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Transport of Proteins Into Yeast Mitochondria

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The amino-terminal sequences of several imported mitochondrial precursor proteins have been shown to contain all the information required for transport to and sorting within mitochondria. Proteins transported into the matrix contain a matrix-targeting sequence. Proteins destined for other submitochondrial compartments contain, in addition, an intramitochondrial sorting sequence. The sorting sequence in the cytochrome c_1 presequence is a stop-transport sequence for the inner mitochondrial membrane. Proteins containing cleavable presequences can reach the intermembrane space by either of two pathways: (1) Part of the presequence is transported into the matrix; the attached protein, however, is transported across the outer but not the inner membrane (eg, the cytochrome c_1 presequence). (2) The precursor is first transported into the matrix; part of the presequence is then removed, and the protein is reexported across the inner membrane (eg, the precursor of the iron-sulphur protein of the cytochrome bc_1 complex).

Matrix-targeting sequences lack primary amino acid sequence homology, but they share structural characteristics. Many DNA sequences in a genome can potentially encode a matrix-targeting sequence. These sequences become active if positioned upstream of a protein coding sequence. Artificial matrix-targeting sequences include synthetic presequences consisting of only a few different amino acids, a known amphiphilic helix found inside a cytosolic protein, and the presequence of an imported chloroplast protein.

Transport of proteins across mitochrondrial membranes requires a membrane potential, ATP, and a 45-kd protein of the mitochondrial outer membrane. The ATP requirement for import is correlated with a stable structure in the imported precursor molecule. We suggest that transmembrane transport of a stably folded precursor requires an ATP-dependent unfolding of the precursor protein.

Key words: origin of presequences, intracellular and intramitochondrial protein sorting, stop-transport sequence, ATP requirement, protein unfolding, translocation machinery

How are proteins sorted between the various intracellular and intramitochondrial compartments? How are proteins transported across membranes? Is there a translo-

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cation machinery and what are its components? What are the characteristics of presequences and what is their evolutionary origin? Much progress has been made in the last few years in answering these questions. Here we review some recent results concerning transport of proteins into mitochondria.

PRESEQUENCES CAN TRANSPORT PROTEINS TO AND INTO MITOCHONDRIA

Most mitochondrial proteins are encoded in the nucleus. They are synthesized in the cytoplasm and can be imported into mitochondria post-translationally. Most imported proteins are initially made as precursor molecules with amino terminal extensions. These presequences are typically about 20–30 amino acids long but may be as long as 80 amino acids. Proteins of the mitochondrial outer membrane are synthesized without cleavable presequences [reviewed in 1–3].

Gene-fusion experiments have shown that several cleavable presequences, and also the noncleavable amino terminus of an outer membrane protein, contain sufficient information for transporting attached "passenger" proteins to mitochondria and into the proper intramitochondrial space. Conversely, mitochondrial proteins without their presequences are not transported to mitochondria [reviewed in 4]. Presequences thus can contain information for intracellular targeting to mitochondria and for intramitochondrial sorting. Presequences can also determine the intracellular sorting of naturally occurring isoenzymes: a cytosolic isoenzyme of alcohol dehydrogenase (ADH II) was imported into the mitochondrial matrix if it was fused to the cleavable presequence of the mitochondrial isoenzyme ADHIII, while ADHIII without its presequence remained in the cytosol [5]. Thus, presequences are essential for transporting authentic mitochondrial proteins to mitochondria; they can transport foreign proteins specifically to mitochondria, and they can determine the intracellular sorting of naturally occurring isoenzymes.

MATRIX-TARGETING SEQUENCES CAN BE CONSTRUCTED BY USING ONLY THREE DIFFERENT AMINO ACIDS, PROVIDED THAT THE RESULTING PRESEQUENCE IS AMPHIPHILIC

Presequences of mitochondrial matrix proteins (matrix-targeting sequences) are generally rich in basic and hydroxylated amino acids and lack acidic residues. They have no apparent homology in their primary amino acid sequences but share several structural characteristics [3,4] (Table I). The entire presequence is not required for intracellular targeting to the mitochondrial matrix. Functional matrix-targeting sequences may be as short as nine or twelve amino acids [6,7] and have also been identified as part of the presequence of an intermembrane space protein or as part of the amino terminal sequence of an outer membrane protein (see below). Matrix-targeting sequences transport proteins specifically to the mitochondrial matrix [4].

