The Yeast ARG7 Gene Product Is Autoproteolyzed to Two Subunit Peptides, Yielding Active Ornithine Acetyltransferase*

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Yeast ornithine acetyltransferase has been purified from total yeast extracts as a heterodimer of two subpeptides (Liu, Y., Van Heeswijk, R., Hoj, P., and Hoogenraad, N. (1995) Eur. J. Biochem. 228, 291–296), confirmed to derive from a single ARG7-encoded precursor (Crabeel, M., Abadjieva, A., Hilven, P., Desimpelaere, J., and Soetens, O. (1997) Eur. J. Biochem. 250, 232–241). By Western immunoblotting, we show that Arg7p is also present as two subpeptides in isolated mitochondria, but that processing occurs before targeting to the mitochondria: deletion of the N-terminal leader peptide results in cytosolic accumulation of N-Arg7p, whereas C-Arg7p partially reaches the organelle by itself. When artificially co-expressed from separate genes, the two subpeptides can complement an arg7 mutation; ornithine acetyltransferase activity is measurable. Matura

ornithine is an important intermediate in the arginine biosynthetic pathway. Its synthesis in five steps starts with acetylation of the α-amino group of glutamate by acetylglutamate synthase (preventing cyclization of glutamate-γ-semialdehyde as occurs in proline biosynthesis) and ends with deacetylation of acetylornithine. In Escherichia coli and a few other bacteria using the linear pathway of arginine biosynthesis, the latter step is catalyzed by acetylornithinase. Most often, however, a more economic cyclic pathway is used in which ornithine acetyltransferase transfers the acetyl group of acetylornithine to glutamate, thereby regenerating acetylglutamate. In this case, the first enzyme of the pathway, acetylglutamate synthase, plays merely an anaplerotic role. Ornithine acetyltransferase (N-acetyl-L-ornithine:L-glutamate N-acetyltransferase, EC 2.3.1.35) is encoded by the bacterial argJ genes and, in the yeast Saccharomyces cerevisiae, by ARG7 (for reviews, see Refs. 1 and 2). In yeast, the acetylated derivatives cycle occurs in the mitochondria (3); ornithine produced in the matrix requires the mitochondrial ornithine carrier Arg11p for its export to the cytosol, where it is further processed to arginine (4).

Ornithine acetyltransferase was purified to homogeneity in the laboratory of Hoogenraad (5). On non-dissociating polyacrylamide gels, purified Arg7p migrated as a single band corresponding to an apparent molecular mass of 57 kDa. The protein in this band, however, turned out to be a heterodimer, since it resolved, when electrophoresed on denaturing polyacrylamide gels, to two subunit peptides with respective apparent molecular masses of 26.3 and 30.7 kDa. The evidence further suggested that the two peptides were derived from a single precursor since (i) a single in vitro translation product of approximately 57 kDa was immunoprecipitated by an antibody raised against the purified small subunit; (ii) the N-terminal sequences of the purified small and large subunits were respectively 40% and 45% identical to the N-terminal and central amino acid sequences of the homologous protein Arg J of Neisseria gonorrhoeae (5).

Our characterization of the ARG7 gene of S. cerevisiae (6) provided confirmation that ornithine acetyltransferase is indeed encoded by a single nuclear gene. It further revealed, by comparison with the N-terminal amino acid sequence determined by Hoogenraad, the presence of a short (8-residue), cleavable mitochondrial targeting leader peptide at the N-terminal end of the pre-enzyme. The calculated molecular mass of the encoded Arg7 protein is 47.8 kDa, lower than the apparent molecular mass determined by gel electrophoresis in Hoogenraad’s laboratory. From the N-terminal sequence of the 30.7-kDa C-Arg7p subpeptide determined by Hoogenraad (XLLG-FIVTD . . . ), it emerges that the proteolysis site lies between alanine 214 and threonine 215 of the DNA-derived amino acid sequence.

The Arg7p acetyltransferase also displays some acetylglutamate synthase activity, enabling it, when the ARG7 gene is overexpressed in either yeast or E. coli, to complement mutations leading to the absence of acetylglutamate synthase activity (6).

