and pYB1+pYB3. The measured activities, respectively, 17.2 and 20 nmol/min/mg of protein, were comparable but only three times as high as the activity corresponding to a single genomic ARG5,6 gene (6 nmol/min/mg of protein in strain 14S31b). An increase factor of at least 10 would be expected for a gene expressed from a strong GAL promoter on a 2 µm-based vector. Thus, even in the (possibly required) presence of overexpressed synthase, there is no direct proportionality between ARG5,6 gene dosage and kinase activity. We can as yet provide no explanation for this, but the absence of a clear correlation prevents us from concluding unambiguously that the same amount of kinase is produced from pYB7 as from pYB3. However, the kinase activity being three times higher in the transformants harboring pYB1+pYB7 than in those harboring pYB1+pYX223, we would expect at least a 3-fold increase in synthase activity in the former case, if the kinase alone were sufficient to activate the synthase, and this was not observed (Table II, rows 9 and 4). It thus seems likely that association of the synthase with the kinase is insufficient to obtain synthase activity.
On the whole then, the data obtained with the artificial ARG5 and ARG6 genes suggest that the synthase is active only if it associates with both the kinase and the reductase. Because unambiguous proof of this is lacking, however, we shall speak in the following sections of association of the synthase with the kinase/reductase as necessary for the development and/or maintenance of synthase activity in vivo.

A Protein with a Molecular Mass Corresponding to Acetylglutamate Kinase Coimmunoprecipitates with HA-tagged Acetylglutamate Synthase—If two or more proteins form a sufficiently stable complex, direct evidence of their association in vitro can be obtained by demonstrating coimmunoprecipitation. To detect proteins that might coimmunoprecipitate with the synthase, we constructed a gene coding for a C-terminally HA-tagged derivative of the Arg2 protein and placed it under the control of the GAL promoter in the same vector as was used to construct pYB1. The resulting plasmid was called pYB2. The tagged synthase behaved enzymatically exactly like the untagged control: pYB2-harboring transformants displayed high synthase activity only if they also harbored pYB3 (Table II, rows 11 and 12). In this case, the activity measured was similar to that observed in transformants harboring pYB1+pYB3.

The synthase was immunoprecipitated with anti-HA antibody from extracts of cells labeled in vivo with [35S]methionine and [35S]cysteine. Cell-free extracts were prepared from strain YeBR6 (pYB2+pYB3), coexpressing ARG5,6 and the gene encoding the tagged synthase. As controls, we used extracts of YeBR6 harboring pYB1+pYB3 (untagged synthase together with the kinase and the reductase) or pOS16+pYB3 (HA-tagged ornithine carbamoyltransferase, product of ARG3, together with the kinase and the reductase).

Analysis of the immunoprecipitated radioactive proteins by SDS-PAGE revealed the presence of two main bands in extracts of cells harboring pYB2+pYB3 (Fig. 5A, lane 2, and Fig. 5B, lane 2). The corresponding molecular masses were about 68 and 52 kDa, in keeping with the respective predicted molecular masses of acetylglutamate synthase and acetylglutamate kinase. Lane 3 in Fig. 5A and lane 1 in Fig. 5B show that precipitated ornithine carbamoyltransferase migrated as a single band when the enzyme was overproduced along with the kinase and reductase. There was thus no trace of coimmunoprecipitation. As expected, nothing precipitated in the absence of HA-tagged enzyme (Fig. 5A, lane 4).

The Protein Coprecipitating with Acetylglutamate Synthase Is Acetylglutamate Kinase—N-terminal amino acid microsequencing confirmed that the ARG5,6-encoded kinase is indeed the protein of ~52 kDa which coprecipitates with the synthase. Fig. 6 shows that immunoprecipitates obtained with HA-tagged antibodies and extracts of galactose-grown YeBR6 (pYB2+pYB3) cells resolved as four protein bands when subjected to SDS-PAGE/Coomassie Blue staining. Despite the presence of a high background throughout the PVDF filter blot, the sequence MWRRIFAHGL corresponding to the N terminus of the unmutated synthase was recognized in band 1 by a program developed for analysis of random combinations (22). In band 2, two sequences were found: XQQLXESGXXLVQ, corresponding to the immunoglobulin heavy chain, and VXSTNGF-SATRXT, corresponding to the acetylglutamate kinase sequence starting at residue 58. In band 3, no sequence emerged above the background noise. In band 4, the program identified sequence KFVLTQAPLSVRV of the immunoglobulin light chain.

