Artificial mitochondrial presequences
(protein transport/mitochondria/targeting sequences)

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ABSTRACT Synthetic oligonucleotides were used to construct artificial mitochondrial presequences that contained, besides the initiator methionine, only arginine, serine, and leucine. The ratio of these three amino acids was adjusted to match that of basic, hydroxylated, and hydrophobic residues in natural mitochondrial presequences. When these sequences were fused to the N terminus of yeast cytochrome oxidase subunit IV lacking its own presequence, they directed the attached subunit IV to its correct intramitochondrial location in vivo. They also mediated import of subunit IV into isolated yeast mitochondria. In contrast, artificial sequences containing glutamine, arginine, and serine residues following the initiator methionine were inactive. Thus, the targeting function of mitochondrial presequences does not depend on specific amino acid sequences but may instead depend on the overall balance between basic, hydrophobic, and hydroxylated amino acids.

Import of nuclear-encoded proteins into mitochondria is usually mediated by a transient presequence (1). In several cases it has been demonstrated that these presequences contain all of the necessary information for targeting the attached “mature” polypeptide chain to its correct intramitochondrial location (2). So far, no significant sequence homology has been detected among the several dozen mitochondrial presequences whose primary structure is known (3). However, these sequences generally lack acidic amino acids; are rich in basic, hydrophobic, and hydroxylated amino acids; and could potentially fold into an amphiphilic $\alpha$-helix (3, 4). This raises the possibility that the function of these peptides does not require a specific primary amino acid sequence but merely a particular balance between positively charged, hydrophobic, and hydroxylated residues, perhaps arranged so as to allow formation of a particular secondary structure.

We have tested this possibility by constructing three artificial peptides composed of only the initiator methionine and the three amino acids leucine, arginine, and serine. These artificial presequences were able to target subunit IV of cytochrome oxidase into mitochondria both in vitro and in vivo.

MATERIALS AND METHODS

Strains. For expression of altered subunit IV genes, the Saccharomyces cerevisiae strain WD1 (ura3 his3 "coad") was used in which the nuclear gene encoding subunit IV of cytochrome oxidase, designated here cos4, had been disrupted (5). WD1 is a derivative of the respiring parental DL1 (5). The S. cerevisiae strain D273-10B (ATCC 25657) was used for isolation of mitochondria for import studies (6, 7).

Oligonucleotides. All oligonucleotides were synthesized by using the fully automated Applied Biosystems 380B DNA synthesizer and purified on HPLC according to the manufacturer’s instructions. The following eight oligonucleotides were made: 1, AATCCACAATGTGT; 2, CTTAGACCATTTGT; 3, CTAGATTGTCTCTAGATTGCT- T; 4, CTAGACAGCAATTCAGAGACA; 5, CTAGCTTGCTGACTTAGACTCT; 6, GACAGAGATCTA- GTTCCAGAACAG; 7, TGTCCTTGCTGTTGAGACTGT- G; and 8, CTAGACAGCTTCAACGCAGAAG. Oligonucleotides 1 and 2 are partially complementary, as are 3 and 4, 5 and 6, and 7 and 8. The DNA duplex from hybridized oligonucleotides 1 and 2 (duplex 1/2) has one 5' protruding end corresponding to that produced by the restriction enzyme EcoRI and another one corresponding to one produced by Xba I. The 24-mer oligonucleotide duplex 3-4 has two Xba I-produced protruding ends. When duplex 5-6 is ligated with duplex 7-8 and then digested with Xba I, a 48-mer duplex was produced overhanging ends results (48-mer duplex 56/78).

