Sequences Distal to the Mitochondrial Targeting Sequences Are Necessary for the Maturation of the F\textsubscript{1}-ATPase \(\beta\)-Subunit Precursor in Mitochondria*

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(Received for publication, April 21, 1986)

The \(\beta\)-subunit of the mitochondrial F\textsubscript{1}-ATPase is synthesized as a precursor in the cytoplasm which is delivered through two bilayers bounding the mitochondria prior to its assembly with other proteins into a functional complex. In order to determine the role of the amino-terminal 50 residues of the precursor on its localization, maturation, and assembly, a set of deletions within this region of the ATP2 gene encoding the \(\beta\)-subunit has been analyzed. These studies reveal that deletions between residue 10 of the F\textsubscript{1} \(\beta\)-presequence and residue 36 can still direct in vivo mitochondrial import and assembly of the mutant subunit into a functional complex. Deletions within ATP2 which contain less than the first 10 residues of the precursor are not imported. Thus, the extreme amino terminus (about half of the transient presequence) of the F\textsubscript{1} \(\beta\)-subunit can direct its mitochondrial import.

The wild-type F\textsubscript{1} \(\beta\)-subunit precursor is matured by the matrix-located metalloprotease at Lys\textsuperscript{19}-Gln\textsuperscript{20}; however, small in-frame deletions up to 17 residues distal to this site fail to be matured either in vitro or in vivo. This nonmatured F\textsubscript{1} \(\beta\)-subunit is also assembled into a functional enzyme and supports growth of its host on a nonfermentable carbon source. These data indicate that maturation of the F\textsubscript{1} \(\beta\)-subunit precursor is dependent on a protein sequence located distal to the proteolytic maturation site which is distinct from the mitochondrial targeting sequence.

The F\textsubscript{1} \(\beta\)-subunit of the mitochondrial ATPase complex contains the active site of the mitochondrial ATPase transducing complex (Cross, 1981). In order for this subunit to function in vivo it must be synthesized on cytoplasmic ribosomes specifically delivered to mitochondria, imported through both membranes of the organelle and assembled with at least 20 other subunits on the inner membrane. All but the last of these events have recently been shown to be under the direction of residues located at the amino terminus of the cytoplasmic subunit (Emr \textit{et al.}, 1986). Indeed, recent studies in both yeast and animal cell mitochondrial systems have convincingly demonstrated that the transient presequence present on cytoplasmically localized precursors is necessary to initiate efficient import into the mitochondrial matrix (Hurt \textit{et al.}, 1984, 1985; Horwich \textit{et al.}, 1985). It is not known if processing of the precursor in the mitochondrial matrix is a prerequisite for assembly of the functional protein. Earlier in vitro studies have shown that the proteolytic removal of the presequence is not required for the completion of the transmembrane delivery event (Zwienski and Neupert, 1983). In addition, recent analysis of trapped "import intermediates" of the matured F\textsubscript{1} \(\beta\)-subunit across both mitochondrial membranes suggests an amino-terminal first delivery of the precursor (Schleyer and Neupert, 1985). These studies indicate that maturation occurs prior to the completion of import and that only amino-terminal sequences are necessary for correct processing in the matrix. The enzyme responsible for this maturation is inhibited by metal chelators and is proposed to participate in the specific processing of at least 20 different presequence-containing peptides which are localized in mitochondria (Reid, 1985). Conditional mutants in yeast have been described which prevent expression or function of this maturation enzyme (Yaffee \textit{et al.}, 1985). To date, however, little is known about the nature of this essential protease recognition or where the actual processing event occurs in relation to its assembly in vivo.

In the present study we have utilized deletion analysis of the F\textsubscript{1} \(\beta\)-subunit and have defined separate determinants for targeting and processing within its amino terminus. These data indicate that a region of the F\textsubscript{1} \(\beta\)-subunit precursor distal to the protease processing site is necessary for efficient precursor maturation both in vitro and in vivo.

These data imply that a conformation of the precursor is recognized by the matrix-localized protease and that residues distal to the processing site are important in the interaction of the precursor with this enzyme. Analysis of the processing sites of proteins matured by the matrix protease reveal no consensus processing sequence for cleavage (Reid, 1985). On the other hand, the cleavage region for presecretory peptides shares some features of primary sequence in common (for review see DuPouy \textit{et al.}, 1985). Thus, the matrix protease of mitochondria may recognize a structure within the amino terminus of the F\textsubscript{1} \(\beta\)-subunit precursor which may be common to other mitochondrial precursors.