These results establish unambiguously that the kinase coimmunoprecipitates with the synthase. Moreover, they also provide information regarding the N-terminal sequences of the mature synthase and mature kinase. The mitochondrial targeting peptide of the synthase seems not to be cleaved upon entry into the mitochondrion, a rare but not unprecedented case. A potential signal peptide comprising at least the first 12 amino acids possesses the expected characteristics for targeting to the mitochondria (22): it is predicted to be able to form an amphipathic helix with a large hydrophobic moment on one side and four positively charged residues on the other; furthermore, the first acid residue is glutamate 13. In the absence of N-terminal maturation, the molecular mass deduced from the DNA sequence is 65,521 kDa. For the HA-tagged derivative used in this immunoprecipitation experiment, the expected...
The presence of multiple did not lead to the expected high synthase activity reflecting the experimental value of about 68 kDa.

The mature acetylglutamate kinase starts at valine 58. In N. crassa, the kinase starts at threonine 45 (12). These two positions are almost superimposed in the Clustal X alignment shown in Fig. 2A. Boonchird et al. (10) previously proposed alanine 66 as the N terminus of the mature yeast enzyme, on the basis of theoretical considerations and a deletion analysis. In the same paper, the N terminus of the reductase generated by maturation of the Arg5,6 precursor protein is predicted to be located between amino acids 531 and 541. On the basis of this last prediction, of a Clustal X alignment of the four available kinase/reductase-encoding genes (not shown) and of knowledge of the reductase N terminus in N. crassa (12), we propose that the yeast kinase counts 475 residues, extending from amino acid 58 to amino acid 533. The corresponding predicted molecular mass of 52,332 kDa is in perfect agreement with the experimental observations.

Acetylglutamate Synthase Is Unstable in the Absence of Coexpressed Kinase and If the Coexpressed ARG5,6 Gene Encodes an N-terminally (Poly)His-tagged Kinase—In parallel with the experiments described in the previous section, we attempted an immunological approach to identify the protein communoprecipitating with the HA-tagged synthase. We constructed a gene encoding an N-terminally (poly)His-tagged kinase (expressed from plasmid pH16) that could be identified with anti-His antibody. Because we did not know, at the time, the precise N-terminal residue of the kinase, we fused the tag to the N-side of valine 84, chosen on the basis of published information (21); the protein tagged at this position was predicted not to lose its tag upon cleavage of the mitochondrial targeting peptide, and the mature enzyme was predicted to be normally active.

The tagged kinase did seem active because pH16 was able to complement the kinase and reductase deficiencies of strain YeBR6, restoring normal growth on galactose medium without added arginine exactly like pYB3 (not shown). Furthermore, the kinase activity measured in strain 14S31b (pHP16) was comparable with that measured in 14S31b (pYB3) (15.3 and 20 nmol·min⁻¹·mg of protein⁻¹, respectively).

However, coexpression of pH16 with either pYB1 or pYB2 did not lead to the expected high synthase activity reflecting the presence of multiple ARG2 gene copies. Strains 14S31b (pYB1+pHP16) and 14S31b (pYB2+pHP16) displayed the same low activity as strains 14S31b (pYB1+pYX223), 14S31b (pYB2+pYX223) (Table II, rows 13 and 4, 15 and 11). The strain YeBR6 as well as the pYB2+pHP16 combination displayed only low synthase activity (Table II, row 17).

This could mean that the (poly)His-tagged kinase and the HA-tagged synthase do not associate efficiently or that the complex formed is nonfunctional. Immuno-Western blot analysis of the proteins immunoprecipitated by rat anti-HA antibody reflects the presence of multiple ARG2 gene copies. Strains 14S31b (pYB1+pHP16) and 14S31b (pYB2+pHP16) displayed the same low activity as strains 14S31b (pYB1+pYX223), 14S31b (pYB2+pYX223) (Table II, rows 13 and 4, 15 and 11). The strain YeBR6 as well as the pYB2+pHP16 combination displayed only low synthase activity (Table II, row 17).

