Processing and Function of a Polyprotein Precursor of Two Mitochondrial Proteins in Neurospora crassa*

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Lilian Parra-Gessert, Kenneth Koo‡, Joaquin Fajardo, and Richard L. Weiss§

From the Department of Chemistry and Biochemistry, UCLA, Los Angeles, California 90095-1569

In Neurospora crassa, the mitochondrial arginine biosynthetic enzymes, N-acetylglutamate kinase (AGK) and N-acetyl-γ-glutamyl-phosphate reductase (AGPR), are generated by processing of a 96-kDa cytosolic polyprotein precursor (pAGK-AGPR). The proximal kinase and distal reductase domains are separated by a short connector region. Substitutions of arginines at positions −2 and −3 upstream of the N terminus of the AGPR domain revealed a second processing site at position −20. Substitution of arginine at position −22, in combination with changes at −2 and −3, prevented cleavage of the precursor and identified two proteolytic cleavage sites, Arg-Gly Tyr-Leu-Thr at the N terminus of the AGPR domain and Arg-Gly-Tyr Ser-Thr located 20 residues upstream. Inhibitors of metal-dependent peptidases blocked proteolytic cleavage at both sites. Amino acid residues required for proteolytic cleavage in the connector were identified, and processing was abolished by mutations changing these residues. The unprocessed AGK-AGPR fusion had both catalytic activities, including feedback inhibition of AGK, and complemented AGK-AGPR mutants. These results indicate that cleavage of pAGK-AGPR is not required for functioning of these enzymes in the mitochondria.

In eukaryotic organisms, arginine biosynthesis is compartmentalized. In Neurospora crassa, the first six steps occur in the mitochondrion and the last two steps in the cytosol. Flux through the arginine biosynthetic pathway is regulated primarily by feedback inhibition of the enzymes that catalyze the first and second reactions. The second and third steps of the pathway are catalyzed by N-acetylglutamate kinase (AGK)1 (EC 2.7.2.8) and N-acetyl-γ-glutamyl-phosphate reductase (AGPR) (EC 1.2.1.38) (1, 2). These enzymes are produced from a polyprotein precursor (pAGK-AGPR), which is targeted to the mitochondria and processed into mature AGK and AGPR (3, 4). The polyprotein consists of a mitochondrial targeting sequence followed by two protein domains, AGK and AGPR, separated by a connector region (Fig. 1A).

Most mitochondrial proteins are synthesized in the cytosol and targeted to the organelle by leader sequences at the N terminus of their precursors. Mitochondrial leader sequences are recognized by specific receptors on the mitochondrial outer membrane and translocated from the receptors to downstream components of the import machinery (5–8). Removal of N-terminal targeting sequences in the matrix is performed by a mitochondrial processing peptidase (MPP), composed of two similar subunits, α-MPP and β-MPP; α-MPP is soluble in the matrix, and β-MPP is associated with the mitochondrial inner membrane (9–12). As import and processing take place, proteins are folded into functional enzymes or assembled into functional multienzyme complexes. We previously showed that two proteins were obtained that comigrated with mature AGK and AGPR upon incubation of in vitro synthesized wild-type pAGK-AGPR with purified MPP (4). However, identification of the precise cleavage site(s) in the connector region of the precursor remained to be determined.

Targeting sequences have the capability to form an amphipathic α-helix (13); however, defined sequences or structural motifs involved in proteolytic processing are not well understood (14–17). The connector of pAGK-AGPR contains an internal processing sequence, which has some of the characteristics of a mitochondrial targeting sequence, although it is not predicted to form an amphipathic α-helix (4). Arginine residues at positions −2 or −3 and positions −10 or −11 relative to the first amino acid in the mature protein are often found in targeting sequences of mitochondrial precursor proteins and appear to form part of the not well defined motifs found at cleavage sites (15, 16). Some similarities between the connector region of pAGK-AGPR and mitochondrial targeting sequences are apparent (Fig. 1B).

Several questions are addressed in this study. What are the sequences or structural motifs that specify the cleavage at the connector region of pAGK-AGPR? How many cleavage events are necessary to process the polyprotein precursor into two proteins? Is processing of the precursor into two independent proteins required for function in the mitochondria? Processing of pAGK-AGPR and its biological function were analyzed in vitro and in vivo. The roles of several amino acid residues as signals for processing were examined by introducing point mutations in the connector region of the precursor. Two sites for proteolytic processing were identified, and processing into two mature proteins was prevented by mutagenesis of these sites. Proteolytic cleavage of pAGK-AGPR in the connector region did not appear to be required for the activity of either enzyme or for feedback inhibition of AGK by arginine. Processing at the connector region of pAGK-AGPR is discussed in the context of putative advantages that targeting of fusion proteins may have versus the targeting of independent proteins.

MATERIALS AND METHODS

Strains and Growth Conditions—Escherichia coli strains DH5α and JM101 were used to propagate plasmid DNA. Strain GM48 was used to

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‡ Present address: Hitachi Chemical Research Center, Inc., Plumwood House, Irvine, CA 92715.

§ To whom correspondence should be addressed: Dept. of Chemistry and Biochemistry, UCLA, Los Angeles, CA 90095-1569. Tel.: 310-825-2065; Fax: 310-206-5213; E-mail: weiss@chem.ucla.edu.