Here we have used various HA epitope-tagged derivatives of Arg7p to re-examine the protein’s structural properties. We confirm that the enzyme is detected as two subunit peptides even in extracts of isolated mitochondria. We show that processing (i) precedes targeting to the mitochondria, (ii) occurs in a heterologous E. coli background, (iii) occurs in vitro, when Arg7p is produced in a coupled transcription/translation system, and (iv) depends on threonine 215. Together, the data strongly suggest that maturation occurs by autoproteolysis.

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We further link maturation with enzyme activity, showing that the unprocessed enzyme remains totally inactive.

**Construction of Various HA Epitope-tagged Derivatives of Arg7p—**

All constructs encoding amino acids 1–214 of Arg7p under the control of the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible lac-lac promoter (7). The full-length *HIS3* gene was amplified on plasmid pYeA7–1 using primers AA37 and AA40.

**Construction of Plasmids Expressing N-Arg7p and C-Arg7p as Separated Peptides—**

Plasmid pH9 was constructed by cloning a PCR fragment encoding amino acids 1–214 of Arg7p under the control of the *GAL1* promoter in vector pYX213 (2μ, URA3) (from R&D Systems). PCR amplification was carried out on plasmid pYeA7–1 as DNA template amplified on plasmid pYeA7–1 using primers AA37 and AA38 (bearing a BamHI restriction site) and pAA40 (bearing a BamHI restriction site).

The PCR inserts of both pH9 and pH97 and the novel junctions were completely DNA sequenced to check for the absence of PCR-induced errors.

**Construction of the T215A Mutant of Arg7p—**

Construction of the T215A mutant of Arg7p was achieved by site-directed mutagenesis of the full-length Arg7p ORF. In this vector expression is driven by the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible T7-promoter (7). The full-length *HIS3* gene was amplified on plasmid pYeA7–1 using primers AA37 and AA40.

**Plasmids—**

- **pYeA7–1** (2μ, URA3, ARG7) and pAA7. (The PCR99A expressing the ARG7 ORF) under the control of the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible lac-lac promoter have been described in (6).
- **Plasmid pHP12** (encoding mitochondrial acetylornithine carboxamidotransferase, and pHP16, bearing ARG2 encoding cytosolic ornithine carboxamidotransferase, are 2μ-based URA3 plasmids described in Ref. 4.

**Oligonucleotide primers are as follows:**

- **AA1, GCCGGGCTCC-CATGGAGATATCATCAACATTGCTTC; AA2, GGCGCGGCGGCGT- GAAGCTTACGCTTATGTTACCTATC; AA3, GGCGGGCCGCGGGCTTACATG
gtAGTTGTTGCTGGTGAC; AA4, GGCGGGGGCTTATATCGAAGCTTACATG
gtAGTTGTTGCTGGTGAC; AA5, GGCGGGGGCTTATATCGAAGCTTACATG
gtAGTTGTTGCTGGTGAC; AA6, GGCGGGGGCTTATATCGAAGCTTACATG.

**Construction of Plasmids pAA31 and pAA32—**

- **pAA2** was derived from pYeF2 (2μ, URA3) into vector pTrc99A and obtained after insertion of an EcoRI restriction site (Promega) and used in a self-priming PCR amplification system, together with the external oligomers AA1 and AA2. Thus, exponentially increasing amounts of multiple DNA (encoding T215A Arg7p) were produced, together with a small amount of wild-type ARG7 DNA linearly amplified from the pAA2 template. After purification, this DNA was cut with BamHI and NotI (5` add-on restriction sites present on AA1 and AA2), purified, and ligated with similarly restricted and purified pYeF2 vector. Plasmid pH12 was obtained from an E. coli transformant and shown by DNA sequencing of the full ARG7 ORF to contain no other modification than the intended G to A mutation causing threonine 215 to be replaced by an alanine.