\textbf{MATERIALS AND METHODS}

\textbf{Strains, Strain Constructions, and Media}—\textit{Saccharomyces cerevisiae} strains used in this study were SEY2102, MATa ura3-52 leu2-3 leu2-112 suc2-\textsuperscript{A9} his3-519 gal2 (Emr \textit{et al.}, 1983), and AVY4-1, an
The Bal31-treated fragments were cut with either EcoRI or BamHI, ATP2 and treated for various periods of time with Bal31 nuclease, which extend in either the 5’ or mid preps were prepared from different pools of the 5’ and 3’ resolved on LGT agarose (Seakem), and then religated in place of the respective wild-type fragments in pVuAV-1 (Fig. 2). Deletions constructed in this fashion yield internal deletions nested at codon 36 internally deleted constructs. Each pool was restricted with Eco and with T4 DNA ligase were performed according to the instructions of the commercial supplier (New England Biolabs or Bethesda Research Laboratories). Digestions with Bal31 nuclease (Bethesda Research Laboratories) were performed as published (Sllivay et al., 1984). Isolation of plasmid DNA, agarose gel electrophoresis, and DNA transformation into E. coli and S. cerevisiae were performed as described (Ito et al., 1983; Maniatis et al., 1982; Birnboim and Doly, 1979). DNA sequence analysis was performed by the dideoxy chain termination method (Sanger et al., 1980) using the oligonucleotide primer described previously (Takeda et al., 1985).

Plasmids and DNA Constructions—Construction of internal deletions in ATP2 utilized a PvuII site located at codon 36. A 2.2-kb EcoRI BamHI fragment of ATP2 (Saltzgaber et al., 1983; Takeda et al., 1985) containing 1100 bp of DNA 5’ of the translation start and 1100 bp of coding DNA was cloned into a derivative of pBR322 in which the PvuII site had been removed by deletion mutagenesis. This construction, pVuAV-1, was then opened at a unique PvuII site within ATP2 and treated for various periods of time with Bal31 nuclease. The Bal31-treated fragments were cut with either EcoRI or BamHI, resolved on LGT agarose (Senkem), and then religated in place of the respective wild-type fragments in PvuAV-1 (Fig. 2). Deletions constructed in this fashion yield internal deletions nested at codon 36 which extend in either the 5’ or 3’ direction in ATP2. In order to select in-frame fusions for further characterization small scale plasmid preps were prepared from different pools of the 5’ and 3’ internally deleted constructs. Each pool was restricted with Eco and with T4 DNA ligase were performed according to the instructions of the commercial supplier (New England Biolabs or Bethesda Research Laboratories). Digestions with Bal31 nuclease (Bethesda Research Laboratories) were performed as published (Sllivay et al., 1984). Isolation of plasmid DNA, agarose gel electrophoresis, and DNA transformation into E. coli and S. cerevisiae were performed as described (Ito et al., 1983; Maniatis et al., 1982; Birnboim and Doly, 1979). DNA sequence analysis was performed by the dideoxy chain termination method (Sanger et al., 1980) using the oligonucleotide primer described previously (Takeda et al., 1985).

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**FIG. 1.** Construction of the yeast ATP2 deletion host. For details, see “Materials and Methods.”

**FIG. 2.** Strategy for the insertion of nested deletions within the 5’ region of ATP2. For details, see “Materials and Methods.”

Bam, and the resulting DNA fragments were cloned into the EcoRI BamHI sites of plasmid pSEY101 (Douglas et al., 1984). This plasmid contains a BamHI site at 39 of lacZ which is in frame with the BamHI site at codon 382 of ATP2. Following transformation into E. coli MC1066, lac+ transformants were picked from plates containing X-gal. Plasmid DNA isolated from each was subsequently screened for β-galactosidase expression in yeast after transformation. Those deletion constructs which expressed β-galactosidase in yeast to the same extent as the wild-type construct pZ1 (Douglas et al., 1984) were subsequently characterized by DNA sequence analysis. To examine the expression and function of the various deletions in an otherwise wild-type ATP2 gene, the reconstruction plasmid pAV010 was constructed as follows. A 700-bp BamHI fragment of ATP2 containing 387 bp of coding DNA and approximately 300 bp of 3’ noncoding DNA was ligated into pSEY101. Restriction analysis confirmed that the fragment was oriented such that the sequence coding the terminal 129 codons of ATP2 was proximal to the unique EcoRI site in pSEY101. This construct was opened at PvuII sites within lacZ which removed all but 72 bp of DNA at the 5’ and 51 bp of DNA at the 3’ end of lacZ. This was followed by brief treatment with exonuclease Bal31 sufficient to delete the BamHI site distal to the ATP2 coding DNA. This construct, approximately 7.1 kb, contains unique EcoRI and BamHI sites for convenient insertion of Eco-Bam fragments of ATP2 containing the various in-frame deletions (Fig. 3). The 3’ region in this construction retains DNA sufficient for correct transcription termination of ATP2.

