Mitochondrial Import of Cytochrome c Oxidase Subunit VIIa in Saccharomyces cerevisiae

IDENTIFICATION OF SEQUENCES REQUIRED FOR MITOCHONDRIAL LOCALIZATION IN VIVO*

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Subunit VIIa of yeast cytochrome c oxidase is a small (59 amino acids) protein of the inner mitochondrial membrane that lacks a cleavable amino-terminal presequence. To identify regions within this polypeptide that are essential for its import, gene fusions were constructed using a leader peptide substitution vector (pLPS) developed in this laboratory (Glaser, S. M., Trueblood, C. E., Dircks, L. K., Poyton, R. O., and Cumskey, M. G. (1988) J. Cell. Biochem. 36, 275–287). In this vector, oligonucleotide sequences encoding all or part of subunit VIIa were fused in-frame with the coding region of mature cytochrome c oxidase subunit Va. The plasmid pLPS is ideal for assaying protein sequences for their ability to direct mitochondrial import in vivo since subunit Va’s leader peptide is essential for import and because subunit VIIa is required for cytochrome c oxidase activity and respiration. Strains containing these fusions but lacking both subunit VIIa genes (COX5a and COX5b) were analyzed to determine whether the chimeric protein is directed to mitochondria. Our findings indicate that the amino-terminal 17 amino acids of subunit VIIa are sufficient to localize subunit VIIa to the mitochondrion and that a 6-amino acid-long region within the amino terminus (Gly¹–Arg¹³) is essential. In addition, some import (~10% of wild type) is observed with the highly charged carboxyl terminus of subunit VIIa, suggesting that the subunit may contain redundancy in its import information.

In yeast, most nuclear encoded mitochondrial proteins are synthesized as preprotein precursors containing an amino-terminal presequence. There are, however, a few proteins that are localized to mitochondria without such amino-terminal extensions. Numerous studies with proteins of the first class have shown that presequences are necessary and, in some cases, sufficient for import (reviewed by Verner and Schatz, 1988). Although presequences lack primary sequence homology, they are enriched for both basic and hydroxylated amino acids, lack both acidic amino acids and long hydrophobic stretches, and may be able to form amphiphilic α-helical and β-sheet conformations (Verner and Schatz, 1988). In contrast, little is known regarding the nature of the import information within proteins that lack presequences.

Proteins lacking a presequence include the ADP/ATP carrier (Smagula and Douglas, 1988), the 70-kDa outer membrane protein (Hurt et al., 1985), cytochrome c (Stuart et al., 1987), and subunit VIIa of cytochrome c oxidase (Wright et al., 1986). So far, no generalizations can be made regarding the disposition of the import-directing information within these proteins. For example, the first 12 amino acids of the 70 kDa outer mitochondrial membrane protein in Saccharomyces cerevisiae can target a heterologous protein to mitochondria, and the sequence of this region conforms to the general characteristics of the presequences mentioned above (Hurt et al., 1985). In contrast, the ADP/ATP carrier from S. cerevisiae (Smagula and Douglas, 1988) or Neurospora crassa (Pfanner et al., 1987) is imported when the polypeptide has been truncated extensively at its amino terminus. The import information in the yeast protein has been shown to be between amino acids 72 and 111 (Smagula and Douglas, 1988). Finally, both the amino- and carboxyl-terminal domains have been implicated in the binding and/or import of cytochrome c by mitochondria (Stuart et al., 1987; Jordi et al., 1989).

In this study we have sought to determine the location and composition of the information within subunit VIIa of cytochrome c oxidase that is required for its import by mitochondria. With a length of only 59 amino acids this polypeptide is the shortest known “leaderless” protein that is imported by mitochondrion. We have utilized the small size of subunit VIIa to dissect the protein and analyze the various regions for their import-directing information.

MATERIALS AND METHODS

RESULTS

Gene Fusions to COX5a of Cytochrome c Oxidase—The plasmid YCpLPS-5a (Fig. 2A) was used to construct gene fusions in which the DNA encoding the 20-amino acid presequence of subunit Va of cytochrome c oxidase was replaced with DNA encoding subunit VIIa sequences. Initially three sets of oligonucleotides, which divide subunit VIIa into three approximately equal regions, were used to replace subunit Va sequences (see “Materials and Methods”). These encode amino acids 1–17 (pDD1), 16–38 (pDD2), or 38–59 (pDD3) of subunit VIIa. Each construct begins with a methionine that is found in the subunit VIIa sequence. The translational contexts around each methionine are similar to those preceding initiator methionines in other S. cerevisiae genes (Ham...
iltron et al., 1987) and are expected to be equivalent with respect to translational initiation (Bairn and Sherman, 1988). Also, in each construct the VIIa sequences are fused in-frame to the 3rd amino acid of mature subunit Va without the insertion of extra amino acids. Expression of all YCpLPS-5a constructs is driven by the COX5a promoter and all have identical 5'-flanking regions.