**Construction of Plasmids pAA31 and pAA32—**

**Wild-type Arg7p and mutant arg7 encoding T215A Arg7p** were PCR-amplified on plasmids pYeA7–1 and pH912, respectively, using oligonucleotide AA69 as forward primer and AA70 as reverse primer. They contain, respectively, an Ndel site followed by a stop codon. The two fragments were inserted into the pET-19b vector from Novagen, under the transcriptional control of the IPTG-inducible T7-RNA polymerase and in translational frame with a N-terminal, enterokinase-removable 10×His tag. The resulting plasmids were called pAA31 and pAA32, respectively.
Fig. 1. List of plasmids used in this study and schematic representation of the proteins encoded by the genes they express. Each protein produced is represented by a line between vertical bars, and the symbol HA indicates the presence of a fused HA epitope. Thick lines represent the proteins detected on Western immunoblots (see "Results"). Masses are derived from previously published nucleotide sequences and do not take into account the presence of the epitope or deletion of the leader peptide (represented by an asterisk) (Arg3p, see Ref. 8; Arg5p, see Ref. 9; Arg7p, see Ref. 6).

| Plasmids | Cloning vectors | Arg7p status |
|----------|-----------------|--------------|
| pAA2     | pYe F2 (2µ, URA3) | N-Arg7p (22.9 kDa) | C-Arg7p (25 kDa + HA) |
| pHPL1    | pYe F1 (2µ, HIS3) | N-Arg7p (22.9 kDa) | C-Arg7p (25 kDa + HA) |
| pAA3     | pYe F1 (2µ, URA3) | N-Arg7p (22.9 kDa) | C-Arg7p (25 kDa + HA) |
| pHPL3    | pYe F1 (2µ, HIS3) | N-Arg7p (22.9 kDa) | C-Arg7p (25 kDa + HA) |
| pHP4     | pY223 (2µ, HIS3) | N-Arg7p (22.9 kDa) | C-Arg7p (25 kDa + HA) |
| pHPL6    | pY223 (2µ, URA4) | N-Arg7p (22.9 kDa) | C-Arg7p (25 kDa + HA) |
| pHPL7    | pY223 (2µ, HIS3) | N-Arg7p (22.9 kDa) | C-Arg7p (25 kDa + HA) |
| pHPL9    | pY223 (2µ, URA4) | N-Arg7p (22.9 kDa) | C-Arg7p (25 kDa + HA) |
| pHPL12   | pYe F2 (2µ, URA3) | N-Arg7p (22.9 kDa) | C-Arg7p (25 kDa + HA) |

| Plasmids encoding subcellular markers | Arg7p p (37.8 kDa + HA) | Arg8 p (46.6 kDa a + HA) |
|--------------------------------------|---------------------------|---------------------------|
| pOS15                               | N-Arg7p (22.9 kDa) | C-Arg7p (25 kDa + HA) |
| pOS13                               | N-Arg7p (22.9 kDa) | C-Arg7p (25 kDa + HA) |

Sonifier, model 250, and microcentrifuged (10 min, 12,000 rpm, 4 °C). Aliquots (10 and 20 µl) of these extracts were used for SDS-PAGE.

Preparation of Mitochondrial and Cytosolic Cell Fractions—The method of Daum et al., as optimized by Yaffe (11) was used to isolate mitochondria, except that "complete" antiprotease mixture (Roche Molecular Biochemicals) was added to the lysis buffer. We usually started with 2 liters of yeast cells grown on galactose medium to OD 2.

SDS-PAGE and Western Immunoblotting—Standard protocols were used for Western immunoblotting (as in Ref. 6). SDS-PAGE was performed on 10%, 12%, or 15% SDS-polyacrylamide gels. Rainbow molecular size standards from Amersham Pharmacia Biotech were used as markers. Proteins were transferred to Millipore Immobilon-P membranes by electroblotting with a Bio-Rad cell. Mouse monoclonal antibodies to HA were used as markers. Proteins were transferred to Millipore Immobilon-P membranes by electroblotting with a Bio-Rad cell. Mouse monoclonal anti-HA (12CA5) from Roche Molecular Biochemicals was used to recognize HA epitope-tagged proteins; this was followed by detection of chemiluminescence with Roche’s Western blotting kit based on peroxidase conjugates of anti-mouse IgG.

Coupled in Vitro Transcription and Translation—The Promega TNT quick-coupled transcription/translation system was used according to the manufacturer’s instructions. For radioactive labeling, we used Revive L-[35S]methionine from Amersham Pharmacia Biotech (1000 Ci/mmol at 10 mCi/ml).