Conservation of specific primary amino acid sequences is not required for a functional matrix-targeting sequence. Allison and Schatz [8] showed that functional matrix-targeting sequences can be constructed by using only three different amino acids: arginine (a basic amino acid), leucine (an apolar amino acid), and serine (a hydroxylated amino acid). These artificial presequences, like most natural presequences, could form positively charged amphiphilic structures [3,8,9]. As shown by

TABLE I. The Functional Presequences Derived From the Mouse DHFR Gene Resemble Natural Presequences More Closely Than Random Sequences Derived From DHFR

	DHFR Functional		DHFR Random				
	Inserts	Presequences	Inserts	Presequences	Authentic DHFR	Average Proteins	Natural Presequences
% of basic amino acids	11.4	13.5	14.5	15.2	13.4	11.1	17.9
% of acidic amino acids	2.5	1.6	8.0	5.0	8.1	11.1	0.0
Range of hydrophobicity	—	−0.23→ +0.34	—	−0.52→ +0.57	—	—	−0.25→ +0.06
% with μmax $\geqslant$ 0.5	—	80	—	40	—	—	90

Short DNA fragments were generated by DNAse I digestion of the DNA encoding mouse DHFR and fused to the 5′-end of a gene encoding a truncated precursor of subunit IV of cytochrome *c* oxidase. The truncated subunit IV precursor lacks most of its presequence and by itself remains in the cytosol. The inserted DNA sequences used in the calculation encoded short peptides that, possibly together with the remainder of the authentic subunit IV presequence, formed functional matrix-targeting sequences (DHFR functional). The characteristics of such experimentally derived composite presequences were compared to a set of 30 theoretically derived random sequences (DHFR random). These random sequences were generated by taking short reading frames of comparable length to the selected ones, starting at randomly chosen points in the DHFR gene sequence. As with the selected sequences, the random sequences were derived from all six possible reading frames. The inserted sequences were analyzed by themselves (inserts), ie, only the amino acids derived from the DHFR gene, and as composite amino acid sequences including the seven amino acids from the residual presequence of subunit IV and two amino acids created at the fusion points of each construct by the cloning procedure (presequences) [see also 11]. Authentic DHFR, the calculated data for all possible open reading frames encoded in the DNA sequence; Average proteins, the average of a pool of 185 proteins taken from reference [34]. Natural presequences, the combined data for ten natural mitochondrial presequences [11]. For each presequence, μ_{max} is the calculated μ value of the ten-amino-acid segment of maximal hydrophobic moment. This value gives an indication of the potential amphiphilicity of the presequence, assuming an alpha-helical conformation [35]. The value for the hydrophobicity of each presequence was obtained by averaging the hydrophobicity of each segment of ten amino acids obtained by moving a ten-amino-acid window through the presequence [35]. The range of these values is indicated. The narrower the range, the more the fragments resemble each other in their hydrophobicity. Negative values, hydrophilic sequences; positive values, hydrophobic sequences. This table summarizes data presented in reference [11].

Roise et al [9], a natural amphiphilic presequence peptide inserts efficiently into lipid monolayers and bilayers and is able to interact with mitochondrial membranes. The functional artificial presequences are also surface active [8]. Thus, a clear correlation exists between the peptide's amphiphilic character and its function as a mitochondrial matrix-targeting sequence. A positively charged amphiphilic structure may therefore be an important characteristic of a functional matrix-targeting sequence.