† The abbreviations used are: AGK, N-acetylglutamate kinase; AGPR, N-acetyl-γ-glutamyl-phosphate reductase; MPP, mitochondrial processing peptidase; PCR, polymerase chain reaction; MOPS, 4-morpholinepropanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; CAPS, 3-cyclohexylamino)propanesulfonic acid.
as specified. Bacterial cultures were grown in LB medium or terrific broth
for the generation of single-stranded plasmid DNA for site-directed mu-
tagenesis. Bacterial cultures were grown in Vogel's minimal medium N (20)
or Neurospora wild-type strain LA1 (74A) and pcP3 (12). Import reactions were performed by incubating a suspension con-
structed from the same sources described above for the first
amplification step. A low melting point agarose gel containing the
second PCR product (−660 ng) was resuspended in 80 µl of× 1
Bsu361-linearized Rh2a-Bsu361 in a final volume of 100 µl of 1×
ligation buffer and incubated with 1 unit of T4 ligase at
room temperature for 12 h (22). The ligation mixture was used to
transform competent cells of E. coli DH5α (23). Constructs pgTPTG4,
pca13PG16, and pcP3 were also generated by asymmetric PCR using
pgP22PG54-Hph. The presence of the desired mutation was
initially screened by digestion of DNA with SacI (new site generated by
the substitutions of Arg 2 and Arg 3 to Gly 2 and Pro 3), KpnI (new
site introduced by the substitutions of Arg 4 and Arg 5 to Gly 14 and
Thr 15), BstXI (new site generated by the change Arg 22 to Gly 22), or
HpaII (new site generated by the change Arg 22 to Pro 22) and subse-
quently by sequencing of the entire amplified regions.

In Vitro Transcription—To analyze processing in vitro, wild-type and mutated precursor proteins were synthesized by in
vitro transcription and translation. Plasmid DNA was linearized with
EcoRV, treated with proteinase K (50 µg/ml) for 30 min at 37 °C,
and precipitated with 2 volumes of ethanol. DNA was resuspended in
DEPC-treated distilled H2O and stored at −20 °C. Proteinase-
treated template was transcribed with T7 RNA polymerase as suggested by the
manufacturer (Promega). Transcripts were visualized by electrophore-
sis, using denaturing agarose gels (1.2% agarose containing 17% form-
alddehyde in MOPS buffer). Translation reactions in rabbit reticulocyte
lysates were carried out in a final volume of 50 µl as suggested by the
manufacturer (Promega). In vitro transcribed RNA (2 µl) was mixed
with 1 µl of Rnasin (10 units/ml), 1 µl of a mixture of amino acids (1
mM) except methionine, 5 µl of [35S]methionine (1,200 Ci/mmol, 10
µCi/ml), 6 µl of H2O, and 35 µl of reticulocyte lysate. The reaction was
incubated at 30 °C for 1 h. A 2.5-µl aliquot was removed by 7.5% SD-
PAGE and analyzed using a PhosphorImager (Molecular Dynam-
ics). The rest of the sample was brought to a final concentration of 0.3
mM sucrose and 0.05 mM methionine and stored at −80 °C.

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Fig. 1. A, domain organization of the polyprotein precursor (96 kDa,
871 amino acids) encoded by the arg-6 locus in N. crassa. The mito-
ochondrial targeting sequence at the N terminus of the precursor (44
amino acids) is indicated as the mitochondrial targeting sequence (MTS).
The kinase domain (52 kDa, GG) and reductase domain (37 kDa,
GG) are indicated as AGK and AGPR, respectively. The internal processing
sequence located in the connector region (−200 amino acids) of the
precursor is indicated as the internal processing sequence (IPS). The other
portion of the connector region is indicated as the eukaryotic domain.
Abbreviations for restriction endonucleases are as follows. B, Bsu361; A, Apal; C, Cia1. B, diagram of internal processing sequence mutations; amino acid substitutions in each construct are indicated in
boldface letters.

prepare nonmethylated DNA for digestion of methylation-sensitive re-
striction sites. E. coli RZ1032 and helper plasmid VCSM13 were used for
the generation of single-stranded plasmid DNA for site-directed muta-
genesis. Bacterial cultures were grown in LB medium or terrific broth
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**Table I**

**Oligonucleotides designed for site-directed and PCR mutagenesis**

| Name (size) | Sequence (5' → 3') |
|-------------|--------------------|
| BsuAGK (forward) (28-mer) | 5'-CCATCATCATTGGAGAAGGATTCCGAG-3' |
| IIB (reverse) (25-mer) | 5'-GGGCCCACCGCAATCTCGGCTGAG-3' |
| PGI (forward) (29-mer)† | 5'-CAGCATGTCTAACCAGGGGTTACTCTA-3' |
| PG2 (reverse) (29-mer)§ | 5'-TTAGGTAGCCCCGCGGTAGAGTCTG-3' |
| GT1 (forward) (35-mer) | 5'-CTCCACTCTGTGCTGTGGCTGCCAAATG-3' |
| GT2 (reverse) (35-mer) | 5'-ACCTGGGAAGGACGAGGTACGACACAGTGAGG-3' |
| OLP18 (forward) (27-mer) | 5'-GTGGCCATTGCTGCTGTGGTAGGAC-3' |
| OLP18 (reverse) (27-mer) | 5'-TTCCACTCTTGTCGCCGCTGCTGGGCTGCCAAATG-3' |
| OLP26 (forward) (33-mer) | 5'-TTGGGAAACGGACGGGGGAGAGAGTGGAGG-3' |
| OLP26 (reverse) (33-mer) | 5'-AAAGATTGGAGTACCGGAAACCTGCTG-3' |
| OLP12 (21-mer)† | 5'-AAAGATTGGAGTACCGGAAACCTGCTG-3' |
| OLP14 (27-mer)§ | 5'-AAAGATTGGAGTACCGGAAACCTGCTG-3' |

† Generates a KpnI site.
§ Generates a Smal site.
§§ Generates a MgI site.
四是 Generates a BstXI site.