Ornithine Acetyltransferase Activity Assay—Yeast cell cultures, cell extracts, and enzymatic reaction conditions were as described previously (6). A modified step was separation of the acetylglutamate formed in the reaction mixture from the [14C]glutamate added to the reaction mixture, by means of prefilled Dowex AG 50-W (X8 resin; 200–400 mesh) chromatography columns from Bio-Rad.

Spot Test Complementation Assay—The different yeast strains were grown overnight on a selective minimal medium. The cells were diluted in fresh medium the next morning and allowed to grow to exactly OD 0.5. Then 1 ml of each culture was centrifuged, washed with sterile water, recentrifuged, and resuspended in 1 ml of sterile water. This suspension was serially diluted down to 10⁻³, and 10-µl aliquots of each dilution were spotted in a row on a Petri dish containing the relevant medium. Plates were incubated for 2 days at 30 °C.

RESULTS

Arg7p Is Obtained as Two Subpeptides in Yeast Total Protein Extracts Prepared under Conditions That Minimize Trivial Proteolytic Digestion—In Hoogenraad’s laboratory, ornithine acetyltransferase was isolated as a heterodimer from a commercial preparation of compressed yeast. We wanted to test whether two subpeptides would also be obtained from ornithine acetyltransferase purified from our Σ1278b-derived laboratory strains and from mutant strain BJ5459 devoid of vacuolar proteinases A and B. The extracts were prepared in the presence of a mixture of protease inhibitors.

To monitor the ARG7 gene product(s) by Western immunoblotting, we constructed a series of gene fusions expected to produce HA epitope-tagged derivatives of the enzyme. pAA2 yields Arg7p bearing the epitope at its C terminus, pHP1 yields Arg7p tagged at the intact N terminus; pAA3 and pHPL3 also encode N-tagged Arg7p, but with partial (pAA3) or total (pHP3) truncation of the mitochondrial leader peptide (Fig. 1).

Western immunoblots showed only subunit peptides. When the harbored plasmid was pAA2, the only peptide to appear was C-Arg7p (apparent molecular mass: 31 kDa), the subunit constituting the C-terminal portion of Arg7p; when the plasmid present was pAA3 or pHPL3, the detected polypeptide was N-Arg7p (apparent molecular mass: 26 kDa), i.e. the smaller, N-terminal portion of Arg7p (data not shown, but see further below for illustrated Western immunoblotting experiments). No product was detected when the tag was on the intact N terminus of Arg7p (use of plasmid pHPL1). This was as expected, since the N-terminal mitochondrial targeting peptide is cleaved off during targeting to the mitochondria.

Thus, Arg7p is processed to two subunit peptides in various yeast genetic backgrounds. A very small quantity of uncleaved precursor, with an estimated apparent molecular mass of 48 kDa, was sporadically observed in overloaded lanes. In some experiments, C-Arg7p appeared as a double band.

Arg7p Is Processed Independently of Its Targeting to the Mitochondrial Matrix—To analyze the mechanism of the proteolytic cleavage of Arg7p, we examined the subcellular localization of N-Arg7p and C-Arg7p produced from wild-type Arg7p and from versions lacking the mitochondrial leader peptide. We expected to observe cytosolic accumulation of the full-length Arg7p precursor if its maturation were catalyzed by a mitochondrial processing peptidase. We used C-terminally HA epitope-tagged derivatives of Arg3p (ornithine carbamoyltransferase, 39 kDa) and Arg8p (acylornithine transaminase, 47 kDa) as markers of the cytosolic and mitochondrial fractions, respectively.