The Amino Terminus of VIIa Directs Vα to the Mitochondrion—The plasmids containing the gene fusions were transformed into a yeast strain, GD5ab, in which the genes, COX5a and COX5b, encoding subunit Vα, and its isolog Vγ, are deleted (Trueblood and Poyton, 1987). This strain is respiratory-deficient because yeast cells require at least one isolog of subunit Vα for cytochrome c oxidase activity. Respiration can be restored to GD5ab by plasmids that carry a wild-type COX5a gene but not by plasmids carrying a COX5a gene in which the presequence has been deleted (Glaser et al., 1988). In a previous study we have demonstrated that presequences from other mitochondrial proteins are capable of directing the Vα subunit to the mitochondrion, where it assembles into a functional holocytochrome c oxidase molecule (Glaser et al., 1988). This provides the basis for an in vivo assay of mitochondrial protein import. All plasmid-containing strains were assayed for their ability to grow on nonfermentable carbon sources (Fig. 3A) and for their level of respiration (Fig. 4). Strains containing plasmids pDD1, pDD2, and pDD3 were grown on a nonfermentable carbon source (YP lactate) and compared with their parent (GD5ab) and the wild-type strain (JM43) (Fig. 3A). It is clear that only strains containing pDD1 showed wild-type growth after 3 days, while the other strains showed no growth (pDD2) or small colonies at 3–6 days (pDD3). The level of respiration in strains carrying pDD1, pDD2, and pDD3 parallel these growth results (Fig. 4). The strain carrying pDD1 respires at essentially a wild-type rate whereas strains carrying pDD3 and pDD2 have little (pDD3) or no respiration (pDD2). The level of respiration in pDD2 is barely greater than that present in untransformed GD5ab, while the level of respiration in pDD3 is only slightly, but reproducibly, greater than that of the strain carrying pDD2.

Western immunoblot analysis was used to identify the cellular location of the chimeric proteins. Strains containing plasmids pDD1, pDD2, and pDD3 were grown under the same conditions used for the oxygen consumption studies (see "Materials and Methods"). The mitochondrial and cytoplasmic fractions were purified from each strain and analyzed using an anti-Vα antiserum. None of the strains showed any evidence of chimeric protein in the cytosolic fraction (data not shown). In Fig. 5A, equal amounts of mitochondrial protein from the various strains are compared with those of JM43 (lane 3) and GD5ab (lane 2). Only the immunoblots of mitochondria containing the chimeric protein encoded by pDD1 (lane 4) are comparable to that of the wild-type Vα signal from JM43 mitochondria. The strains containing pDD2 and pDD3 show little or no evidence of chimeric protein in the mitochondrial fraction. Since the processing site for the presequence of Vα is removed in the chimeric proteins, it is not surprising that the protein encoded by pDD1 has a higher molecular weight than mature subunit Vα.

Together, these results demonstrate that the chimeric protein encoded by pDD1 is both imported by mitochondria and assembled in cytochrome c oxidase at or near wild-type levels, while the chimeric proteins from pDD2 and pDD3 are either not imported (pDD2) or imported inefficiently (pDD3) into mitochondria.

Localization of Sequences Essential for the Import of the Protein Encoded by pDD1—The subunit VIIa sequence encoded in pDD1 has a cluster of three basic amino acids (Lys⁻⁸—Arg⁻¹⁰) that is reminiscent of the positive charges found in presequences of nuclear-encoded mitochondrial proteins. Since mutations and deletions of basic amino acids found in presequences affect their ability to direct import (Horwich et al., 1985, 1986), it was of interest to determine whether this cluster of basic amino acids in subunit VIIa is similarly required. This was tested by analyzing a strain carrying a plasmid with a deletion of a 6-amino acid region from pDD1 (including the three basic charges, see "Materials and Methods"). This plasmid, pDD1Δ6, was transformed into GD5ab and tested as before. Unlike the protein that contains these 6 residues, the fusion protein lacking residues 8–13 of subunit VIIa is not localized to the mitochondria (Fig. 5A) and does not allow growth on YP lactate or support respiration (Figs. 3A and 4).