**Results and Discussion**

Mitochondrial membranes were removed by centrifugation at 15,000 g for 10 min at 4 °C. Supernatants were brought to 400 µl with 1% TNET, and a 40-µl aliquot of undiluted anti-AGPR antiserum was added for each 5 mg of starting mitochondrial protein. Samples were incubated overnight with gentle rotation at 4 °C. Immunocomplexes were precipitated with the equivalent of two volumes of *Staphylococcus aureus* extract as the source of protein A. Incubation was continued for 60 min on ice. Samples were centrifuged at 15,000 × g for 1–2 min, and pellets were washed four times with 1 ml of TX/SDS (25 mM Tris-Cl, pH 7.0, 150 mM NaCl, 5 mM EDTA) and once with 1 ml of TBS (25 mM Tris-Cl, pH 7.4, 100 mM NaCl, 1 mM KCl). Washed pellets were solubilized in 50 µl of SDS/sample buffer without β-mercaptoethanol. Immunocomplexes were resolved by 7.5% SDS-PAGE and blotted onto polyvinylidene difluoride membranes (Bio-Rad) in CAPS transfer buffer (10 mM CAPS, 10% methanol, pH 11) at 4 °C (27). Proteins on the membrane were stained with Coomassie blue R250, the band of interest was cut out of the membrane, and the amino terminus was sequenced by the UCLA Sequencing Facility.

**Enzyme Assays—N-Acetylglutamate kinase and N-acetyl-γ-glutamyl-phosphate reductase activities of the uncleaved precursor were assayed using mitochondria purified by sucrose step gradient centrifugation (3). Acetylglutamate kinase was assayed by a radioactive procedure modified from Wolf and Weis (28). Reactions contained 0.15 µM Tris, pH 8.5, 60 mM MgCl₂, 30 mM ATP, 3.75 mM [¹⁴C]Acetylglutamate, and 0.2 mM NH₄OH and were incubated for 80 min at 30 °C.**

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Acetylglutamyl-phosphate reductase activity was assayed by following the increase in fluorescence as NADP⁺ was converted to NADPH (3). The reaction mixtures contained 0.1 mM glycine, pH 9.3, 1.33 mM acetylglutamate 5-semialdehyde, 25 mM K₃HPO₄, pH 9.3, 0.67 mM NADP⁺ ( freshly prepared), and 100, 200, or 300 µl of mitochondrial extract in a final volume of 3 ml. The reaction was initiated by the addition of the substrate, acetylglutamate semialdehyde, and the increase in fluorescence was followed using a Gilson Spectra/Glo filter fluorometer (excitation filter, 330–380 nm; emission filter, 430–600 nm). The reactions were carried out at 25 °C. The activity is expressed as the change in fluorescence/min and represents the average from three independent determinations.

Methylglyoxal was purchased from Promega and New England Biolabs. *Taq* polymerase was purchased from Pro-
When the arginine residues at positions 2 and 3 were changed to glycine and the arginine pair at position 14 to alanine in a background where Arg2 to Gly2 and Arg3 to Gly3 were not cleaved (34, 35), to determine if the presence of a proline residue at position 2 and 3 is located in a sequence motif that resembles the recognition site for MPP (14–17) but did not appear to function as a signal for processing. Analysis of amino acid sequences flanking cleavage sites of several hormonally and proteolytic cleavage sites of several hormone and protein precursors revealed that most peptides that contain a Pro residue at position +1 are not cleaved (34, 35). To determine if the presence of a proline residue at position −13 disrupted a possible MPP cleavage site, the proline residue at position −13 was changed to an alanine in a background where the arginine residues at −2 and −3 have been disrupted a possible MPP cleavage site, the proline residue at position −13 was changed to an alanine in a background where the arginine residues at −2 and −3 have been changed to Gly and Pro, shows that processing of the precursor was prevented by these substitutions, since no mature AGK or AGPR products were observed. The uncleaved precursor derived from pcP22PG54 was imported into mitochondria, since it was protected from proteinase K digestion. Some degradation of the precursor may have occurred in the matrix, suggested by the smear under the precursor band.

The role of arginine at position −22 can also be observed with construct pcG22PG10 (Fig. 2B, lane 4). The truncated precursor (T) was imported into mitochondria (protected from proteinase K) but was not cleaved (higher molecular mass than wild-type AGK). A full-length precursor containing the substitution at Arg22 to Gly22 (pcG22PG42; Fig. 2C, lanes 4 and 9) yielded similar results to those obtained with the precursor derived from pcP22PG54 (Fig. 2C, lanes 5 and 10); both precursors were imported (protected from proteinase K digestion) but were not processed in the mitochondria. However, some proteolytic degradation in the mitochondrial matrix was observed. These results indicate that proteolytic cleavage occurs at two different positions in the connector region of the precursor (Fig. 2D).