MANY SEQUENCES IN EUKARYOTIC AND PROKARYOTIC DNA POTENTIALLY ENCODE MITOCHONDRIAL MATRIX-TARGETING SEQUENCES; THEY CAN BE ACTIVATED BY TRANSPOSITION IN FRONT OF A PROTEIN-CODING SEQUENCE

What is the evolutionary origin of presequences? According to the endosymbiont theory [reviewed in 10], mitochondria and chloroplasts developed from free-living bacteria that entered into symbiosis with other organisms. With time, many of the genes originally present in the bacterial genome were transferred to the nucleus, and the former bacterial genome evolved into the present-day mitochondrial or chloroplast genomes. After gene transfer, the nuclear-encoded proteins had to be reimported into the organelles from the cytosol and, therefore, had to acquire presequences.

Since presequences share only general characteristics, it was not unreasonable to expect that moderately complex genomes such as those of a protoeukaryotic cell or a present-day bacterium might encode many sequences that could function as mitochondrial presequences if placed at the amino terminus of a protein. This idea was indeed correct. Random DNA fragments of about 60–100 bases long were generated from the *Escherichia coli* genome or from the coding region for the mouse cytosolic enzyme dihydrofolate reductase (DHFR). These fragments were fused to the 5′-end of the gene encoding a truncated precursor of subunit IV of cytochrome *c* oxidase. This truncated subunit IV precursor lacked most of its presequence and, as a result, remained in the cytosol. Many DNA fragments were found that restored a functional presequence to the truncated subunit IV precursor. These presequences transported subunit IV into the yeast mitochondrial matrix [11]. Table I summarizes the characteristics of 20 presequences obtained this way from the DNA-encoding mouse DHFR. Most of the reading frames that restored a functional presequence were derived from DNA sequences that are normally not expressed in their natural environment [11]. As with many naturally occurring presequences, these presequences were low in acidic amino acids and neither extremely hydrophobic nor hydrophilic. All contained at least two positively charged residues. Many of these sequences were also predicted to be able to form an amphiphilic alpha-helix (Table I). Clearly, these sequences had general characteristics also found in naturally-occurring presequences. The primary sequences of these presequences are published elsewhere [11]. The functional presequences derived from the *E. coli* DNA had characteristics very similar to those listed in Table I [11].

X-ray crystallography data show that the mouse cytosolic enzyme DHFR contains an amphiphilic alpha-helix whose apolar face is not exposed to the outside of the folded molecule [12]. By gene-fusion experiments, Hurt and Schatz [12] placed this sequence at the amino terminus of either DHFR or of mature cytochrome oxidase subunit IV and showed that the resulting fusion proteins were specifically transported

into the mitochondrial matrix in living yeast cells [12]. Thus an amphiphilic helix within a folded protein can be made to function as a mitochondrial matrix-targeting presequence. We believe that many such sequences exist in eukaryotic and prokaryotic genomes, but that most of these sequences are not expressed, or if expressed are buried within the folded structure of proteins. However, such sequences represent a large pool of potential targeting sequences that can be activated by transposition and then refined by natural selection.

THE PRESEQUENCE OF A CHLOROPLAST PROTEIN FUNCTIONS AS A MATRIX-TARGETING SEQUENCE IN YEAST

The presequence of the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco, a chloroplast protein) is surprisingly similar to mitochondrial matrix-targeting sequences [13]. In plants, chloroplasts and mitochondria are present in the same cell and each of these organelles has a specific set of proteins. How similar are import sequences of chloroplast and mitochondrial precursor proteins and how are proteins sorted between these organelles? Hurt et al [13] fused part of the presequence of Rubisco to DHFR or to the mature subunit IV of cytochrome *c* oxidase. In yeast cells, the chloroplast presequence fragment transported both proteins into the mitochondrial matrix. Hurt et al [14] also showed that the mature part of the small subunit of Rubisco can be transported into the mitochondrial matrix by a mitochondrial presequence: it lacks a signal that would interfere with its transport into yeast mitochondria. Plants must have a system to distinguish between chloroplast and mitochondrial precursor proteins. How this distinction is made, however, is not known.