Coupled in Vitro Transcription Translation—In vitro synthesis of ATP2 specific mRNA utilized an SsrI-HindIII fragment containing the ATP2 gene inserted into the like sites of the polylinker in pT7-1 (Tabor and Richardson, 1980). This construction places the T7 promoter approximately 200 bp 5’ of the ATP2 initiation codon. The HindIII-linearized pT7-ATP2 DNA was then utilized to generate a capped full length ATP2 transcript as follows. The transcription reaction included in a 50-μl reaction, 40 mM Tris-HCl, pH 8.0, 15 mM MgCl₂, 5 mM dithiothreitol, 500 μg/ml bovine serum albumin, 50 units of RNAasin, 5 mM each of ATP, CTP, UTP, 10 μM GTP, 200 μM GpppG, 2-3 μg of linearized DNA, and 60 units of T7 polymerase (International Biotechnologies Inc.). Following an incubation of 10
min at 37 °C to allow the formation of cap structures GTP was added to 5 mM (Konarska et al., 1984), and the mixture was incubated for an additional 30 min. The RNA was processed for translation and translated in a nuclease-treated reticulocyte lysate (Pelham and Jackson, 1978).

**Determination of the Metalloproteinase Cleavage Site within the F1-β-Subunit Precursor—**Sequence analysis of the amine terminus of isolated (mature) F1-ATPase α and β subunits revealed that they were blocked (Todd et al., 1980). Therefore, an in vitro processing system was utilized to define the amino terminus of the mature β-subunit. The F1-β-subunit precursor synthesized from a coupled transcription-translation of ATP2 was as described in the previous section. The translated rabbit reticulocyte lysate (100 μl) containing [35S]methionine F1-β-precursor was mixed with 30 μl of soluble mitochondria extract (McAda and Douglas, 1982) in 10 mM Tris-SO4, pH 7.0, 25 mM MnCl₂. Following incubation for 1 h at 24 °C the reaction was terminated by the addition of SDS gel-loading buffer and processed as described (Hunkapiller et al., 1983). The samples were distributed into 19 wells flanked by the purified yeast F1-β-subunit which served as a molecular weight standard to locate the mature labeled subunit from the transcription-translation reaction to determine the extent of processing. One lane from the gel was dried for autoradiography. Approximately 70% of the F1-β-subunit in the reaction mixture was processed and migrated as the mature subunit. The remainder of the gel was not fixed as usual with acid but rapidly stained with fresh Coomassie Blue R in 10% methanol and destained prior to removing the region of the gel containing the processed subunit for electroelution and dialysis (Hunkapiller et al., 1983). The bluish powder obtained following lyophilization (29,780 cpm) was mixed with sequence grade apomyoglobin and sequenced on a Beckman model 890 M liquid phase Sequencer. The repetitive yield was 93% based on apomyoglobin. Samples were also counted for 3H. Background counts 90–111 cpm were observed for the first 20 cycles with the exception of cycle 14 with 1580 cpm. Based on the content of methionine (7 for ATP2) and assuming equal labeling of each methionine present this represents greater than 90% of the counts expected for a labeled methionine at this position in the sequence.

Since the only methionine present within the F1-β-subunit relative to the processing site was located at residue 33 (Fig. 4) the processing site determined by this method was Lyv-Gln³.