Plasmid Constructions. A 1.1-kilobase EcoRI/HindIII fragment from pMC4 (see below) containing the yeast gene encoding the precursor of cytochrome oxidase subunit IV was first ligated into the phage vector M13mp11 (8) to create the recombinant phage DNA M13mp11-R/HCOX-IV. In that plasmid, the unique EcoRI site is 23 base pairs upstream of the initiating ATG of subunit IV, and the single Xba I site is located at a position corresponding to amino acids 21 and 22 of the presequence of subunit IV (Fig. 1). M13mp11-R/HCOX-IV was first digested with EcoRI/Xba I; the large fragment was isolated and ligated with 5'-phosphorylated oligonucleotide duplex 1-2 to create M13mp11-CXII. This procedure in effect resulted in a deletion of DNA corresponding to amino acids 3-20 in the subunit IV presequence (oligonucleotide designated d3-20; in Fig. 1) and created an Xba I restriction site 6 base pairs downstream from the initiator methionine codon. M13mp11-CXII was digested with Xba I, dephosphorylated, and ligated either with the phosphorylated 24-mer duplex 3-4 or with the 48-mer duplex 56/78. Altered subunit IV genes with one insert in each orientation of duplex 3-4 (subunit IV gene with presequence Syn A1 or Syn B1; Fig. 1) or two inserts (Syn A2 or Syn B2) were identified by DNA sequencing (8). Similarly, mutant subunit IV genes with one insert of the 48-mer duplex 56/78 in each orientation were identified (presequence Syn C or Syn D; Fig. 1). The recombinant subunit IV genes were then excised from the phage M13 vector as EcoRI/HindIII fragments and ligated into the EcoRI/HindIII site of the in vivo expression plasmid pMC4 (11), a single-copy yeast shuttle plasmid from which the subunit IV genes are expressed by the promoter for the yeast alcohol dehydrogenase I gene (12). For in vitro expression, the EcoRI/HindIII fragments were ligated into pDS 5/2-1 (7, 13), a plasmid carrying a phage T5 promoter. All DNA manipulations were carried out essentially as described by Maniatis et al. (14).

Growth of Yeast Cells and Subcellular Fractionation. The yeast strain WD1 was transformed (15), and transformants were selected on the basis of uracil prototrophy. For subcellular fractionation, transformed WD1 cells were grown at 30°C in 1% yeast extract/2% peptone/0.2% glucose to an OD600 of about 1. Cells were fractionated into a mitochondrial
FIG. 1. Amino acid sequences of authentic [wild type (WT)] subunit IV presequence and of artificial presequences. (Upper) Linear sequences. The arrow designates the site of cleavage by the mitochondrial matrix processing protease. The complete sequence of the subunit IV precursor is shown in ref. 9. (Lower) Representation as α-helical wheel diagrams (10). See Results for details.

and postmitochondrial supernatant fraction essentially as described (16), except that the postmitochondrial supernatant was not further fractionated.

Mitochondrial Protein Import. Mitochondria were isolated, and import experiments were performed as described (6), except that the various subunit IV proteins were synthesized by using a coupled in vitro transcription/translation system (7, 13). Published procedures were used for protease protection (17), NaDodSO₄ gel electrophoresis and fluorography (18), and quantitation of bands on x-ray films (19).

Miscellaneous. Proteins were extracted from yeast cells, and immunoblots were prepared as described (20).

RESULTS

Construction of Cytochrome Oxidase Subunit IV Fused to Artificial Presequences. The strategy we used for building artificial presequences for subunit IV was to replace most of the DNA encoding the authentic presequence of subunit IV with synthetic DNA duplexes. These manipulations replaced IV amino acids 4–20 of the authentic subunit IV presequence with the artificial peptides shown in Fig. 1. The “Syn A” set of artificial presequences contained one (Syn A1) or two (Syn A2) copies of an octapeptide composed of only leucine, arginine, and serine (Fig. 1). The percentage of hydrophobic, basic, and hydroxylated residues of this Syn A series approximated that of natural mitochondrial presequences (Table 1). In addition, the amino acids in Syn A presequences were arranged such that an α-helical conformation of the peptide would be amphiphilic (Fig. 1 Lower). The Syn C peptide had the same amino acid composition as the Syn A2 peptide but lacked the potential to form an amphiphilic α-helix (Fig. 1). The Syn B1, Syn B2, and Syn D sequences (Fig. 1 Upper) were composed predominantly of glutamine, arginine, and serine residues and, thus, were significantly more hydrophilic than were natural presequences (Table 1). All of these sequences were fused to the amino terminus of mature subunit IV and were expressed either in yeast cells (using a single copy yeast plasmid with a strong yeast promoter) or by coupled transcription/translation in vitro.