Fig. 2A shows what we actually observed when the plasmid insert (pHP3) encoded an N-terminally tagged Arg7p lacking...
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**FIG. 2.** Western immunoblots showing that when the Arg7p N-terminal mitochondrial targeting peptide is deleted, the N-Arg7p subpeptide accumulates in the cytosol while most of the C-Arg7p subpeptide reaches the mitochondria on its own. Panels A and B show two distinct 12% SDS-polyacrylamide gel electrophoreses where cytosolic (Cy) and mitochondrial (Mt) extracts of strains bearing different combinations of HA-tagged fusion proteins are run in parallel. Panel A, lanes 1 and 2, cytosolic and mitochondrial fractions of a strain producing the Arg3p cytosolic marker and N-terminally tagged Arg7p deleted of amino acids 1–16; lanes 3 and 4, cytosolic and mitochondrial fractions of a strain producing the Arg3p mitochondrial marker and N-terminally tagged Arg7p deleted of amino acids 1–16. Panel B, lanes 1 and 2, as in panel A; lanes 3 and 4, cytosolic and mitochondrial fractions of a strain producing the Arg3p cytosolic marker and C-terminally tagged Arg7p deleted of amino acids 1–16. Roche’s Rainbow molecular mass markers were used as molecular size standards (not shown here, but as in Fig. 6A).

**TABLE I**

| Strains | Generation time on Gal minimal medium | Ornithine acetyltransferase activity (nmol · min⁻¹ · mg protein⁻¹) |
|---------|--------------------------------------|-------------------------------------------------------------|
| 14S40c (pYX213 + pYX223) | 14 h 40 min | 10.2 |
| 14S40c (pHP6 + pYX223) | >15 h | 418 |
| 14S40c (pYX213 + pH7) | 14 h 40 min | 128 |

Arg7p is active in vivo as a heterodimer consisting of an N-Arg7p and a C-Arg7p subpeptide, a functional enzyme might possibly be reconstituted from the two peptides produced separately in the same cells through co-expression of two distinct artificial genes. To test this possibility, we constructed pHP6, expressing a “gene” encoding N-Arg7p under the control of the GAL1 promoter of plasmid pYX213 (2μ, HIS3), and pH7, expressing a “gene” encoding C-Arg7p under the control of the GAL1 promoter of plasmid pYX223 (2μ, URA3). We then transformed strain 14S40c (ura3⁺, his3⁺, arg7⁺) with all combinations of empty vectors and of the plasmids just mentioned. Table I shows that the arg7⁻ strain’s bradytrophic growth can be almost fully remedied when both Arg7p subunits are produced in the same cells. Neither subunit produced separately has any complementing effect. Furthermore, ornithine acetyltransferase activity was detectable only in cases of complementation. The measured enzyme activity amounted to 2.5% of the activity displayed by cells expressing a full-length ARG7 gene likewise driven by the GAL1 promoter (pHP9), and to about 10% of the activity measured in cells expressing ARG7 from its own promoter.
mally as separate domains with sufficient affinity for each other to allow their proper association and correct targeting to the mitochondria. The fact that complementation is only partial and enzyme activity not fully restored might have several causes, among which the presence of a terminal methionine in the C-Arg7 peptide seems to us the most probable. No activity was detected when we tried to reconstitute the enzyme by mixing two separate extracts, one presumably containing N-Arg7p and the other C-Arg7p. This suggests that at least one of the subunits might be unstable when produced singly.

The Fully Conserved Threonine 215 at the N Terminus of C-Arg7p Is Absolutely Required for Processing—A large number of ornithine acetyltransferases have been characterized to date and their alignment (Fig. 4) shows that the most conserved region in this family overlaps with the maturation site predicted from the data by Hoogenraad’s team. Threonine 215 in particular, the predicted N-terminal amino acid of C-Arg7p, is conserved in all members of the family. To evaluate the role of this residue in ornithine acetyltransferase maturation, we constructed a T215A mutant protein, i.e., one in which threonine 215 is changed to an alanine. The mutant enzyme, produced from plasmid pHP12 in strain BJ5459, was shown in total protein extracts to accumulate exclusively as an uncleaved precursor (Fig. 5A). However, it was shown by subcel-

![ClustalW alignment of the 17 characterized ornithine acetyltransferases whose sequences are available in data banks.](http://www.jbc.org/)