**Miscellaneous—**Mitochondria were prepared from spheroplasts as published (Daum et al., 1982). Yeast cells harboring different plasmids were grown at 28–30 °C to 2–4 × 10⁶ cells/ml on YNB (Yeast Nitrogen Base Dextrose) medium containing the required amino acid supplements (Douglas et al., 1984). F1-ATPase was released from isolated mitochondria by treatment with chloroform (Douglas et al., 1977). ATPase assays were performed at 30 °C with an ATP-regenerating system as previously described (Todd et al., 1979). Soluble matrix protease was prepared from mitochondria and was utilized immediately in an in vitro processing reaction containing 25 mM MnCl₂ (McAda and Douglas, 1982). Treatment of isolated mitochondrial ATPase with low concentrations of the detergent Lubrol WX, to release nonmembrane-bound protein, was performed with minor modification of published methods (Schmitman and Greenawalt, 1968). Mitochondria at 2 mg/ml in 0.6 M sorbitol, 0.02 M Tris/SO₄, 0.002 mM ATP, 0.001 mM EDTA were combined with 0.1 volume of Lubrol WX to give a final ratio of 0.3 mg of Lubrol/mg of mitochondrial protein. After incubating for 10 min on ice the extracts were centrifuged for 10 min at 12,900 × g. Mitochondrial pellet and supernatant fractions were analyzed for protein distribution by enzyme assays and gel electrophoresis. Succinate dehydrogenase activity (Singer, 1974) and malate dehydrogenase activity (Bergmeyer and Bernt, 1974) were determined as published. Published procedures were used for SDS-polyacrylamide gel electrophoresis (Douglas et al., 1979). Immunodetection of proteins transferred to nitrocellulose with F1-ATPase β-subunit antiserum (rabbit plus horseradish peroxidase-conjugated goat anti-rabbit serum) was performed as previously described (Emr et al., 1986). Protein was measured by a modification of the Lowry method (Douglas et al., 1977). All nucleotides were purchased from Pharmacia-P-L Biochemicals. RNAsin (PreMega), oligomycin, Lubrol WX (Sigma), and X-gal (IBI) were used without further purification. [35S]Methionine (1105 Ci/mmol) was obtained from New England Nuclear.

**RESULTS**

**Construction of Deletions to Localize ATP2 Targeting Signals—**We have demonstrated that information to direct the intracellular delivery and import of the F1-β-subunit precursor protein is located at its extreme amino terminus (Emr et al., 1986). In order to further define the minimum sequence necessary for correct targeting and assembly of a functional β-subunit protein, a host-vehicle system was designed to examine specific deletions within the amino-terminal region. A PuuII site which restricts at codon 36 of ATP2 was utilized to construct a set of nested deletions extending in either the
Using the hybrid protein-dependent phenotype and subcellular localization as an assay for mitochondrial delivery (Table I) we observe that deletions which extend forward from codon 36 up to codon 16 progressively reduce but do not abolish mitochondrial localization of the hybrid βZ protein. Deletions which retain the first 15 residues or less of F1 β-presequence further reduced the amount of hybrid localized to mitochondria to the point where normal growth of the host on glycerol was observed. Internal deletions from codon 36 extending away from the region encoding F1 β-presequence expressed a hybrid protein which was localized to mitochondria with reduced efficiency although the level of hybrid protein delivered was sufficient to block growth on glycerol.

**Table I**

| Construct | Deletion junction | Lys + Arg groups at amino terminus | Growth of host SEY2102 on glycerol | % of β-galactosidase localized in mitochondria |
|-----------|------------------|----------------------------------|-----------------------------------|-----------------------------------------------|
| pβZ1      | No deletion      | 4                                | -                                 | 30-95                                         |
| pSEY101   | Vector           | +                                |                                   |                                               |
| pAV297T37Z1 | Trp39-Thr-Ala37  | 4                                | -                                 | 60-65                                         |
| pAV353-37Z1 | Pro35-Ala37      | 4                                | ±                                 | 55-60                                         |
| pAV316P37Z1 | Lys97-Pro-Ala37  | 3                                | ±                                 | 40-45                                         |
| pAV107T37Z1 | Leu127-Thr-Ala37 | 2                                | +                                 | 5-10                                          |
| pAV100-37Z1 | Thr116-Ala37     | 1                                | +                                 | 5-10                                          |
| pAV44-37Z1  | Pro114-Ala37     | 0                                | <5                                |                                               |
| pAV*37Z1   | *-Ala37          | +                                | <5                                |                                               |
| pAV361-41Z1 | Ala32-Pro41      | 3                                | -                                 | 90-95                                         |
| pAV55V45Z1  | Ser28-Val-Lys35  | 3                                | -                                 | 75-80                                         |
| pAV55V450Z1 | Ser28-Gly-Lys85  | 3                                | -                                 | 60-65                                         |