Artificial Prequences Direct Cytochrome Oxidase Subunit IV into Yeast Mitochondria in Vivo. In order to test the ability of the artificially constructed presequences to transport attached proteins into mitochondria, a subunit IV-deficient yeast strain (5) was transformed with plasmids encoding subunit IV attached to the various peptides (Fig. 1). The untransformed strain lacked cytochrome oxidase and, thus, was unable to grow on a nonfermentable carbon source. Plasmids that restore growth on a nonfermentable carbon source must encode a subunit IV protein that is at least partially targeted to its correct intramitochondrial location (31). As shown in Fig. 2, subunit IV lacking most of its own presequence (oligonucleotide d3-20; see Fig. 1) did not restore growth on glycerol, even though the truncated pre-
Table 1. Percentage of positively charged residues and average hydrophobicity of natural and artificial presequences

<table>
<thead>
<tr>
<th>Presequence</th>
<th>Positively charged residues, %</th>
<th>Average hydrophobicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average of 10 natural presequences</td>
<td>21</td>
<td>-0.12</td>
</tr>
<tr>
<td>Syn A2</td>
<td>26</td>
<td>-0.09</td>
</tr>
<tr>
<td>Syn B2</td>
<td>26</td>
<td>-0.69</td>
</tr>
<tr>
<td>Syn C</td>
<td>26</td>
<td>-0.09</td>
</tr>
<tr>
<td>Syn D</td>
<td>22</td>
<td>-0.45</td>
</tr>
</tbody>
</table>

*Positively charged residues include the amino-terminal methionine residue.
1Hydrophobicity values were calculated by using a normalized consensus hydrophobicity scale (21) in which arginine is the most hydrophilic (-2.53) residue, and isoleucine, the most hydrophobic (1.38).
2The 10 mitochondrial presequences are those of known or inferred length of 40 or fewer residues. These sequences are from precursors of: yeast (9) and bovine (22) cytochrome oxidase subunit IV; yeast cytochrome oxidase subunit VI (23); yeast superoxide dismutase (24); yeast alcohol dehydrogenase (25); rat (26) and human (27) ornithine transcarbamylase; rat carbamoyl-phosphate synthetase (28); Neurospora crassa Rieske FeS protein (29); and yeast cytochrome oxidase subunit V (30).
3Range, 16 to 28.
4Range, -0.20 to -0.12.

cursor accumulated in cells after growth on glucose (Fig. 3, lane d3-20). In contrast, all artificial presequences consisting of mostly leucine, arginine, and serine (Syn A1, Syn A2, and Syn C) could target subunit IV to its correct intramitochondrial location: the corresponding transfectants exhibited cytochrome oxidase activity (not shown) and grew on glycerol (Fig. 2). During logarithmic phase in liquid glycerol-containing medium, these transfectants grew as rapidly as those expressing the authentic subunit IV precursor (not shown). In contrast, subunit IV proteins with artificial presequences composed of mostly glutamine, arginine, and serine (Syn B1, Syn B2, and Syn D) did not restore growth of the mutant cells on glycerol, even though the corresponding fusion proteins were readily detected within the transformed cells (Fig. 3).

In order to quantify the amount of subunit IV protein within the mitochondria of the various transfectants, mitochondria were isolated and subjected to immunoblotting with antiserum against subunit IV. Mitochondria from cells expressing subunit IV with artificial presequences composed mostly of leucine, arginine, and serine (Syn A1, Syn A2, and Syn C) accumulated various cleaved intermediates of each respective precursor subunit IV protein (Fig. 4). Although there was very little mature protein detected, previous work had indicated that artificially constructed subunit IV precursors need not be processed to the normal mature form in order to assemble into and function in the cytochrome oxidase complex (31). In each of the three mitochondrial preparations, all of the subunit IV protein was protected from digestion by externally added proteinase K unless a detergent was also added; thus, the mitochondria-associated subunit IV was localized inside the organelle (not shown). The amount of Syn A-subunit IV or Syn C-subunit IV protein found inside isolated mitochondria was about one-fifth of the amount found when cells expressing the authentic subunit IV precursor. This could reflect a decreased rate of expression, increased degradation, or less-efficient targeting into mitochondria. The last possibility was supported by the observation that subunit IV protein with the presequence Syn A2 was found not only in the mitochondrial fraction but also in the postmitochondrial fraction (Fig. 5). We did not attempt to quantify the relative amounts of the different subunit IV proteins in different cellular compartments because we considered it very likely that their stability differed significantly depending on their location within the cell (31). Cells syn-