FIG. 7. Immuno-Western blot analysis of proteins immunoprecipitated by rat anti-HA antibody from total protein extracts of strain YeBR6 containing the plasmids indicated above the lanes. Panel A, primary detection with mouse anti-His antibodies and peroxidase-coupled anti-mouse secondary antibodies. An arrow shows cross-species detection of the rat immunoglobulin heavy chain by the anti-mouse antibodies. Panel B, secondary detection, using mouse anti-HA antibody, detects the 68-kDa synthase band.

FIG. 8. Immuno-Western blot analysis of total protein extracts of strain YeBR6 containing the plasmids indicated above the lanes.

Moreover, no synthase at all was visible in the absence of coexpressed kinase (lane 6).

These data clearly show that the stability of the synthase is reduced if no kinase or only the tagged kinase is present and that tagged kinase associates inefficiently with the synthase.

The reduced stability of the synthase in the absence of the kinase was also demonstrated directly on immuno-Western blots obtained with total protein extracts. Fig. 8 shows the synthase band in lane 4, containing YeBR6 (pYB2+pYB3) extract, and no band in lane 3, loaded with YeBR6 (pYB2) extract. The results were the same in several independent experiments.

DISCUSSION

The Yeast ARG2 Gene Encoding Acetylglutamate Synthase Is Clearly the Homolog of the Corresponding N. crassa and S. pombe Genes—Our sequence comparisons in Fig. 2 clearly show that Ascomycete and prokaryote acetylglutamate synthases constitute two distinct families of orthologous genes. A common evolutionary origin for the Ascomycete and prokaryote genes seems highly improbable.

Prokaryotic acetylglutamate synthases are proposed to have derived from an ancestral kinase gene by duplication. This was initially suggested by a dot matrix analysis comparing the
translated products of \textit{N. crassa arg-6} (kinase and reductase domains) and \textit{E. coli argA} (acetylglutamate synthase) \cite{12}. The three identified conserved domains were, as expected, confined to the N-terminal half of the fungal kinase, which is conserved in all acetylglutamate kinases across the domains of living organisms and is therefore inferred to constitute the catalytically active region. In keeping with this, the \textit{E. coli} synthase used as a query in a BLAST search identifies the same three domains in the acetylglutamate kinases of \textit{S. cerevisiae} and \textit{S. pombe} as well as other bacterial acetylglutamate kinases (not shown). There thus seems to be no doubt that prokaryotic acetylglutamate synthases are related to the interdomain acetylglutamate kinase family.

In support of the view that the Ascomycete and prokaryotic acetylglutamate synthases are evolutionarily unrelated, there appears no clear relationship between the Ascomycete synthase sequences and the acetylglutamate kinase family. Actually, when the \textit{N. crassa} and \textit{S. cerevisiae} synthases were used as queries in BLAST data base searches, some sequence similarities were detected between their C-terminal regions and the Ascomycete-specific C-terminal domain of Ascomycete kinases, but a multiple Clustal alignment of the concerned regions identified no generally shared similarity, which suggests that the similarities were not significant (not shown).

A probable relevant feature of the yeast synthase sequence is the presence of a potential membrane anchor (spanning amino acids 433–449 in the yeast synthase and 546–563 in the \textit{N. crassa} synthase, and boxed in Fig. 2B). This sequence is highly conserved among the three Ascomycete synthases. This observation may relate to data showing a loose mitochondrial membrane attachment of the \textit{N. crassa} and yeast synthases \cite{5,7}. Evidence for the Existence of a Yeast Metabolon Involving at Least Acetylglutamate Synthase and Acetylglutamate Kinase—We have shown that to be active in \textit{vivo}, the yeast acetylglutamate synthase must associate directly with acetylglutamate synthase and/or acetylglutamyl-phosphate reductase. This is indicated clearly by the fact that synthase activity does not increase in proportion to the number of \textit{ARG2} gene copies unless the number of \textit{ARG5,6} gene copies is increased similarly. This suggests that the synthase and at least one of the \textit{ARG5,6} gene products interact stoichiometrically. It appears to rule out a mere catalytic role of the \textit{ARG5,6} gene product(s).

A similar situation seems to prevail in \textit{N. crassa}, where synthase activity is lost not only in \textit{arg-14} mutants affected in the structural gene, but also in some \textit{arg-6} mutants, affected in the gene encoding the kinase and the reductase. This again suggests a role for acetylglutamate kinase and/or acetylglutamyl-phosphate reductase in the proper processing or functioning of acetylglutamate synthase \cite{7,16}. Although these data, obtained in the context of single gene copies, are open to more interpretations than the more compelling yeast data, the nature of the \textit{arg-6} mutants affecting synthase activity, specifically nonsense mutations resulting in the loss of the kinase and reductase proteins, is certainly in agreement with the requirement for protein complex formation in \textit{Neurospora} as well.