MITOCHONDRIAL PRESEQUENCES CONTAIN SEPARATE DOMAINS FOR INTRACELLULAR TARGETING AND FOR INTRAMITOCHONDRIAL SORTING

The presequence of cytochrome c_1 (an inner membrane protein exposed to the intermembrane space) and the amino terminus of the 70-kd outer membrane protein (a protein anchored in the mitochondrial outer membrane and exposed to the cytosol) contain potential matrix-targeting sequences at their amino termini: the first 12 amino acids of the 70-kd protein and the first 16 amino acids of the cytochrome c_1 presequence transport the mouse cytosolic enzyme DHFR into the mitochondrial matrix [15–17]. The entire cytochrome c_1 presequence (61 amino acids), however, transports DHFR and cytochrome *c* oxidase subunit IV, lacking its own presequence (mature subunit IV), into the intermembrane space [16–18], while the first 41 amino acids of the 70-kd protein anchor *E. coli* beta-galactosidase to the mitochondrial outer membrane [19]. Both transport sequences contain, in addition to a matrix-targeting sequence, a sequence responsible for intramitochondrial sorting [see also 4]. These sorting sequences each include a stretch of uncharged amino acids, which is 19 amino acids long for the cytochrome c_1 presequence [20] and 28 amino acids for the 70-kd protein [21]. Each of these stretches is long enough to span a lipid bilayer if an alpha-helical structure is assumed.

Deletions have been made in the cytochrome c_1 presequence to define the matrix-targeting and intramitochondrial sorting domains more precisely [17]. The results are summarized in Figure 1. The first 16 amino acids (the first quarter) of the

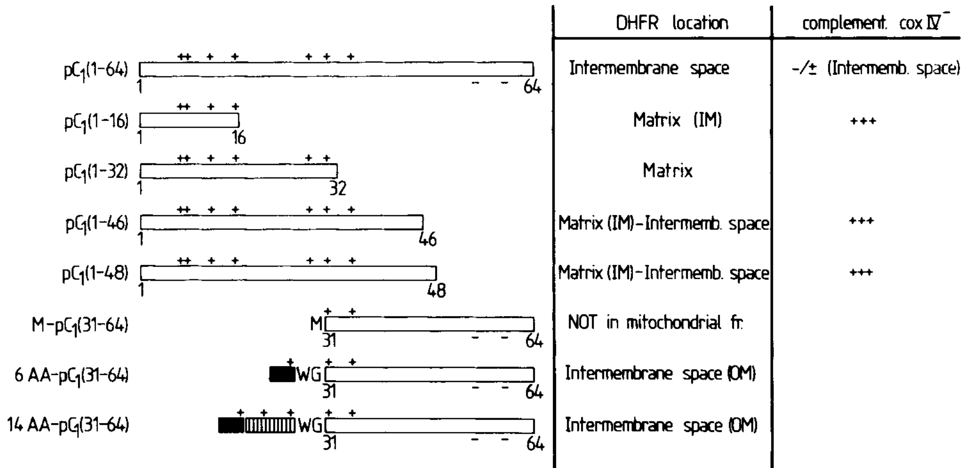

Fig. 1. The cytochrome c_1 presequence contains separate matrix-targeting and intramitochondrial sorting domains. By gene manipulation, parts of the cytochrome c_1 presequence (open boxes) were deleted or replaced by other sequences to determine the functions of these parts in intracellular and intramitochondrial sorting. Each of the sequences shown in the left panel was fused to the amino terminus of mouse DHFR. Some were also fused to the amino terminus of a cytochrome oxidase subunit IV protein lacking most of its own presequence. The intracellular and intramitochondrial locations of the fusion proteins were assayed in yeast cells expressing these proteins and/or after protein import into isolated mitochondria. The fusions to subunit IV were also tested for their ability to complement the growth deficiency of cells lacking the gene for the authentic subunit IV precursor (cox IV$^-$ cells). Thus far, all subunit IV proteins transported into the matrix complement the mutant's inability to grow on glycerol. The positions of basic (+) or acidic (−) amino acids are indicated. Filled box, amino acid sequence M-L-S-R; hatched box, amino acid sequence L-S-L-R-L-L-S-R. 6AA and 14AA, artificial matrix-targeting sequences containing 6 and 14 amino acids, respectively. Matrix (IM), protein bound to inner membrane but exposed to the matrix; intermembrane space (OM), mature-sized DHFR in intermembrane space and uncleaved precursor at the outer surface of the outer membrane. This figure summarizes data presented in references [16] and [17].