![Fig. 2. Growth on glycerol medium of transformed yeast cells expressing cytochrome oxidase subunit IV (COX IV) with various presequences. A yeast strain (W1) with a disrupted subunit IV gene was transformed with single-copy yeast plasmids carrying subunit IV genes with different presequences as shown in Fig. 1. Transformants were selected on uracil-free medium, individual colonies were streaked onto YPG medium (1% yeast extract/2% peptone/2% glycerol), and the plates were incubated at 30°C for 4 days. All of the yeast transformants shown above are derived from W1 (ura3 his3 cop7); the untransformed resiping COX IV' strain is DL1 (ura3 his3). WT, wild type.](image)

![Fig. 3. Subunit IV-related proteins present in yeast cells transformed with various subunit IV genes. Transformed yeast cells were grown in 0.67% yeast nitrogen base/2% glucose/20 mg of L-histidine per liter to an OD600 of about 1. Total protein was extracted, and equal amounts were electrophoresed through a NaDodSO4/14% polyacrylamide gel and subjected to immunoblotting by using antiserum against cytochrome oxidase subunit IV (see COX IV). The various presequences of each subunit IV protein are indicated above each lane and correspond to those shown in Fig. 1. m, Mature subunit IV; WT, wild type.](image)

![Fig. 4. Subunit IV-related proteins in mitochondria from yeast cells transformed with various subunit IV genes. Transformed yeast cells were grown in 1% yeast extract/2% peptone/0.2% glucose to an OD600 of about 1. The cells were converted to spheroplasts and homogenized, and mitochondria were isolated. Aliquots (50 μg) of mitochondria were subjected to NaDodSO4/14% polyacrylamide gel electrophoresis and immunoblotting using antisem against cytochrome oxidase subunit IV (see COX IV) and citrate synthase (see CS), a mitochondrial matrix enzyme. m, Position of mature subunit IV; WT, wild type. See Fig. 1 for the structure of the different presequences.](image)
thesizing subunit IV protein attached to peptides containing mostly glutamine, arginine, and serine (Syn B and Syn D) lacked any detectable subunit IV protein within their mitochondria (Fig. 4), in agreement with their inability to grow on glycerol. However, the apparently unprocessed subunit IV fusion proteins could be detected in the postmitochondrial fractions (Fig. 5 and data not shown).

**Artificial Presequences Import Subunit IV into Isolated Mitochondria.** In order to estimate the rates at which the artificial presequences directed subunit IV into mitochondria, the various subunit IV fusion proteins were synthesized *in vitro* in the presence of [35S]methionine and were added posttranslationally to either energized or de-energized isolated yeast mitochondria. The authentic subunit IV precursor bound to de-energized mitochondria (WT in Fig. 6, lane 2) but was not imported, as shown by its susceptibility to externally added proteinase K (WT in Fig. 6, lane 3). Upon addition to energized mitochondria, about 30% of the authentic subunit IV precursor was processed to its mature form by the matrix-located protease during a 20-min incubation (WT in Fig. 6, lane 4) and became resistant to externally added proteinase K (WT in Fig. 6, lane 5) unless a detergent also was added (WT in Fig. 6, lane 6). Subunit IV lacking most of its own presequence was neither bound nor imported by isolated mitochondria (d3-20 in Fig. 6). All of the subunit IV proteins with artificial presequences composed mostly of leucine, arginine, and serine (Syn A1, Syn A2, and Syn C) bound efficiently to the surface of de-energized mitochondria (Syn A1, Syn A2, and Syn C in Fig. 6, lanes 2). With energized mitochondria, 20% (Syn A1), 5% (Syn A2), or 20% (Syn C) of the added subunit IV protein became resistant to externally added proteinase K (Syn A1, Syn A2, and Syn C in Fig. 6, lanes 5) and was, thus, presumably imported. The rates of import mediated by Syn C and Syn A2 were about two-thirds and one-sixth, respectively, of the rate mediated by the authentic subunit IV presequence (Fig. 7). Imported subunit IV containing artificial presequences was not significantly processed by the matrix protease, even though the normal

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**Fig. 5.** Subcellular distribution of subunit IV-related proteins in yeast cells transformed with Syn A2 or Syn B2 presequences. Yeast cells were grown as described in Fig. 4, converted to spheroplasts, and fractionated into homogenate (H), mitochondrial (M), and postmitochondrial (PM) fractions. Aliquots of homogenate and postmitochondrial fraction [equivalent to 5 mg (wet weight) of cells] and mitochondria (equivalent to 15 mg of cells) were subjected to NaDodSO4/14% polyacrylamide gel electrophoresis and immunoblotting with antisera against cytochrome oxidase subunit IV (see COX IV), citrate synthase (see CS; a mitochondrial matrix marker), and hexokinase (see HK; a cytosolic marker). Citrate synthase migrates slightly faster in the homogenate fractions than in the mitochondrial fractions, probably because of protein overloading in the homogenate fraction.