**RESULTS**

**Role of the Arginine Pairs at Positions −2 and −3 and Positions −14 and −15 from the N Terminus of the AGPR Domain**—The importance of an arginine residue at position −2 or −3 relative to the cleavage site of mitochondrial targeting sequences has been demonstrated (13–16). Preliminary analysis indicated that replacement of arginine with glycine at position −2 relative to the N terminus of the AGPR domain (Arg2 to Gly2) did not prevent cleavage of the polyprotein in vitro. A precursor containing the changes Arg2 and Arg3 to Gly2 and Pro3 (pcPG16) was used as a substrate for an in vitro import assay (Fig. 2A). Postimport mitochondria were reisolated and treated or not treated with proteinase K to digest bound precursor. Processing of the wild-type precursor (lanes 1 and 4) resulted in two protein bands; the upper protein band corresponds to mature AGK (52 kDa), and the lower protein band corresponds to mature AGPR (37 kDa) (29). Processing of the mutant precursor derived from pcPG16 resulted in an AGK protein that comigrated with wild-type AGK, but the AGPR protein (AGPR+) appeared to have a higher molecular mass than wild-type AGPR (Fig. 2A, lanes 2 and 5). This result suggested that processing at the N terminus of the AGPR domain, between amino acids −1 and +1, was prevented by the amino acid substitutions and that one or more proteolytic cleavages took place upstream of the −1/+1 site in the connector region of the precursor.

To investigate the role of the arginine residues at positions −14 and −15, mutations were introduced at these sites in a background that contained the previous changes at −2 and −3. Fig. 2A (lanes 3 and 6) shows that processing of the precursor derived from pcGTPG5, which contains the changes Arg15, Arg14 to Gly15, Thr14 and Arg3, Arg2 to Pro3, Gly2, resulted in an AGK protein band that comigrated with wild-type AGK (compare with lane 1) and an AGPR band with a higher molecular mass than wild-type AGPR. The larger AGPR band comigrated with the AGPR+ observed in the processing of the precursor from pcPG16 (lanes 2 and 5). Thus, substitution of the arginine pair at −14 and −15 had no effect on processing at the second cleavage site.

**Role of the Arginine Residue at Position −22 from the N Terminus of the AGPR Domain**—Since mutations at the arginine pairs upstream of the −1/+1 cleavage site identified a second processing site in the connector region, the role of the arginine at position −22 was examined. Arg22 was chosen because it is in a context that resembles the cleavage site at −1/+1. Analysis of the precursor derived from pcP22PG54 (Fig. 2B, lane 5), in which arginine at −22 was changed to Pro in a background where the arginine pair at −2 and −3 have been changed to Gly and Pro, shows that processing of the precursor was prevented by these substitutions, since no mature AGK or AGPR products were observed. The uncleaved precursor derived from pcP22PG54 was imported into mitochondria, since it was protected from proteinase K digestion. Some degradation of the precursor may have occurred in the matrix, suggested by the smear under the precursor band.

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A nucleoside-treated rabbit reticulocyte lysate kit was obtained from Promega. 35S-labeled [35S]E. coli hydrolysate labeling reagent (Tran[35S] label, 1274 Ci/mmol, 11.74 mCi/ml) was purchased from ICN. A nucleoside-treated rabbit reticulocyte lysate kit was obtained from Promega.

The role of arginine at position 2 relative to the cleavage site identified a possible MPP cleavage site, the proline residue at position 2 relative to the N terminus of the AGPR domain (Arg2 to Gly2) did not prevent cleavage of the polyprotein in vitro. A precursor containing the changes Arg2 and Arg3 to Gly2 and Pro3 (pcPG16) was used as a substrate for an in vitro import assay (Fig. 2A). Postimport mitochondria were reisolated and treated or not treated with proteinase K to digest bound precursor. Processing of the wild-type precursor (lanes 1 and 4) resulted in two protein bands; the upper protein band corresponds to mature AGK (52 kDa), and the lower protein band corresponds to mature AGPR (37 kDa) (29). Processing of the mutant precursor derived from pcPG16 resulted in an AGK protein that comigrated with wild-type AGK, but the AGPR protein (AGPR+) appeared to have a higher molecular mass than wild-type AGPR (Fig. 2A, lanes 2 and 5). This result suggested that processing at the N terminus of the AGPR domain, between amino acids −1 and +1, was prevented by the amino acid substitutions and that one or more proteolytic cleavages took place upstream of the −1/+1 site in the connector region of the precursor.

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**Effect of Proline after an Arginine Pair on Processing**—The arginine pair at positions −14 and −15 is located in a sequence motif that resembles the recognition site for MPP (14–17) but did not appear to function as a signal for processing. Analysis of amino acid sequences flanking cleavage sites of several hormone and protein precursors revealed that most peptides that contain a Pro residue at position +1 are not cleaved (34, 35). To determine if the presence of a proline residue at position −13 disrupted a possible MPP cleavage site, the proline residue at position −13 was changed to an alanine in a background where the arginine residues at −2 and −3 have been changed to Gly and Pro, shows that processing of the precursor was prevented by these substitutions, since no mature AGK or AGPR products were observed. The uncleaved precursor derived from pcP22PG54 was imported into mitochondria, since it was protected from proteinase K digestion. Some degradation of the precursor may have occurred in the matrix, suggested by the smear under the precursor band.