We tried to demonstrate in yeast the loss of synthase activity in strains possessing a normal \textit{ARG2} gene but lacking part of the \textit{ARG5,6} gene. Synthase activity was indeed undetectable in strains YeBR6 (\textit{ura}^3, \textit{ArgB}:\textit{genR}/\textit{arg5}''), and YeBR5 (\textit{ura}^3, \textit{ArgB}:\textit{genR}/\textit{arg6}''), but the activity was also hardly above background in the wild-type mother strain MG471 grown under the same conditions as the mutants, \textit{i.e.} in the presence of arginine (data not shown). Our interpretation, knowing that \textit{ARG2} is not repressed by arginine \cite{24,2}, is that the synthase in MG471 extracts is too strongly retroinhibited by arginine to allow measurement of its activity (arginine is probably not removed efficiently from the enzyme by a simple run through a Sephadex column). It is noteworthy in this context that the yeast enzyme is 8-fold more sensitive to arginine feedback control than the \textit{N. crassa} enzyme; the former is half-inhibited by 0.02 mM arginine \cite{5}, whereas the latter requires 0.16 mM arginine for half-inhibition \cite{7}.

We have obtained clear \textit{in vitro} evidence for the formation of a complex between acetylglutamate synthase and acetylglutamate kinase. By N-terminal amino acid microsequencing, we unambiguously identified the kinase as the 52-kDa protein co-immunoprecipitating with a C-terminally HA-tagged synthase.

We also constructed an N-terminally (poly)His-tagged kinase with a double aim in mind: to identify the kinase immunologically in immunoprecipitates of the synthase and to test for the presence of the synthase in immunoprecipitates of the kinase. Although the tagged kinase retained quasi-normal catalytic activity in enzymatic assays, it proved unable to associate properly with the synthase and was therefore unsuitable for the intended experiments. Improper association was deduced from 1) the failure of the pHP16-encoded tagged kinase to increase the acetylglutamate synthase activity in cells also harboring pYB1 or pYB2; 2) the fact that less synthase is immunoprecipitated from extracts of strains containing pYB2+pHP16 than from extracts of strains harboring pYB2+pYB3; and 3) our inability to detect coprecipitating synthase in immunoprecipitates of the tagged kinase (these last data are not shown).

We did not construct a C-terminally tagged kinase for two reasons. First, construction of such a derivative is problematic today because the precise C terminus of the kinase, arising from the maturation of the kinase-reductase precursor, has not yet been determined accurately \cite{10}. Second, it can reasonably be suspected that the C-terminal domain of the kinase is involved in association with the synthase. This belief is based on the existence, first pinpointed by Gessert \textit{et al.} \cite{12}, of a large Ascomycete-specific domain at the C terminus of the fungal kinases. The domain appears clearly when prokaryotic kinases are aligned with the kinase/reductase precursors of \textit{N. crassa}, \textit{S. pombe}, \textit{S. cerevisiae}, and now also \textit{C. albicans} \cite{11}. This extra domain of about 200 amino acids showing up in the precursor protein between the active kinase and reductase domains was initially called the connector domain \cite{12}. That it is actually part of the kinase was deduced from knowledge of the internal maturation site of the \textit{N. crassa} precursor and from the molecular mass of the corresponding kinase \cite{12}. This view now also tallies with the molecular mass of the yeast kinase.

The hypothesis that the C-terminal domain of the Ascomycete kinase is a synthase association domain can now be tested easily in yeast using the relevant constructs. The fact that a yeast synthase highly expressed from an IPTG-induced pTrc promoter was unable to complement the synthase deficiency of an \textit{E. coli} strain may be caused by the absence of the Ascomycete-specific C-terminal domain in the \textit{E. coli} kinase, preventing association of the prokaryotic kinase with the Ascomycete synthase. The hypothesis that the Ascomycete-specific C-terminal domain is the synthase association domain would be confirmed if adding this domain to the \textit{E. coli} kinase proved sufficient to allow complementation of the \textit{argA} mutation by the yeast synthase. Of course, it might also be necessary to express the first three enzymes of the yeast arginine pathway

\footnote{O. Soetens, K. Pauwels, and M. Crabeel, unpublished results.}
to complement the argA mutation in *E. coli*.