presequence transport attached DHFR or mature subunit IV efficiently to mitochondria and into the matrix: it is a matrix-targeting sequence. The second half of the cytochrome c_1 presequence by itself (starting at amino acid 31) does not contain sufficient information for transporting an attached protein to mitochondria: it lacks information for intracellular targeting. The last quarter of the presequence (starting at amino acid 49) is essential for intramitochondrial sorting. Our data suggest that the entire stretch of uncharged amino acids (amino acids 36–54) may be part of the intramitochondrial sorting domain. The matrix-targeting and intramitochondrial sorting functions are, thus, located in different parts of the cytochrome c_1 presequence.

The first half of the cytochrome c_1 presequence (30 amino acids) can be replaced by artificial matrix-targeting sequences as short as six amino acids without abolishing transport of attached DHFR into the intermembrane space (Fig. 1). This shows that the first 30 amino acids of the authentic cytochrome c_1 presequence do not contain a hidden sequence motif which acts together with the second half of the presequence in intramitochondrial sorting.

THE INTRAMITOCHONDRIAL SORTING SEQUENCE IN THE CYTOCHROME c_1 PRESEQUENCE IS A STOP-TRANSPORT SEQUENCE SPECIFIC FOR THE INNER MEMBRANE

How does the intramitochondrial sorting sequence in the cytochrome c_1 presequence work? Is it a stop-transport sequence specific for the inner membrane as suggested [4,20]? If so, then the attached protein should cross the mitochondrial outer, but not the inner, membrane. Alternatively, the precursor protein may first be completely transported into the mitochondrial matrix by the matrix-targeting sequence and subsequently be reexported into the intermembrane space. This latter model was recently invoked [22] for the iron-sulphur protein precursor of the *Neurospora crassa* cytochrome bc_1 complex. This protein, like cytochrome c_1, is assembled into the transmembrane cytochrome bc_1 complex in such a way that it is partly exposed toward the intermembrane space [22].

To test which pathway is followed by the cytochrome c_1 presequence, we compared the import of (1) a fusion protein between the cytochrome c_1 presequence and mature subunit IV of cytochrome c oxidase (pC_1 (1–64)-COX IV), (2) the authentic subunit IV precursor, and (3) the authentic yeast iron-sulphur protein precursor, into both mitochondria and mitoplasts (mitochondria in which the outer membrane has been disrupted). Both the authentic subunit IV precursor (not shown) and iron-sulphur protein precursor (Fig. 2, bottom panel) and their cleavage intermediates became protease resistant in mitochondrial and mitoplast import: they were transported across the inner membrane. In contrast, the pC_1 (1–64)-COX IV precursor and its cleavage intermediate became protease resistant upon import into mitochondria but not upon import into mitoplasts (Fig. 2, top panel), although mitoplasts generated as much of the intermediate as mitochondria did [18]. Thus, the iron-sulphur precursor and the pC_1 (1–64)-COX IV precursor use different pathways to reach the intermembrane space. The intramitochondrial sorting sequence in the cytochrome c_1 presequence indeed behaves as a stop-transfer sequence specific for the inner membrane.