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**Cleavage of the Connector Region of the Precursor Is Inhibited by 1,10-Phenanthroline and EDTA—MPP cleaves mitochondrial targeting sequences during or after import of precursor proteins into the mitochondria. In N. crassa, removal of the targeting sequence by MPP can occur in more than one step (30). In some cases in yeast and mammals, processing of mitochondrial targeting sequences that are cleaved in two steps is carried out by two different enzymes; the first cleavage is performed by MPP, and the second cleavage is performed by a mitochondrial intermediate peptidase that produces the mature protein (31–33). MPP and mitochondrial intermediate peptidase are metal-dependent proteases inhibited by the chelators 1,10-phenanthroline and EDTA (9, 10, 30), which do not affect the import of precursor proteins (24). Thus, it was of interest to investigate a possible role of a mitochondrial metal-dependent peptidase in the processing at the upstream cleavage site in pAGK-AGPR.

Import reactions with wild-type and mutated precursors were performed in the presence of inhibitors of metal-dependent processing peptidases (Fig. 3). To ensure that no precursor remained associated with the mitochondrial outer membrane and to evaluate the efficiency of proteolytic cleavage at the second site, postimport mitochondria were reisolated and treated with proteinase K. Wild-type precursor (Fig. 3, lanes 1 and 6) showed processing into two mature proteins, AGK and AGPR, in the absence of inhibitors (lane 1). Processing of the wild-type precursor was inhibited in the presence of 1,10-phenanthroline and EDTA (lane 6), as inferred from the presence of a protein band corresponding to uncleaved precursor and the almost complete absence of bands corresponding to mature AGK and AGPR. Processing of mutant precursors derived from pcPG16 (lanes 2 and 7) and pcGTPG5 (lanes 3 and 8) was also inhibited by 1,10-phenanthroline and EDTA, indicating that proteolytic cleavage at both processing sites is blocked by inhibitors of metal-dependent peptidases. Wild-type pAGK-AGPR is cleaved by purified MPP in the connector region to generate mature AGK and AGPR proteins (4). The importance of arginine at position −2 or −3 relative to the cleavage sites and the sensitivity to metal ion chelators suggest that MPP is likely to be responsible for the two-step proteolytic processing of the connector region of pAGK-AGPR.
completely inhibited in 1,10-phenanthroline/EDTA pretreated mitochondria (Fig. 3, lane 9). This result indicates that substitution of Pro for Ala in the wild-type sequence RRPAL is not sufficient to generate a cleavage site at RRA

Role of Thr in Recognition by MPP—A threonine residue is often found 2 or 3 residues downstream from the cleavage site of mitochondrial targeting sequences. Interestingly, a threonine residue is also present 2 residues downstream from the cleavage site at the N terminus of the mature domain of the distal AGPR. To investigate the role of Thr\textsuperscript{13} in the AGPR
domain in processing of the connector region, this residue was changed to a proline in a wild-type background to generate the construct pcP3. In vitro processing of the resulting precursor resulted in three protein bands (Fig. 3, lane 5). The upper band corresponded to remaining unprocessed precursor; the two lower bands corresponded to wild-type AGK and to a larger sized AGPR (AGPR*) containing the N-terminal extension indicative of processing exclusively at the second cleavage site. Processing of the precursor was completely inhibited in 1,10-phenanthroline/EDTA pretreated mitochondria (Fig. 3, lane 6; 7). These results suggest that threonine is a critical residue of the processing motif in the connector region of the precursor. The large fraction of unprocessed precursor (~35% as measured by scanning densitometry) protected from proteinase K digestion suggests that processing at the second site is much less efficient than processing at the N terminus of AGPR.

Western Blot Analysis of in Vivo Expressed Constructs Containing Mutations in the Connector Region of the Precursor—To analyze processing of pAGK-AGPR in vivo, strain LA358 (AGK AGPR*) was transformed with constructs containing different amino acid substitutions in the connector region of pAGK-AGPR. Fig. 4 shows the results of immunoblot analysis of transformants obtained with the construct pgGTPG4-Hph (Arg\(^{15}\), Arg\(^{16}\), Arg\(^{17}\), and Arg\(^{22}\) to Gly, Pro, and Arg) and pgGTPG5 (Arg\(^{22}\), Arg\(^{23}\), Arg\(^{24}\), and Arg\(^{25}\) to Gly and Pro). These results are consistent with the in vitro analysis of processing that showed generation of a wild-type AGK for the corresponding construct. The results suggest that threonine is a critical residue of the processing motif in the connector region of the precursor. The large fraction of unprocessed precursor (~35% as measured by scanning densitometry) protected from proteinase K digestion suggests that processing at the second site is much less efficient than processing at the N terminus of AGPR.

processing of the complex on the import and processing of wild-type and mutant precursors. Isolated mitochondria were preincubated in import buffer without (lanes 1–5) or with (lanes 6–10) 5 mM EDTA and 2.5 mM 1,10-phenanthroline (o-Phe) for 5 min at 25 °C. 35S-Labeled precursor was added, and incubation was continued for 30 min at 25 °C. Mitochondria were reisolated and treated with proteinase K (50 μg/ml) at 0 °C for 30 min. To stop proteolysis, 1 mM phenylmethylsulfonyl fluoride was added, and the mixture was incubated for 5 min at 0 °C. Postmitochondrial supernatants were separated on 7.5% SDS-PAGE and fluorographed. Lanes 1 and 6, wild-type precursor; lanes 2 and 7, precursor from pcG16; lanes 3 and 8, pcGTPG5; lanes 4 and 9, pGAP13PG16; lanes 5 and 10, pcP3.