Chimeric Proteins That Contain All of Subunit VIIa Sequences in YCpLPS-5a—We wished to verify that amino acids 8–13 are important and that the results found with pDD1 and

![Fig. 3. Growth on a nonfermentable carbon source. GD5ab strains containing the leader peptide substitution vectors were tested for growth on a nonfermentable carbon source (YP lactate) and compared with their parent strain (GD5ab) and the wild-type strain (JM43). Single colonies were selected and streaked onto YPLactate plates and grown for either 3 (A) or 6 (B) days at 28 °C.](http://www.jbc.org/)

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Mitochondrial Import of Subunit VIIa

<table>
<thead>
<tr>
<th>STRAIN</th>
<th>PLASMID</th>
<th>SUBUNIT VIIa DOMAIN IN pLPS</th>
<th>RATE OF RESPIRATION</th>
</tr>
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<tbody>
<tr>
<td>JM43</td>
<td>----</td>
<td>---------------------------</td>
<td>100%</td>
</tr>
<tr>
<td>GD5ab</td>
<td>----</td>
<td>---------------------------</td>
<td>4%</td>
</tr>
<tr>
<td>GD5ab</td>
<td>pDD1</td>
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<tr>
<td>GD5ab</td>
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<tr>
<td>GD5ab</td>
<td>pDD3</td>
<td>^38 MDKINKREKFAELAERKKQEN</td>
<td>11%</td>
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<tr>
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<td>^1 MTPAPIT--- ^14 ^17 VIMD</td>
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<tr>
<td>GD5ab</td>
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</tr>
<tr>
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<td>27%</td>
</tr>
<tr>
<td>GD5ab</td>
<td>pDD1,2,3,318-13</td>
<td>^1 MTPAPIT---- ^14 VIMD</td>
<td>13%</td>
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</tbody>
</table>

FIG. 4. Respiration rates of strains carrying COX9/COX5a gene fusions. The strains used in these studies and the plasmids they contain are listed. The amino acid sequences of the subunit VIIa regions follow the plasmid names. The rate of respiration of each strain is listed in percent of the rate of respiration of the wild-type JM43 strain (100% = 29.6 pmO_2 consumed per min/µg of cells, dry weight).

pDD1,8-13 are not artifacts created by placing the first 17 amino acids of subunit VIIa next to mature subunit Va. Therefore, we constructed YCpLPS-5a derivatives, which contain either the first 38 amino acids of subunit VIIa fused in-frame with subunit Va (pDD1,2), all of subunit VIIa fused in-frame with mature Va (pDD1,2,3), or a deletion of amino acids 8-13 from pDD1,2,3 (pDD1,2,318-13) (see “Materials and Methods”). These YCpLPS-5a derivatives were then introduced into the strain GD5ab and tested as before.

The mitochondrial import of the chimeric proteins encoded by both pDD1,2 and pDD1,2,3 is less efficient than pDD1, as shown by Western immunoblotting (Fig. 5A), growth in YP-lactate, and respiration rates (Figs. 3B and 4). At present, it is uncertain whether the observed targeting inefficiency is due to polypeptide instability, masking of import information, or some less obvious factor. The results with pDD1,2,318-13 mimicked those of pDD1,318-13 in that its mitochondrial localization, as measured by growth on YP-lactate (Fig. 3B) and respiration (Fig. 4), dropped dramatically when compared with its undeleted counterpart, pDD1,2,3.

The small amount of import seen with the carboxyl terminus of subunit VIIa is not an artifact created by the partition of subunit VIIa sequences. In analyzing the results with the various strains it was noted that pDD1,2,318-13 was remarkably similar to pDD3 in both growth on YP-lactate and oxygen consumption (Figs. 3 and 4). Under the conditions used for Western immunoblot analysis of all the strains little (pDD1,2,318-13) or no protein (pDD3) was seen in mitochondria.
Mitochondrial Import of Subunit VIIa

dria from strains carrying pDD3 or pDD1,2,3,4,5,13 (Fig. 5A). In order to
determine if the increased respiratory rates and growth on YP lactate were
reflections of a small amount of chimeric protein in mitochondria of strains
carrying pDD3 we looked for a more sensitive probe for the protein.
Western immunoblots were performed on mitochondria from pDD3 and
pDD1,2,3,4,5,13 with an anti-VIIa antisera which demonstrated that the chimeric
proteins were present in near equal amounts (Fig. 5B). It is assumed that the highly charged
carboxyl terminus of subunit VIIa was more antigenic than the bovine serum albumin-peptide
conjugate giving a higher titer of antibodies in the antisera, thereby making it a more
sensitive probe. While it was surprising that the immunoblots with the two antibody probes
did not show the same specific bands, we interpret the results from all three assays as suggesting
that the small amount of mitochondrial localization seen with the carboxyl terminus of
VIIa is not an artifact created by a change in its relative location in the chimeric protein.
When amino acids 8-13 are removed from pDD1,2,3 the import information remaining in
the carboxyl terminus is sufficient to direct import of the chimeric protein, hence the similarities to pDD3 in growth,
oxygen consumption, and (potentially) immunoblot analysis. It is interesting to note that the carboxyl terminus of subunit
VIIa has a region with three consecutive basic amino acids (Arg84-Lys85-Lys86) similar to the amino terminus.