TRANSPORT OF PROTEINS INTO MITOCHONDRIA REQUIRES ATP AS WELL AS AN ENERGIZED INNER MEMBRANE

Transport into chloroplasts and the endoplasmic reticulum, and protein export from bacteria, requires ATP. Is ATP also required for protein import into mitochondria? To test this, a fusion protein consisting of the first 22 amino acids of the subunit IV presequence fused to DHFR was purified to near homogeneity (pCOX IV(1–22)-DHFR) [23]. The availability of a radiochemically pure precursor protein allowed the study of protein import in the absence of a cell-free protein synthesizing system and, thus, of ATP. Efficient import into isolated mitochondria was obtained if the respiratory substrates succinate and malate were used for generating the membrane potential and for ATP synthesis (Fig. 3, lanes 2 and 3). If ATP synthesis and transport of ATP into the matrix were specifically inhibited with oligomycin (an inhibitor of the mitochondrial ATPase) and carboxyatractyloside (an inhibitor of adenine nucleotide translocation across the mitochondrial inner membrane), no import was observed (Fig. 3, lanes 6 and 7). Addition of ATP (Fig. 3, lanes 8 and 9) or GTP, but not of

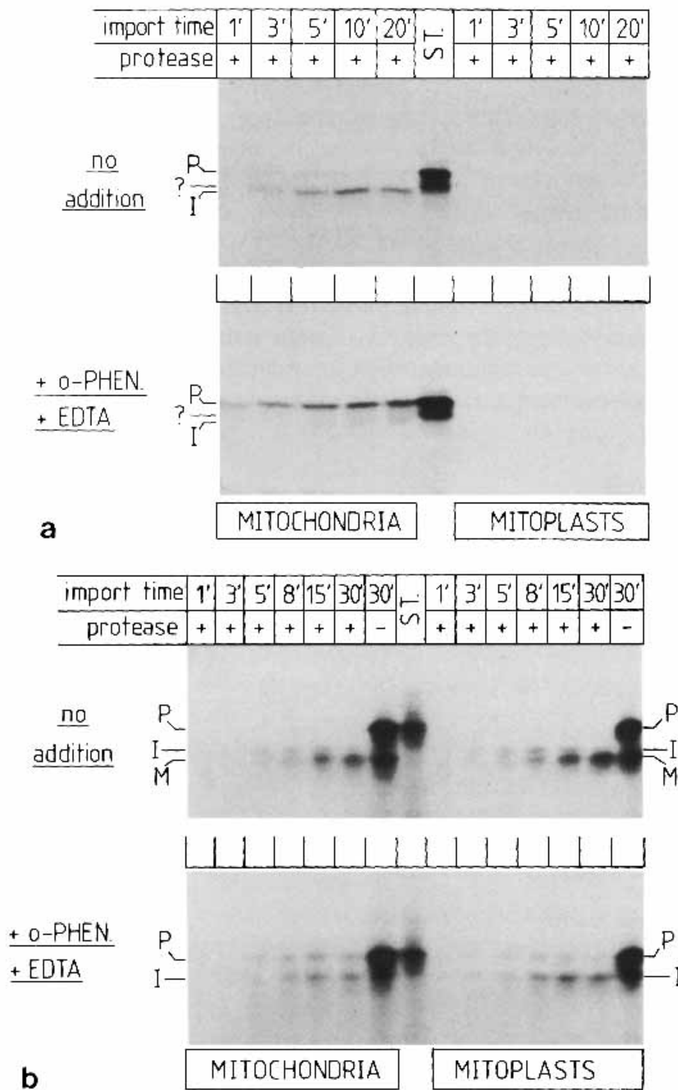

Fig. 2. The intramitochondrial sorting sequence in the cytochrome c_1 presequence is a stop-transport sequence specific for the inner membrane. The radiolabeled precursor of the iron-sulphur protein of the cytochrome bc_1 complex (bottom panel) or the pC_1(1–64)-COX IV fusion protein (top panel) were incubated with energized mitochondria or mitoplasts (mitochondria whose outer membranes have been broken) either in the absence (no addition) or the presence of ortho-phenanthroline plus EDTA (+o-PHEN. +EDTA) to inhibit the matrix-located processing protease. At the indicated times, samples were taken and diluted into ice-cold buffer containing various inhibitors that interfere with the functions of the respiratory chain, the mitochondrial ATPase, and the matrix-located processing protease. Where indicated, the buffer also contained trypsin to remove all proteins bound to the mitochondrial surface or outside the inner membrane in mitoplasts. After incubation, the organelles were reisolated and analyzed for the presence of radiolabeled proteins by SDS-polyacrylamide gel electrophoresis and fluorography. ST., 20% of the radiolabled precursor added to each of the other incubations; P, precursor; I, cleavage intermediate; M, mature-size iron-sulphur protein; ?, an unknown radiolabeled contaminant present in this particular preparation of the pC_1(1–64)-COX IV precursor. This contaminant does not bind to mitochondria and its electrophoretic mobility is distinctly lower than that of the cleavage intermediate. This figure summarizes data presented in reference [18].