**FIG. 4.** ClustalW alignment of the 17 characterized ornithine acetyltransferases whose sequences are available in data banks. From top to bottom and in alphabetical order: *Aquifex aeolicus*, *Archaeoglobus fulgidus*, *Bacillus amyloliquefaciens*, *Bacillus stearothermophilus*, *Bacillus subtilis*, *Corynebacterium glutamicum*, *Lactobacillus plantarum*, *Methanococcus jannaschii*, *Methanobacterium thermoautotrophicum*, *Mycobacterium tuberculosis*, *N. gonorrhoeae*, *S. cerevisiae*, *Streptomyces clavuligerus*, *Schizosaccharomyces pombe*, *Synechocystis*, *Thermus thermophilus*, and *Thermotoga neapolitana*. The conserved sequence around the *S. cerevisiae* threonine 215, on the N-side of which proteolysis occurs, is underlined, and the conserved threonine 215 is indicated by a triangle.
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lular fractionation to be correctly targeted to the mitochondrion (Fig. 5B).

The fact that threonine 215 is absolutely required for Arg7p processing suggests that an autoproteolytic mechanism could be responsible for maturation. Such self-processing events mediated by the -OH group of a threonine or serine, or by the -SH group of a cysteine, have been shown to be quite frequent (12, 13). If maturation of Arg7p does rely on autoproteolysis, it should not be restricted to the yeast background.

Arg7p, Which Is Functional When Produced in E. coli, Is Processed in This Heterologous Background as Well—If Arg7p maturation is autocatalytic rather than catalyzed by a distinct endoprotease, the enzyme could be correctly processed in a heterologous context as well. We have shown previously by complementation and enzymatic assays that Arg7p is functional in E. coli when its ORF is expressed from a prokaryotic promoter (6). To see whether Arg7p maturation occurs in this heterologous background, we produced E. coli transformants harboring either the empty pTrc99A vector or the derived pAA7 plasmid expressing functional Arg7p. The strains were induced with IPTG, total protein extracts prepared, and SDS-PAGE was used to separate the extracted proteins. Coomassie Blue staining revealed two extra protein bands appearing only in the presence of pAA7 (estimated apparent molecular mass: 31 and 26 kDa). This shows that Arg7p is cleaved in E. coli as well (data not shown). We repeated the same experiment with E. coli strains containing either the vector pET19b or a derived plasmid expressing the gene for either wild-type Arg7p (pAA31) or the T215A variant, under the control of an IPTG-inducible T7-RNA polymerase. As shown in Fig. 6A, the wild-type enzyme is processed to the usual 31- and 26-kDa subpeptides, while the mutant enzyme accumulates as an unprocessed precursor with an apparent molecular mass of 47 kDa, corresponding to the molecular mass of 47.8 kDa calculated for Arg7p on the basis of the DNA sequence.

Arg7p Is Cleaved in an in Vitro System—Although Liu et al. (5) obtained results showing that ornithine acetyltransferase produced from yeast mRNA in a reticulocyte lysate was uncleaved, showing up as a 57-kDa precursor, we decided to reassess the structure of the enzyme produced in vitro.

Transcription/translation reactions were carried out in a rabbit reticulocyte system using pET19b, pAA31, or pAA32 plasmid DNA as the template in the presence of 35S-labeled methionine. As shown in Fig. 6B, only a 47-kDa precursor (not a 57-kDa protein) was obtained from the mutant template, while the wild-type template gave rise to 31- and 26-kDa subunits, with only a faint band at 47 kDa. The 31-kDa band appeared fainter than the 26-kDa band. This is explained in part by the presence of only 4 methionines in C-Arg7p versus 8 in N-Arg7p. Furthermore, C-Arg7p appeared as a double band, a fact not yet understood.

The T215A Mutant Displays No Detectable Ornithine Acetyltransferase Activity and Does Not Complement an arg7 Mutation—In enzyme assays, we measured the ornithine acetyltransferase activity of the yeast mutant strain JD1(arg7A) harboring either plasmid pAA2, encoding wild-type Arg7p, or plasmid pH12, encoding the T215A Arg7p mutant. After growth on galactose medium to induce GAL1-promoter-driven expression of the enzyme, we detected no activity in the extract expected to contain the mutant Arg7p. The background level was the same as for the same strain carrying the empty pYeF2 cloning vector. This is in sharp contrast with the activity measured (300 nmol of acetylglutamate formed/min/mg of protein) when extracts of pAA2-harboring cells were used (Table II).