* Lysine plus arginine residue numbers refer to the number present within the first 20 residues of the F1 β-presequence. The Δ*-37Z1 is a gene fusion that uses a non-ATP2 start codon approximately 300 bp upstream of ATP2.
Targeting and Processing Signals in Mitochondrial Import

ATP2 recombined to an otherwise wild-type gene in pAVO10 into the Aatp2:LEUZ host, AW4-1, and the transformants were subsequently monitored for growth on solid medium with glycerol as the carbon source. This reduction in growth rate most likely reflects the amount of active energy-transducing enzyme present in the host.

Based on this growth assay, the Δ4-37 deletion was not able to target a functional F1 β-subunit into mitochondria. However, the deletion which expressed only the first 10 residues of the ATP2 (Δ10-37) targeted and assembled an active F1 β-subunit. This same 10-37 deletion, however, was only about 10% as effective as the wild-type construct in targeting the ATP2-lacZ hybrid to mitochondria. This apparent difference in the delivery of the two proteins may be explained by our previous observation that the large β-galactosidase protein will interfere with the sequences at the extreme amino terminus which directs mitochondrial delivery (Emr et al., 1986).

Assembly of Internally Deleted F1 β-Subunit into a Functional ATPase Complex—The specific activity of the mitochondrial ATPase or the F1 particle released from mitochondria indicated that the complex containing a deleted F1 β-subunit was in some cases not as active as the wild-type complex (Table III). The wild-type F1 β (p@OK) restored an oligomycin-sensitive ATPase to the deletion mutant. The lower specific activities observed for the modified enzyme complexes were due to a change in the catalytic efficiency of the modified β-subunit rather than the amount of the subunit present in the complex. Quantitation of the steady state level of F1 β-subunit in these mitochondria indicated that the amount of immunodetectable subunit changed less than 10% (Fig. 5) for the constructs which support growth on a nonfermentable carbon source. The two deletions which expressed only a background level of mitochondrial ATPase activity did not exhibit a detectable level of the β-subunit protein in mitochondria (see Fig. 5).

The βΔ35G50 protein was the only β-subunit deletion which assembled an active F1-ATPase of high specific activity that was unable to support growth of its host on a nonfermentable carbon source. The oligomycin sensitivity of the mitochondrial ATPase containing the Δ5G50 β-subunit was only 5%, whereas that of the wild type was greater than 90% for inhibiting the hydrolysis of ATP (Table III). Oligomycin interacts with integral membrane subunits of the ATPase complex to inhibit the hydrolysis of ATP within the β-subunit. Thus, the sensitivity of ATP hydrolysis to this antibiotic is a convenient indicator of correct subunit interactions within a functional enzyme complex. The deletion Δ5G50 which retains an intact targeting sequence but extends into the subunit beyond residue 46 assembles an F1-ATPase particle which is not coupled in some manner to the remainder of the enzyme. These data define the limit of deletions within the F1 β-subunit beyond the targeting and processing sequences for the formation of a catalytically active complex.

Some deletions within the amino terminus of ATP2 are still able to complement an ATP2 deletion host

| Construction | Plasmid-dependent growth on glycerol, °C | Doubling time on YPG, 30°C | Doubling time on YPG, 37°C |
|--------------|------------------------------------------|-----------------------------|-----------------------------|
| p@OK         | +                                       | 205                         | 235                         |
| pΔ29T-37     | +                                       | 235                         | 235                         |
| pΔ16P37      | +                                       | 245                         | 245                         |
| pΔ15T37      | +                                       | 260                         | 280                         |
| pΔ10-37      | +                                       | 285                         | 285                         |
| pΔ4-37       | +                                       | 245                         | 275                         |
| pΔ36-41      | +                                       | 245                         | 275                         |
| pΔ35G50      | +                                       | 275                         | 275                         |
| pAVO10       | -                                       | -                           | -                           |

Deletions which do not block targeting of the F1 β-precursor to mitochondria assemble a partially active energy-transducing complex