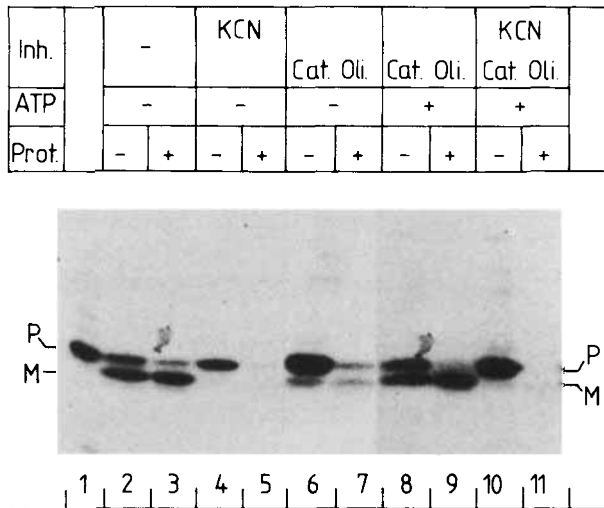

Fig. 3. Import of a purified precursor protein into mitochondria requires both a membrane potential and ATP. Purified radiolabeled pCOX IV(1–22)-DHFR fusion protein was incubated with isolated yeast mitochondria in the presence of the respiratory substrates succinate and malate, and, where indicated, with ATP and/or the inhibitors (inh.) mentioned below. Mitochondria were either left untreated (lanes 2, 4, 6, 8, 10) or treated with proteinase K (Prot.) after import (lanes 3, 5, 7, 9, 11), to digest all proteins bound to the mitochondrial surface. The mitochondria were then reisolated by centrifugation and analyzed for the presence of radiolabeled proteins by SDS-polyacrylamide gel electrophoresis and fluorography. Lane 1 contains a standard corresponding to 10% of the amount of precursor added to each of the other incubations. Cat., carboxyatractyloside (an inhibitor of adenine nucleotide translocation across the mitochondrial inner membrane); Oli., oligomycin (an inhibitor of the mitochondrial ATPase); P, precursor; M, mature-size DHFR protein. This figure summarizes data presented in reference [24].

TTP, CTP, or a noncleavable ATP analogue, restored import [24]. The restoration of import by ATP could not be explained by an effect on the membrane potential: ATP also supported import if it was added in the presence of oligomycin and carboxyatractyloside (Fig. 3, lanes 8 and 9). In the absence of a membrane potential, ATP did not support protein import: inhibition of respiration by cyanide blocked import in the absence and in the presence of ATP (Fig. 3, lanes 4, 5, 10, and 11). Similar results were presented recently by Pfanner and Neupert [25] and Chen and Douglas [26]. Differences were only observed in the nucleotides that could replace ATP. These results all demonstrate that, in addition to the membrane potential, ATP is required for transporting proteins across mitochondrial membranes.

TRANSPORT OF PROTEINS ACROSS MITOCHONDRIAL MEMBRANES CAN BE INHIBITED BY STABILIZING THE PROTEIN'S STRUCTURE

Many precursor proteins can be imported into mitochondria post-translationally [reviewed in 1]. What is the structure of the precursor protein prior to its transport into mitochondria? The purified pCOX IV(1–22)-DHFR protein is enzymatically active and has K_m values very similar to that of authentic DHFR without a presequence [23]. This purified protein is efficiently imported by isolated mitochondria. Mouse DHFR found in yeast mitochondria subsequent to import is enzymatically active [7,15–17]. The crystal structure of the highly homologous chicken DHFR