**Fig. 6.** Efficacy of artificial presequences and of authentic [wild type (WT)] subunit IV presequence in directing import of subunit IV into isolated yeast mitochondria. Each subunit IV protein was synthesized by *in vitro* transcription/translation in the presence of L-[35S]methionine. Immediately after synthesis, the translation products were added to isolated mitochondria, the mixture was incubated at 30°C for 20 min and the mitochondria were reisolated by centrifugation, followed by NaDodSO4/14% polyacrylamide gel electrophoresis and fluorography. Lanes: 1, 20% of the translation products that were added to mitochondria in lanes 2–6; 2, incubation with de-energized mitochondria (valinomycin/K+); 3, incubation with de-energized mitochondria followed by digestion with proteinase K; 4, energized mitochondria; 5, energized mitochondria followed by proteinase K addition; 6, energized mitochondria followed by proteinase K and Triton X-100 addition. p, Authentic subunit IV precursor; m, mature subunit IV.

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**DISCUSSION**

The data reported here make it extremely improbable that targeting of a protein to mitochondria requires a specific amino acid sequence within the targeted protein. We could
build functional mitochondrial presequences consisting almost exclusively of leucine, arginine, and serine. None of the natural presequences known so far exhibits such a simple amino acid composition (3). When these artificial presequences were fused to cytochrome oxidase subunit IV lacking its own presequence, they directed subunit IV to its correct intramitochondrial location in vitro. Uptake experiments with isolated yeast mitochondria revealed that the efficiency of import mediated by some of the artificial presequences approached that of the authentic subunit IV presequence. Three artificial presequences differing in length and/or amino acid sequence proved to be active; this makes it unlikely that we had fortuitously created part of a natural presequence and implies that specific sequence motifs within presequences are not necessary for targeting function. Therefore, the mitochondrial import machinery must recognize more general features of these amino-terminal peptides. That a minimum hydrophobicity may be important is suggested by the inability of artificial peptides Syn B1, Syn B2, or Syn D to import subunit IV (Table 1). The cleavable signal peptides of secreted pro- or eukaryotic proteins also lack sequence homology (32); indeed, the hydrophobic core of a bacterial signal peptide can be largely replaced by leucine residues without loss of function (33). In contrast to mitochondrial presequences, however, presequences of secreted proteins share at least two distinct domains: an amino-terminal charged region and a subsequent hydrophobic region (32). Experimental (4) and statistical (3) evidence indicates that most natural mitochondrial presequences have the potential to form amphiphilic α-helices. To test whether this feature is essential for the targeting function of these presequences, we designed artificial presequences with (Syn A1 and Syn A2) and without (Syn C) a well-defined α-helical amphiphilic structure (Fig. 1 Lower). We found that both types of sequences could target subunit IV into mitochondria. Thus, an amphiphilic α-helix is not essential for mitochondrial targeting. It is possible, however, that our "nonamphiphilic" artificial presequences might be able to fold into alternative secondary structures with amphiphilic character (34). Thus, it remains unclear why most natural mitochondrial presequences are strongly biased toward amphiphilic α-helix; perhaps this feature could be related to the stability of the precursor in vivo or be required for recognition of the presequence by the specific processing protease located in the mitochondrial matrix.

In summary, our data suggest that the function of mitochondrial targeting sequences might be largely determined by overall amino acid composition. There may be additional parameters, such as perhaps the ability to form certain secondary structures (3, 4, 34). However, it now appears clear that the import function of a mitochondrial presequence is not dependent on a specific amino acid sequence.

Note Added in Proof. Roise et al. (4) recently have found that the chemically synthesized Syn A2 and Syn C peptides are as surface-active as the authentic cytochrome oxidase subunit IV prepeptide. Thus, amphiphilicity may be important for the function of these artificial peptides.

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