We have shown previously that a strain deleted of the ARG7 ORF has an arginine-leaky phenotype (6). In keeping with the results of the enzyme assays, we detected no improvement of

![Fig. 6. Proteolysis of wild-type Arg7p in the heterologous E. coli background (panel A) and in vitro (panel B). Panel A, SDS-PAGE (10% polyacrylamide) of total protein extracts of IPTG-induced E. coli HMS174(DE3)pLYSs transformed with the vector pET19b (lane 2), the pET19b-derived plasmid pAA31 bearing wild-type ARG7 (lane 3), or the pET19b-derived plasmid pAA32 bearing a mutated ARG7 encoding the mutant protein T215A Arg7p (lane 4). A mixture of protein standards (Rainbow molecular mass) was run in lane 1, and their molecular mass values (in kDa) are indicated. Panel B, the products of four in vitro coupled transcriptions/translations in rabbit reticulocyte lysates were run in parallel on the same gel. The following templates were added to the reaction mixtures: lane 1, no DNA; lane 2, pAA31; lane 3, pET19b; lane 4, pAA32. Arrows between the two panels indicate the positions, from top to bottom, of unmaturated Arg7 precursor protein and of the C-Arg7p and N-Arg7p subpeptides.

**Table II**

| Strains | Protein expressed from the plasmid | Ornithine acetyltransferase activity (growth on galactose) |
|---------|----------------------------------|----------------------------------------------------------|
| JDI (pYeF2) | Empty vector                   | <0.3                                                    |
| JDI (pAA2)  | Wild-type Arg7p                | 300                                                     |
| JDI (pHP12) | T215A–Arg7p                    | <0.3                                                    |

![Fig. 7. The mutant protein T215A Arg7p is unable to complement a strain deleted of its chromosomal ARG7 gene. Yeast strain JD1 transformed with the empty vector pYeF2 (row 1), with pAA2 expressing wild-type Arg7p (row 2), or with pHP12 expressing the mutant protein T215A Arg7p was grown on inducing galactose medium and spotted after serial dilution on solid galactose medium without (left) or with (right) added arginine.](http://www.jbc.org/)

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growth in the absence of arginine in JD1(pYeF2) or JD1(pHP12),
but observed full complementation with JD1(pAA2) (Fig. 7).

Thus, T215A Arg7p is inactive, and this absence of catalytic
activity correlates with the absence of maturation. This sugges-
ting that processing could be directly required to render orn-
ithine acetyltransferase catalytically active.

**DISCUSSION**

**Mechanism of Arg7p Maturation—** Ornithine acetyltrans-
ferase is encoded by the ARG7 gene (6), expected to produce a
single 441-amino acid protein. However, the enzyme is isolated
from yeast extracts as a heterodimer consisting of the N- and
C-Arg7p subpeptides.

A priori, three different cleavage mechanisms can be consid-
ered: (i) Arg7p proteolysis could occur trivially during extract
preparation, (ii) the Arg7p precursor protein could be matu-
rated in vivo by a specific endoproteolytic processing enzyme,
and (iii) the Arg7p protein could undergo intrinsic autoproteo-
lysis. In the first case, processing should be physiologically irrel-
levant while, in the latter two, maturation is likely to have
biological significance. As explained below, although we pro-
vide no compelling data for ruling out the possibility of trivial
proteolysis, our results as a whole strongly favor the hypothesis
of autamaturation, a hypothesis further supported by similar
results obtained with bacterial ornithine acetyltransferases
(see below).