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FIG. 3. Effect of inhibitors of the mitochondrial processing peptidase on the import and processing of wild-type and mutant precursors. Isolated mitochondria were preincubated in import buffer without (lanes 1–5) or with (lanes 6–10) 5 mM EDTA and 2.5 mM 1,10-phenanthroline (o-Phe) for 5 min at 25 °C. 35S-Labeled precursor was added, and incubation was continued for 30 min at 25 °C. Mitochondria were reisolated and treated with proteinase K (50 μg/ml) at 0 °C for 30 min. To stop proteolysis, 1 mM phenylmethylsulfonyl fluoride was added, and the mixture was incubated for 5 min at 0 °C. Postmitochondrial supernatants were separated on 7.5% SDS-PAGE and fluorographed. Lanes 1 and 6, wild-type precursor, lanes 2–5, processed with 1,10-phenanthroline/EDTA treatment for 5 min at 25 °C. 35S-Labeled precursor was added to lanes 1–5 or with 1,10-phenanthroline/EDTA pretreated mitochondria (Fig. 3, lane 6). Lanes 3–5 show the results of immunoblot analysis of transformants obtained with the construct pgGTPG4-Hph. DNA from pcGTPG5 (Arg\(^{22}\), Arg\(^{23}\), Arg\(^{24}\), and Arg\(^{25}\) to Gly and Pro) was subcloned into a vector containing the Hyg' selective marker (see “Materials and Methods”). The resulting construct, pgGTPG4-Hph, was transformed into strain LA358 (arg-6, allele CD118), which lacks AGK and AGPR proteins. A, Western blot analysis of initial heterokaryon transformants probed with anti-AGK antisera. Control lanes are wild-type strain, 74A (lane 1), and recipient strain, LA358 (lane 2). B, the same blot reprobed with anti-AGPR antisera.

Processing of precursors derived from pgG22PG42-Hph (Arg\(^{22}\), Arg\(^{23}\), Arg\(^{24}\), and Arg\(^{25}\) to Gly and Pro) was subcloned into pgGTPG4-Hph, which was transformed into strain LA358 (arg-6, allele CD118), which lacks AGK and AGPR proteins. A, Western blot analysis of initial heterokaryon transformants probed with anti-AGK antisera. Control lanes are wild-type strain, 74A (lane 1), and recipient strain, LA358 (lane 2). B, the same blot reprobed with anti-AGPR antisera.
son of the sequence RG YLT (first cleavage) and RGY ST (second cleavage) reveals that the scissile bonds are in a sequence flanked by well conserved arginine and threonine residues (in boldface type). This suggests important roles for arginine and threonine as part of the cleavage site. Both residues appear to be critical for processing, since substitution of either of them results in misprocessing at the AGPR N terminus (see above).

Enzyme Activities and Feedback Inhibition in Uncleaved AGK-AGPR Precursor—A question of major interest is whether processing of pAGK-AGPR into two mature proteins in the mitochondrial matrix is required for acetylglutamate kinase and acetylglutamyl-phosphate reductase to be active. Transformants expressing AGPR* with the N-terminal extension and those expressing an uncleaved precursor were able to grow in minimal medium at a rate comparable with that of wild type (not shown). This result indicated that processing was not essential for the functioning of these enzymes in the mitochondrial matrix. To obtain more direct evidence that proteolytic processing was not required, AGK and AGPR activities were measured in purified mitochondria from transformants expressing the uncleaved AGK-AGPR precursor. Transformants expressing uncleaved precursors derived from pgG22PG42-Hph (Arg-22, Arg-3, and Arg-2 to GPG) and pgP22PG54 (Arg-22, Arg-3, and Arg-2 to PPG) exhibited activities equal to or greater than wild type (Table II). In addition, feedback inhibition of AGK was not significantly affected by the lack of cleavage. These results show that the two-step proteolytic processing at the connector region of the pAGK-AGPR precursor to generate two mature proteins is not required for the biological activity of the two protein domains.

DISCUSSION

The 871-amino acid AGK-AGPR polyprotein precursor of N. crassa is organized as two protein domains separated by a 200-amino acid connector region with a 45-amino acid mitochondrial targeting sequence at the N terminus, which is cleaved into two mature proteins in the mitochondrion (4). Proteolytic cleavage of leader sequences of mitochondrially targeted proteins has been shown to be performed in one or two steps by MPP (13–17) or in more than one step by two unrelated enzymes: MPP and a mitochondrial intermediate peptidase (31–33). MPP and mitochondrial intermediate peptidase recognize and cleave different amino acid sequences. Processing of the internal processing sequence of the AGK-AGPR precursor involves removal of 22 residues upstream of the N terminus of the AGPR domain. Both cleavage events are inhibited by 1,10-phenanthroline and EDTA (this report) and take place upon incubation of in vitro synthesized precursor with purified MPP (4).