Mitochondria were prepared from the indicated yeast host strain harboring the indicated pΔA plasmid. Partially purified F1-ATPase was prepared from each mitochondrial preparation according to Boutry and Douglas, 1983. ATPase activities in isolated F1-ATPase and in mitochondria, plus or minus 5 μg/ml of the inhibitor oligomycin were determined as described under “Materials and Methods.” The activities are expressed as nmol of inorganic phosphate released per min per mg of protein. ND designates undetectable activity.

| Construction | Host     | -Oligomycin | +Oligomycin | Inhibition | Specific activity | Wild type |
|--------------|----------|-------------|-------------|------------|------------------|-----------|
| pAVO10       | SEY2102  | 0.94        | 0.21        | 67         | 17.7             | 73        |
| pAVO10       | AVY4-1   | 0.03        | 0.02        | ND         | ND               | ND        |
| pΔOK        | AVY4-1   | 3.33        | 0.32        | 90         | 24.3             | 100       |
| pΔ29T37     | AVY4-1   | 3.04        | 0.62        | 80         | 20.9             | 86        |
| pΔ10-37     | AVY4-1   | 0.89        | 0.23        | 74         | 16.2             | 62        |
| pΔ4-37      | AVY4-1   | 0.04        | 0.01        | ND         | ND               | ND        |
| pΔ36-41     | AVY4-1   | 2.84        | 0.71        | 75         | 13.2             | 54        |
| pΔ35G50     | AVY4-1   | 3.01        | 2.85        | 5          | 27.1             | 111       |

Table II

Table III
Mitochondria were prepared from yeast strain AVY4-1 harboring the indicated pΔ or pΔOK constructs. For these analyses mitochondrial proteins were resolved on 10% polyacrylamide-SDS gels, electrophoretically transferred to nitrocellulose, and probed with antiserum for the F1 β-subunit. Panel A, twenty μg of mitochondria from the strain expressing the indicated construct was loaded into duplicate lanes. pΔOK mitochondria were loaded in addition into the right lane of each pair. Purified mature F1 β-subunit (0.5 μg) was loaded into the lanes designated β for each gel. Panel B, samples from each mitochondrial preparation treated in the following manner were run in three adjacent lanes. The left lane of each set contained 30 μg of mitochondrial protein. The middle lane contained the 12,800 g pellet of 60 μg of the same mitochondria extracted with 0.3 μg of Lubrol WX/μg of mitochondrial protein (see “Materials and Methods”). The right lane contained the entire 12,800 g supernatant from each detergent extraction. These extraction conditions released 85–90% of the soluble matrix malate dehydrogenase activity and 5–10% of the membrane-bound succinate dehydrogenase activity in the mitochondrial preparations.

protease were not processed and still retained the presequence. Fig. 5A compares different internally deleted F1 β-subunits which fractionate with mitochondria. Only in two deletions, Δ29T37 and Δ36-41, was any mature form of the protein detectable in addition to the precursor. The mature Δ36-41 and Δ29T37 proteins exhibited a slightly greater mobility than the mature wild-type F1 β-construct (βOK) suggesting that processing had occurred at the same site as the wild type. The nonprocessed form of the Δ36-41 subunit was firmly associated with mitochondrial membrane and could not be released even under conditions in which a detergent wash of the membranes was employed (Fig. 5B). These conditions are sufficient to strip nonassembled F1 proteins and other soluble proteins from the membrane surface.

Taken together these data show that deletions introduced within the F1 β-precursor distal to the targeting sequences do not significantly affect the steady state level of the protein assembled into the membrane-bound complex. On the other hand, any modification to the F1 β-subunit sequence starting 17 residues down from the maturation site either partially or completely prevented processing of the protein within mitochondria. Thus, a region of the F1 β-subunit precursor has been defined which is necessary for correct processing by the matrix protease that is distinct from the region required for delivery of the protein to the organelle. It is considered very likely that the transient presequence, which itself contains the mitochondrial targeting signal, is part of the structure formed at the end of the β-subunit that is recognized by the processing protease. The function of the mitochondrial targeting/import signal and the formation of the structure for maturation within the amino terminus of the F1 β-subunit precursor may be distinct events in vivo since they occur on opposite sides of the membrane. The presence of processed and nonprocessed forms of the assembled Δ36-41 subunit may reflect conditions in which the rate of processing is slower than the delivery of the precursor into the matrix.