In fact, in an attempt to demonstrate unambiguously that
Arg7p is matured in vivo, we did observe, in a series of kinetic
pulse-chase and immunoprecipitation experiments, the ap-
pearance (and dilution) of radioactive full-length Arg7p before
the appearance of labeled N- and C-Arg7p subpeptides, as
expected in the case of an in vivo precursor (data not shown).
The low amount of precursor, however, makes this approach
technically very difficult. Nevertheless, the body of indirect
evidence makes trivial proteolysis, and even maturation by a
distinct endoprotease, seem highly improbable. The observa-
tions in favor of autoproteolysis are the following: (i) the yeast
enzyme is also matured in the heterologous E. coli back-
ground, (ii) in vitro coupled transcription/translation of ARG7
also yields processed enzyme, (iii) a threonine plays a key role
in proteolysis, (iv) processing occurs in the region most con-
served among the sequenced ornithine acetyltransferases and
is required for activity, and (v) the recombinant ornithine
acetyltransferases of *Bacillus stearothermophilus*, *Thermotoga
neapolitana*, and *Methanococcus jannaschii* have also been iso-
lated from *E. coli* as heterodimers produced by cleavage up-
stream from the conserved threonine. Furthermore, the threo-
nine of the B. stearothermophilus enzyme is also essential to
maturation and catalytic activity.2

Numerous cases of in vivo self-catalyzed protein rearrange-
ments have been discovered in the 1990s. They are all initiated
by an acyl rearrangement in which a crucial threonine, serine,
or cysteine forms a (thio)ester bond by nucleophilic attack on a
backbone carbonyl which can be adjacent, distal, or even situ-
ated on another molecule (12, 13). Such autoprocessing reac-
tions relate to a variety of biological functions ranging from
protein splicing (of inteins, for example), to protein targeting
(of certain hedgehog proteins, for example), proenzyme activa-
tion (for instance, formation of pyruvoyl enzymes and of N-
terminal nucleophilic hydrolases), and to generation of en-
zyme-bound prosthetic groups (in ubiquitin transfer, for exam-
ple) (reviewed in Ref. 13).

Structural data to be obtained on purified ornithine acetyl-
transferase by x-ray crystallography will be required to fully
understand the mechanism of Arg7p maturation.

**Function of Arg7p Processing—** Because the T215A mutation
totally prevents processing of Arg7p and completely abolishes
its enzymatic activity, it is tempting to speculate that post-
translational proteolytic maturation of Arg7p is directly re-
quired to activate the catalytic site of an inactive proprotein,
especially since cleavage occurs in the domain most conserved
among the ornithine acetyltransferases. Alternatively, matu-
rated could be required indirectly, for example in a structural
reorganization needed to activate enzyme function. Whether
directly or indirectly, maturation is clearly required for the
enzyme to function. Structural comparisons between wild-type
Arg7p, the T215A mutant, and the two subpeptides crystallized
separately will shed light on these matters.

The following 14-amino acid consensus sequence surrounds
the site of proteolytic cleavage: KGXXGM/LP/MXPX(M/L)-
ATX215(M/L). It will, of course, be most interesting to deter-
mine the role of these amino acids in enzyme maturation
and catalytic activity by comparing the x-ray crystallog-
graphic structures of purified wild-type Arg7p, T215A Arg7p,
and other Arg7p variants mutated in this region.

**Why Are the Observed Apparent Molecular Masses of the
Subunit Peptides Higher than the Calculated Molecular Mass?—**
In contrast to Hoogenraad (5), who estimated the ap-
parent molecular mass of the Arg7p precursor at 57 kDa (the
sum of the molecular mass values of the two subunit peptides),
we found for Arg7p a molecular mass of 48 kDa, which is in
agreement with the molecular mass calculated from the DNA
sequence of ARG7 (6). However, the apparent molecular mass
values we observe for the subunit peptides (26 and 31 kDa for
N- and C-Arg7p, respectively) are the same as determined by
Hoogenraad, i.e. greater than the calculated values (22.9 for
N-Arg7p and 25 kDa for C-Arg7p). This has not been elucidated
yet, but could mean that an additional posttranslational modi-
fication is involved in the maturation of Arg7p. It might also
explain the occasional observation of double bands for C-Arg7p.
However, the molecular masses of the subpeptides appear the
same in yeast extracts, *E. coli* extracts, and when produced in
vitro in reticulocyte lysates. Further investigation is needed to
understand these aspects.

In summary, we show that formation of a functional yeast
ornithine acetyltransferase requires maturation of an inactive
precursor protein to two subunit peptides, and that threonine
215, the N-terminal amino acid of the posttranslationally re-
leased C-Arg7p, plays a key role in enzyme processing.

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