Kinetic studies of processing using oligopeptides with differ-

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**Fig. 5.** Expression and processing of precursor derived from constructs pgG22PG42-Hph and pgP22PG54-Hph. DNAs from constructs pgG22PG42-Hph (Arg-22, Arg-3, and Arg-2 to Gly, Pro, and Gly) and pgP22PG54-Hph (Arg-22, Arg-3, and Arg-2 to Pro, Pro, and Gly) were used to transform strain LA358 (arg-6, allele CD118), and transformants were selected by hygromycin resistance (see "Materials and Methods"). Crude extracts were prepared from initial hygromycin transformants with pgG22PG42-Hph and pgP22PG54-Hph are shown (as indicated at the top). Wild-type protein is indicated by AGK, and uncleaved precursor is indicated by P.

**A** wild-type

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**B** derivative pgPG15-Hph

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**C** derivative pgGTP4-Hph

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**Fig. 6.** Determination of second cleavage site in the connector region of the precursor by N-terminal sequencing of mutant AGPR proteins. Mutant proteins were purified by immunoprecipitation with anti-AGPR antiserum from crude extracts of the corresponding transformants and blotting to a polyvinylidene difluoride membrane. A second cleavage site was identified upstream of the N terminus of wild-type AGPR. A, wild-type sequence upstream of the N terminus of AGPR. B, mutant sequence from pgPG15-Hph (Arg-22 and Arg-2 to Pro and Gly). C, mutant from pgGTP4-Hph (Arg-22 and Arg-14 to Gly and Thr, and Arg-3 Arg-2 to Pro and Gly). The positions of cleavages are indicated by arrows. Positively charged residues are indicated in boldface letters. Numbers under the amino acid indicate position relative to the N terminus of the AGPR domain. Italics indicate amino acid sequence data.
ent amino acid sequences and lengths showed the necessity for at least 16 residues consisting of 11 residues upstream and 5 residues downstream from the cleavage site for effective hydrolysis by MPP (36). Analysis of amino acid sequences around the cleavage site of mitochondrial targeting peptides cleaved by MPP has revealed no consensus amino acids; however, several motifs conserved within various subgroups have been identified (14, 15). In one subgroup, arginine residues were often observed at positions −2, −3, −10, and −11, relative to the scissile bond. In the connector region of pAGK-AGPR, arginine residues are present at positions −2, −3, −14, and −15 relative to the N terminus of mature AGPR. No motif resembling a cleavage site for mitochondrial intermediate peptidase was identified. Although both MPP and mitochondrial intermediate peptidase are metal-dependent proteases inhibited by 1,10-phenanthroline and EDTA, analysis of the cleavage sites suggests that the two-step cleavage is carried out by MPP alone. Thus, maturation of pAGK-AGPR involves three proteolytic cleavage events: cleavage of the leader (targeting) peptide and two cleavages removing the internal processing sequence.

Cleavage at the connector region of the AGK-AGPR precursor occurs at sequences RGY, ST and RG, YLT, 20 amino acids apart. The results of in vitro import and processing of the mutant precursors support an important role for arginine at −2 or −3 and revealed the importance of a threonine at +2 or +3 relative to the cleavage site. A third sequence motif containing two arginines (RRPAL) occurs between the two cleavage sites. Substitution of alanine for proline, creating the sequence RRAAL, did not result in a new cleavage site.

The involvement of secondary structure in recognition by processing peptidases has been reported (34, 35). Processing sites are believed to be in exposed and flexible regions of the precursors situated in, or immediately next to, the C terminus of the AGPR domain (pcPG16; Fig. 7B) decreased the predicted length of the β-turn at −3; the rest of the structure was not affected. Mutations at the arginine pairs at positions −2 and −3 from the N terminus of the AGPR domain (pcPG16; Fig. 7B) decreased the predicted length of the β-turn at −3; the rest of the structure was not affected. Mutations at the arginine pairs at positions −2 and −3 and positions −14 and −15 from the C terminus of the AGPR domain (pcPG16; Fig. 7C) eliminated the turn predicted to be centered at −19. Substitution of Arg residue with Gly (32), combined with the substitutions at −2 (32) and −3 by Pro (32PG42PG42; Fig. 7D) increased the length of the turn centered at −19. Substitution of Arg residue with Gly (32), combined with the substitutions at −2 and −3 by Pro (32PG42PG42; Fig. 7E) shifted the turn centered at −19 toward the C terminus. Substitution of Pro residue for Ala (32) in combination with changes at −2 and −3 resulted in the same structure predicted in Fig. 7B, where only changes at −2 and −3 were made (pcPG16; Fig. 7F). Substitution of Thr with Pro (34, Fig. 7G) resulted in complete loss of the turn centered at −3 and the β-sheet at +2. Therefore, specific amino acid replacements in the connector region of the precursor are predicted to affect the local secondary structure of the region. We found that in general, loss of proteolytic processing between the −1 and +1 residues could be correlated with a decrease in the length or the complete loss of the β-turn centered at −3. However, loss of the β-turn centered at −19 did not affect processing at −20 (Fig. 7C). Loss of processing at position −20 is likely to result from the substitution of the arginine residue at −22. Thus, the predicted changes in the secondary structure caused by the amino acid substitutions consisted of variations in the length and position of β-turns, and some of these changes may be correlated with loss of proteolytic cleavage. Protein secondary structure as well as specific amino acid residues have been shown to be important for the processing of mammalian and plant precursors of mitochondrial proteins (38, 39).