It is noteworthy that the Ascomycete-specific C-terminal domain is not necessarily a general eukaryotic trait. It is lacking, in fact, in an acetylglutamate kinase-like sequence found in the *Arabidopsis* data base. In keeping with this, an acetylglutamate synthase-like sequence of *A. thaliana* displayed a significant alignment with the *E. coli*, but not with the yeast, acetylglutamate synthase sequence.

A fact that remains totally unexplained is why, specifically in Ascomycetes, the kinase and the reductase are encoded by a single gene instead of the two separate homologous genes found in prokaryotes, especially because the fungal bifunctional precursor is processed anyway to two distinct mature enzymes.

Predictions concerning mammalian acetylglutamate synthases might be made on the basis of the fact that in yeast the synthase must associate with the kinase/reductase to be active. In ureotelic organisms, acetylglutamate synthase produces the acetylglutamate needed for carbamoyl phosphate synthesis, the rate-limiting entrance step of the urea cycle (3). Because neither the kinase nor the reductase seems to exist in mammals, the mammalian synthase enzyme might be expected to be active in the absence of any association with a kinase/reductase, and thus to be of the prokaryotic type rather than the Ascomycete type. Alternatively, an equivalent of the Ascomycete kinase/reductase gene might be present in mammals. BLAST searches of the human genome revealed no significant alignments with either yeast or *E. coli* acetylglutamate sequences. Also an *E. coli* kinase query against the human genome produced no relevant hits.

**In Yeast, the in Vivo Stability of Acetylglutamate Synthase Depends on Its Association with the Kinase/Reductase**—Acetylglutamate synthase activity is well known to be unstable in total protein extracts. For example, the specific activity is halved if the enzyme is incubated at 30 °C for 30 min instead of 10, and it decreases by 40% if the enzyme is incubated for 10 min at 37 °C instead of 30 °C. Synthase activity is also lost rapidly in frozen extracts.

The data presented here suggest that the synthase is even unstable *in vivo* whenever it is produced in excess of the kinase/reductase. In repeated experiments, we failed to detect any HA-tagged synthase on immuno-Western blots when total protein extracts of strain YeBR6, expressing pYB2 alone, were compared with extracts of YeBR6 (pYB2+pYB3). Moreover, as already discussed above, reduced quantities of synthase are immunoprecipitated from extracts producing the synthase in the presence of a kinase bearing a (poly)His tag at its N terminus instead of a wild-type kinase. This fact suggests that the N-terminal domain of the kinase might be involved in forming a complex with the synthase, in addition to the Ascomycete-specific C-domain mentioned above as a likely association domain. *In vivo* instability of the synthase when not associated with the kinase/reductase might also explain why high synthase activity cannot be restored *in vitro* by combining extracts of strains overproducing the synthase and the kinase/reductase separately.

It is worth stressing that association of the synthase with the kinase/reductase might have another role in addition to synthase stabilization: it could be necessary to make the synthase catalytically active. In some *arg-6* *N. crassa* mutants, no acetylglutamate synthase activity is detected, despite detection of the synthase on Western blots (14). Molecular level understanding of the function of the complex will have to await enzyme purifications and structural analyses.

**Is the Reductase Also Required in the Complex?**—Our data appear to rule out the possibility that the reductase alone promotes the maintenance/activation of the synthase by associating with it. First, the reductase produced from a plasmid bearing a synthetic *ARG5* gene has no activity-enhancing effect in cells harboring a multicopy *ARG2*-bearing plasmid. This contrasts with the activity-enhancing effect of the whole *ARG5,6* gene under the same conditions, and we have checked that in the two cases similar amounts of reductase are produced (see in Fig. 4 the band at 38 kDa in the SDS-PAGE analysis of extracts of strains harboring YP3 or YP8).