The F1 β-Subunit Modified at Sites Distal to the Processing Site Cannot be Processed in Vitro—The presence of unprocessed forms of the F1 β-subunit in a functional ATPase complex suggested that either a specific conformation required for processing of the subunit might not present or that the processing site was not accessible to the metalloprotease in the mitochondrial matrix. To distinguish between these possibilities a linked transcription-translation system was employed to examine the processing of deletion constructs in vitro. The wild-type ATP2 coding sequence and selected deletion constructs were positioned next to the promoter for the T7 RNA polymerase (see “Materials and Methods”). These constructs were then utilized to generate capped mRNA specific for the wild-type and mutant forms of the F1 β-subunit precursor. As shown in Fig. 6, the wild-type F1 β-precursor was approximately 40% processed by isolated matrix protease. The addition of o-phenanthroline to this processing assay prevented maturation of the wild-type gene product (not shown). In the same experiment no detectable processing was observed for F1 β-subunit harboring internal deletions either distal to or encompassing the site of processing. Thus, the isolated protease was unable to process the soluble F1 β-precursor molecule harboring internal deletions distal to the processing site. This observation is consistent with the proposal that the matrix protease recognizes some conformation of the precursor rather than a specific sequence at the processing site. Deletions distal to the processing site prevent or diminish maturation of the precursor in vivo and in vitro.

Although the processing of the deletion constructs still did not occur under conditions in which the matrix protease was freely accessible to the precursor subunit, the present data do not rule out the possibility that the deletions may bury the processing site in some fashion within the completed protein. To address this point the experiments were performed to partially denature the wild-type and deletion constructs with mild detergent or heat prior to in vitro processing. However, these conditions did not promote any processing of the deletion constructs and partially inhibited the processing of the wild-type subunit. The requirement for a conformation may explain why one metalloprotease within the mitochondrial matrix can act to process a variety of precursor proteins imported into mitochondria at sites which lack any apparent sequence homology.

DISCUSSION

Deletions within the amino terminus of the F1 β-subunit precursor have defined a region required for its processing that is distinct from its targeting sequence. Small deletions, 17 residues beyond the processing site, prevent protease-dependent removal of the presequence yet have no apparent effect on the level of the β-subunit imported into mitochondria. This region which is required for processing of the F1 β-precursor is necessary for maturation either in vivo or in vitro.

In vivo analysis has demonstrated that the first 10 residues of the F1 β-precursor constitute the bulk of the sequence
case an equal volume of lysate (20 µl) was treated for SDS-gel autoradiographic analysis. Samples treated in the same
manner of the nascent maltose-binding protein precursor which were responsible for this interference were located within the ma-
ture portion of the precursor distal to the presecretory signal. Thus, like the post-translational delivery of these mutant proteins in E. coli, the import of mitochondrial proteins in yeast may involve the interaction of targeting sequences with other proteins.

The enzyme which catalyzes the maturation of the F, β-
precursor is located in the mitochondrial matrix (McAda and
Douglas, 1982; Bohni et al., 1983). The processing of precursors following their entry into mitochondria is proposed to complete the vectorial events of transport into the matrix and to potentially assist assembly of the protein with its partner proteins. Several earlier reports have demonstrated that the transport of proteins into mitochondria is not dependent on their maturation (Zwijinska and Neupert, 1984; Hurt et al., 1985). In the present study, the fact that in vivo assembly still occurred in the absence of apparent maturation raises the question of what prevents the subunit from prematurely as-
sembling with itself or other F, ATPase precursors in the wrong space. The most straightforward explanation for this is that during steady state growth in yeast of imported proteins is concentrated in mitochondria for assembly. The F, β-
subunit precursor may be normally cotranslationally delivered into the organelle in vivo and not present in the cytoplasmic
compartment. During logarithmic growth in yeast extra-
mitochondrial precursor forms of the F, β-subunit precursor are not observed and can only be seen under special conditions of mitochondrial uncoupling (Reid and Schatz, 1982). On the other hand, a small cytoplasmic pool of nascent precursor protein, if present, may be complexed with other proteins to maintain the precursor for import. In this regard, several groups have now defined various soluble factors present in both reticulocyte lysate (Miura et al., 1983; Argan et al., 1983) or in a yeast cytosolic fraction (Ohta and Schatz, 1984) which may be necessary for import of proteins into mitochondria. Thus, an efficient import process ensures the maintenance of protein concentrations favorable for their assembly within the organelle. Several imported mitochondrial proteins have been described which are not apparently processed (Hampsey et al., 1983) yet are still assembled within the mitochondrial matrix. These nonmaturable precursors like the F, β-
subunit deletion mutants described in the present study probably do not form a "maturation-competent" structure which is rec-
ognized by the processing enzyme. Thus, an import scenario may be envisioned for the F, β-precursor in which the secondary
structure of a targeting sequence is recognized by mito-
chondria for import. However, within the organelle, a struc-
ture defined in part by residues distal to the processing site of the F, β-precursor is required for its proteolytic maturation.