**TABLE II**

| Strain/Transformant | Enzyme activities | Reduction in activity |
|---------------------|-------------------|----------------------|
| pgP22PG42-Hph T4    | 5.3               | 1.1                  |
| pgP22PG42-Hph T6    | 7.5               | 2.3                  |
| pgP22PG42-Hph T8    | 8.3               | 2.3                  |
| pgP22PG42-Hph T9    | 6.8               | 1.1                  |

**Fig. 7.** Predicted secondary structure of the connector region of wild-type and mutant precursors based on the combined algorithms of Chou-Fasman and Robson-Garnier. Predictions for α-helices (Hix), β-sheets (Shit), or β-turns (Trn) starting at position −51 (T) to position +11 (V), are indicated by shaded and black boxes. A window size of 7 was used. A, wild-type sequence; B, substitution of Arg −2 and Arg −3 by Gly and Pro; C, substitution of Arg −2 and Arg −3 by Gly and Pro, and Arg −14 and Arg −15 by Thr and Gly; D, substitution of Arg −2 and Arg −3 by Gly and Pro, and Arg −22 by Gly; E, substitution of Arg −2 and Arg −3 by Gly and Pro, and Arg −22 by Pro; F, substitution of Arg −2 and Arg −3 by Gly and Pro, and Arg −13 by Ala; G, substitution of Thr −13 by Pro.
The biological advantage of targeting a fusion protein versus two independent proteins is not obvious; several possible reasons have been postulated (3, 4). Facilitation of a multi-enzyme complex formation in the mitochondrial matrix for the channeling of labile intermediates is one explanation. In yeast, the Hiss4 locus encodes a multifunctional protein with three different functional domains (40). Proteolytic processing of the Hiss4 protein is not required for function, since the purified native protein contains the three activities.

AGK and AGPR activity assays using transformants expressing only an unprocessed pAGK-AGPR revealed that proteolytic cleavage in the connector region is not required for activity. In addition, the functions of AGK and AGPR in vivo were not affected as assessed by the ability of the unprocessed precursor to support growth of AGK-AGPR mutants. We conclude that conformational changes that may be associated with the lack of proteolytic processing of the fused proteins did not have a dramatic effect on the function of the uncleaved enzymes. Moreover, the lack of processing did not affect feedback inhibition of AGK by arginine. However, whether interaction with other proteins in the mitochondrial matrix has been affected by the lack of processing remains to be studied.

The results shown here indicate that two proteolytic cleavages in the connector region of the precursor occur to release the two protein domains. Since cleavage in the connector region of the precursor takes place at positions −19 and −20 and positions −1 and +1 from the N terminus of the mature AGPR, we propose that the enzyme responsible, possibly MPP, scans the connector region as the precursor reaches the mitochondrial matrix. The enzyme recognizes a first sequence for cleavage at Arg-22 and it cleaves two residues toward the C terminus. The scanning continues as import of the precursor progresses. The enzyme skips the arginine pair at positions −15 and −14, probably due to the absence of a threonine residue in the recognition sequence. As scanning continues, the enzyme recognizes the arginine pair at positions −3 and −2 and cleaves 2 residues C-terminal. Since some unprocessed precursor is still observed after cleavage at the second site, cleavage at the N terminus of AGPR seems to be more efficient than the cleavage at the upstream position. At this point, folding of the processed distal domain into a functional protein begins, and the enzyme falls off the substrate.

What is the role of processing of the pAGK-AGPR precursor in the metabolism of arginine? It has been suggested that this precursor resulted from the fusion of two genes for independently targeted proteins. It has been hypothesized that this arrangement may result in a more efficient delivery of the proteins to the mitochondria or to stabilization of one or both proteins prior to their assembly into their mature functional forms (3, 4, 29). The increased activity of AGPR in transformants expressing the uncleaved polypeptide (Table II) suggests that possible effects on protein stability can be further enhanced by retaining the two enzymes as a polyfunctional protein. Because such transformants grow normally, the advantage of cleavage must be subtle, and its identification will require more extensive analysis of the properties of the polypeptide and independent enzymes.

Identification of the internal processing sites allowed identification of the precise C terminus of AGK. This confirmed the existence of a −15-amino acid subdomain that is absent from prokaryotic homologs (4). A role for this subdomain may be related to the feedback inhibition properties of AGK in the mitochondrial matrix. Another possibility is that this subdomain plays a role in protein-protein interaction; mutations in arg-6 can affect the activity or feedback sensitivity of acetylglutamate synthase encoded by the unlinked arg-14 gene (28, 41, 42). Processing of the precursor may be needed for this interaction to occur. Kinetic analysis of the uncleaved precursor and examination of its interaction with other proteins may reveal new aspects of the role of proteolytic processing of the AGK-AGPR precursor on the metabolism of arginine.

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Lilian Parra-Gessert, Kenneth Koo, Joaquin Fajardo and Richard L. Weiss

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