DISCUSSION

By using gene fusions that replace the DNA encoding the presequence of subunit Va of cytochrome c oxidase with DNA
encoding subunit VIIa sequences, we have identified regions of VIIa that direct the import of the protein to mitochondria.
Import of the chimeric proteins was measured by assaying strains carrying these fusions for growth on YP lactate,
cellular oxygen consumption, and, by Western immunoblot analysis. These studies show that the first 17 residues of VIIa are
capable of directing a heterologous protein, subunit Va, to mitochondria in a way that permits its subsequent assembly
into a functional complex. In the amino terminus, deletion of a 6-amino acid region containing 3 positive residues leads to
the loss of import. We have also demonstrated that the middle region of VIIa is unable to direct the chimeric protein to
mitochondria and that the carboxyl terminus is capable of directing a lower level of import and assembly. Although the fusion protein encoded on pDD1,2,3 was imported at a lower efficiency than expected, a 6-amino acid deletion in its coding
region (pDD1,2,3,4,5,13) decreased its import to near that of the protein encoded on pDD3, confirming results with pDD1 and
its deletion.

The localization of import information in the amino terminus of subunit VIIa is similar to the results found with the
70-kDa outer membrane protein (Hurt et al., 1985) but dissimilar to the results with ADP/ATP carrier (Smagula and Dougal,
1988; Pfanner et al., 1987). Together, the results with these proteins may indicate that mitochondrial proteins with-
out presequences can be subdivided into those proteins with amino-terminal import information (distinct from that found
in presequences) and those proteins with more internal import information. Another interpretation is that some of these
proteins have essentially uncleaved presequences, while proteins with more internal import information belong to a
distinct group of their own. Further studies of mitochondrial import must be undertaken in order to classify import
information in proteins that lack amino-terminal presequences.

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Mitochondrial Import of Subunit VIIa


MATERIALS AND METHODS

Protein Sources and Methods

The Saccharomyces cerevisiae strains used in this study were: JRA85 (MATa, his3Δ2, leu2Δ1, hec4Δ, spo11Δ, URA3,ade2-1, ura3-52, trp1-901, met15), JRA220 (MATa, his3Δ2, leu2Δ1, hec4Δ, spo11Δ, URA3, ade2-1, ura3-52, trp1-901, met15, cys8Δ), and JRA221 (MATa, his3Δ2, leu2Δ1, hec4Δ, spo11Δ, URA3, ade2-1, ura3-52, trp1-901, met15, cys8Δ, tyr1Δ). Yeast strains were grown in YP media unless otherwise stated. Yeast transformants were identified by Southern blot analysis, using a PCR fragment corresponding to the 5′ portion of the mitochondrial gene as a probe.

Plasmid Construction and Propagation

All plasmids were propagated in E. coli JM109 cells. BMH (P. Renner and R. Pantel, unpublished observations) was a gift from Dr. Renner and Dr. Pantel. pPIC9K (Pichia pastoris) was a gift from Dr. Pantel. All plasmid DNA was prepared by the alkaline lysis method. Yeast strains used were grown in selective media as previously described (see Table 1). The CaCl2 pulse was performed at 37°C.

RESULTS

The two cytochrome c oxidase subunits were identified by the method of Dubil et al. (1995), and the mitochondrial origin of the subunit VIIa gene was confirmed by Northern blot analysis using a probe corresponding to the 3′ end of the gene. The subunit VIIa gene contains a single open reading frame encoding a polypeptide of 669 amino acids. The amino acid sequence of the protein is identical to that of the mitochondrial enzyme.

MATERIALS AND METHODS

The Saccharomyces cerevisiae strain used in this study was: JRA85 (MATa, his3Δ2, leu2Δ1, hec4Δ, spo11Δ, URA3, ade2-1, ura3-52, trp1-901, met15). All plasmid DNA was prepared by the alkaline lysis method. Yeast strains used were grown in selective media as previously described (see Table 1). The CaCl2 pulse was performed at 37°C.

SUPPLEMENTAL MATERIAL

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