Acknowledgments—We gratefully acknowledge the expert technical assistance of M. Britten and L. Vallier in these studies.

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βOK Δ29T37 Δ35V45 Δ4-37

FIG. 6. Mutant ATPase β-subunit cannot be processed in vitro by isolated mitochondrial matrix protease. Wild-type and
mutant constructs of ATP2 were inserted into the transcription vector pTT-1 and then utilized to generate specific messenger RNAs in vitro. These transcripts were then used to program the synthesis of [35S]-methionine-labeled subunits in a rabbit reticulocyte lysate. In each case an equal volume of lysate (20 µl) was treated for 30 min at 22 °C with 20 µl of partially purified matrix protease (1 mg/ml) (+) followed by SDS-gel autoradiographic analysis. Samples treated in the same
manner but without added protease (−) were resolved in the adjacent lane. The lower translation product common to each sample results from internal translation initiation of ATP2 at methionine 97.

necessary for correct delivery of the protein. The βΔ10-37
subunit was targeted to mitochondria and assembled into a functional ATPase whereas the βΔ4-37 subunit was not. The mitochondrial targeting signal defined by this analysis, as well as that defined for other proteins (Hurt et al., 1985; Horwich et al., 1985), is relatively small and approaches the size of the protein signals defined previously for sorting and localization of proteins to the nucleus (Hall et al., 1984; Moreland et al., 1985). The sequences defined thus far for the import of different proteins into the mitochondrial matrix lack any apparent direct sequence homology. The only common struc-
tural feature among these targeting signals is that they contain
positively charged residues at an interval such that an
amphiphilic structure may be formed (Douglas et al., 1986).
In the present study the Δ4-37 deletion removes residues such that only one lysine residue is expressed within the first 20 residues and the potential for formation of an amphiphilic structure is much reduced. This construction expresses a protein which is not targeted to mitochondria in vivo or imported into isolated mitochondria.2 However, cis mutations can be selected which restore correct localization and assem-
bly of the βΔ4-37 protein. These plasmid-linked suppressor
mutations are located within the gene coding for the amino-
terminal region of the βΔ4-37 protein to partially restore an
amphiphilic structure.

The efficiency with which the targeting signal located at
the extreme amino terminus functions to direct mitochondrial
import may be determined in part by the protein sequences
located carboxyl-terminal to it. In this study, deletions some distance from the targeting region within the F, β decreased the amount of hybrid βZ protein delivered to mitochondria. These deletions probably modify the primary sequence in such a way that the accessibility of the targeting signal at the extreme amino terminus of the precursor protein to the mito-
chondrial import apparatus is somehow restricted. Other
studies have demonstrated that gene constructions which
fused E. coli β-galactosidase too close to the mitochondrial
targeting sequence interfered with their function in targeting
into the organelle (Hase et al., 1984; Emr et al., 1986; Keng et
al., 1986; Adrian et al., 1986). Thus, the post-translational
delivery of proteins to mitochondria which is directed by signals near the extreme amino terminus of the protein can be influenced by additional sequences in the nascent polypep-
tide. In E. coli, the accumulation of mutant maltose-binding
protein containing signal sequence mutations has been shown to interfere with the export of other proteins to the periplasm and outer membrane (Bankaitis and Bassford, 1984). Regions of the nascent maltose-binding protein precursor which were
responsible for this interference were located within the ma-
ture portion of the precursor distal to the presecretory signal. Thus, like the post-translational delivery of these mutant proteins in E. coli, the import of mitochondrial proteins in yeast may involve the interaction of targeting sequences with other proteins.

3 W. Chen and M. Douglas, manuscript in preparation.

4 A. Vassarotti, R. Stroud, and M. Douglas, submitted for publica-
tion.