We cannot, on the other hand, rule out the possibility that the kinase alone might be responsible for the activity-enhancing effect of the *ARG5,6* gene. Although plasmid pYB7, bearing a synthetic *ARG6* gene, was also unable to increase the measured synthase activity when coexpressed with pYB1, we have no formal proof that the amounts of kinase produced from pYB7 and by pYB3 are comparable because no extra band corresponding to the kinase was detectable on SDS-polyacrylamide gels. We did observe the same kinase activity levels in 14S31b (pYB1+pYB3) and 14S31b (pYB1+pYB7), but this does not necessarily imply that similar amounts of kinase were present because, for an unknown reason, the kinase activity measured was not proportional to the number of gene copies. It was nevertheless three to four times higher in the presence of pYB7 or pYB3 than in the presence of the genomic gene copy alone, 14S31b (pYB1+pYB7) might be expected to display 3-fold higher kinase activity than 14S31b (pYX1+pYX223). As this is not the case (Table II, rows 9 and 4), the most likely hypothesis seems to be that the appearance of measurable synthase activity requires association of the synthase with both the kinase and the reductase. This, however, remains to be proven definitely.

We have shown that the kinase coimmunoprecipitates with HA-tagged synthase. That the reductase was not clearly shown to do so certainly does not allow us to exclude its participation in a multienzyme complex. In fact, a faint protein band with the molecular mass expected for the reductase (38 kDa) did show up in one synthase immunoprecipitation (Fig. 6), but the amount was nevertheless three to four times higher in the presence of pYB7 or pYB3 than in the presence of the genomic gene copy alone, 14S31b (pYB1+pYB7) might be expected to display 3-fold higher kinase activity than 14S31b (pYX1+pYX223). As this is not the case (Table II, rows 9 and 4), the most likely hypothesis seems to be that the appearance of measurable synthase activity requires association of the synthase with both the kinase and the reductase. This, however, remains to be proven definitely.

**Biological Significance of the Metabolon**—The term metabolon was introduced by Scherer (25) to describe the organization of enzymes of a given metabolic pathway in supramolecular associations of sequentially acting enzymes and structural components. Such associations of enzymes can for example confer protection of labile intermediates.

*N*-Acetylglutamate, product of acetylglutamate synthase and substrate of acetylglutamate kinase, is not a labile intermediate. *N*-Acetylglutamyl phosphate, product of the kinase, is labile, and this might justify complex formation between acetylglutamate kinase and acetylglutamyl-phosphate reductase, but for such an interaction there is no evidence so far.

However, careful regulation of the first step of arginine biosynthesis is important to prevent wasteful consumption of acetyl-CoA. The step catalyzed by acetylglutamate synthase is needed to feed the acetylated derivatives cycle with newly synthesized acetylglutamate when the arginine pool becomes too low, but only until enough “cheap” recycled acetylglutamate is available.

Our present data clearly show that the synthase is catalytically active only when it can associate with the kinase/reductase and that it is unstable in the nonassociated state. This suggests a model where the kinase would be the “sensor” of arginine and acetylglutamate, assuming different configurations according to the sizes of the arginine and acetylglutamate pools. These changes would modulate the protein’s interaction with the synthase and thus control the latter’s activity. In this
model, the catalytic activity of the synthase would be optimal when neither arginine nor acetylglutamate was bound to the kinase. Only then would a productive protein complex occur, enabling the activated synthase to produce acetylglutamate at the expense of acetyl-CoA. This “expensive” acetylglutamate would be “channeled” to the kinase, promoting a configuration modification reducing the catalytic efficiency of the synthase. As soon as the ideal arginine concentration was reached, or if arginine was provided from outside, the synthase activity would be reduced even more drastically and eventually inactivated completely. This would account for the synergistic feedback inhibition of synthase activity by arginine in the presence of acetylglutamate, observed by Wipf and Leisinger (6).

Association between the two first enzymes could further allow coordination of the concentrations of these enzymes. When arginine is plentiful, expression of the ARG5,6 gene is repressed 5–6-fold (26, 27). In addition, the kinase is a limiting step in arginine biosynthesis (28). Hence the synthase, whose expression is not repressed by arginine (5)3 might be produced in molar excess under repressing conditions. Down-regulation of this undesirable excess could result from the intrinsic instability of the free synthase protein or even possibly from programmed, targeted degradation.

As concluding remark, we like to stress that the regulation of the synthase catalytic activity by the kinase/reductase activities via a required protein association is not the only strategy allowing the coordination of the enzymes at the entrance of the pathway; indeed, there is no evidence of its existence in prokaryotes, even those where arginine biosynthesis follows the same cyclic pattern as in fungi and where arginine exerts feedback inhibition on the first two steps of the pathway.

3 A. Abadjieva, K. Pauwels, P. Hilven, and M. Crabeel, unpublished results.