shows that the enzyme has a very stable globular structure. Is this structure maintained during transport across the membranes? To answer this question, import of various DHFR-containing precursors into isolated mitochondria was studied. Import of each of these precursors could be inhibited by methotrexate (Fig. 4, top panel shows this for a fusion protein between the first 27 amino acids of the ADHIII precursor and DHFR). This inhibitor binds very tightly to the active site of DHFR and "freezes" the folded conformation of the protein. With the purified pCOX IV(1–22)-DHFR protein, inhibition of import and DHFR enzyme activity was observed with similar methotrexate concentrations (not shown). The inhibition was specific for DHFR-containing fusion proteins and was not observed for several other imported proteins (Fig. 4, bottom panel shows this for the authentic pADHIII precursor). The inhibition

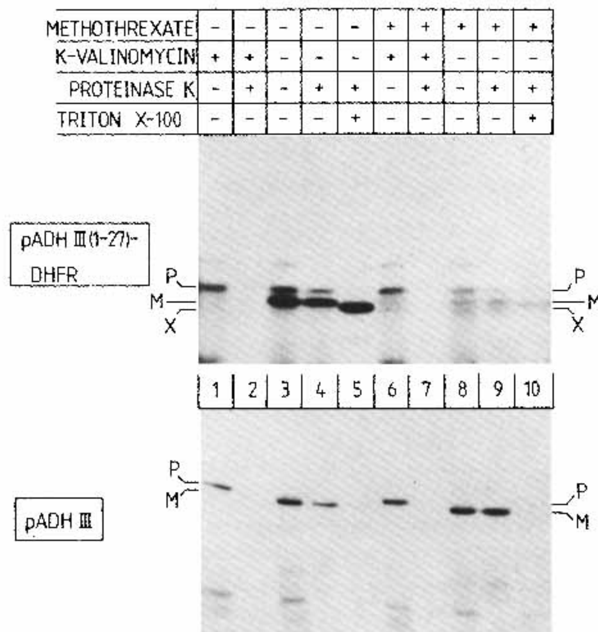

Fig. 4. Methotrexate inhibits the import into isolated mitochondria of the pADHIII(1–27)-DHFR fusion protein but not that of the authentic pADHIII precursor. The pADHIII(1–27)-DHFR fusion protein (top panel) and the authentic pADHIII precursor (bottom panel) were synthesized by transcription-translation in the presence of ^{35}S-methionine. The radiolabeled precursors were incubated with energized mitochondria (lanes 3–5 and 8–10) or with mitochondria deenergized with valinomycin and KCl (lanes 1, 2, 6, 7). The incubation was done in the absence (lanes 1–5) or presence (lanes 6–10) of methotrexate, an inhibitor of DHFR which binds tightly to the enzymatically active protein. After incubation, some samples were treated with proteinase K in the absence (lanes 2, 4, 7, 9,) or presence of the detergent Triton X-100 (lanes 5, 10). The mitochondria were then reisolated by centrifugation and tested for the presence of radiolabeled proteins by SDS-polyacrylamide gel electrophoresis and fluorography. P, precursor; M, mature-size protein. Band X is a proteinase K-resistant degradation product of DHFR which is of distinctly lower molecular weight than imported "mature-sized" DHFR. It is not generated upon protase treatment of intact mitochondria but only upon protease treatment of detergent-lysed mitochondria [16–18, 23, 24, 31]. Band X is also generated if a purified, DHFR-containing precursor protein is treated at low protease concentrations in the absence of mitochondria. Further digestion of band X then requires high protease concentrations (T. Endo and S. Hwang, unpublished). This figure summarizes data presented in reference [23].

of import is not the result of the inhibitor's inability to be transported across membranes. An apolar inhibitor, which by itself can freely cross membranes, also inhibited import of DHFR-containing precursor molecules [23]. Thus, the protein may have to undergo a structural change and at least partially unfold in order to move across the mitochondrial membranes.

ATP MAY BE NEEDED TO CHANGE PRECURSOR STRUCTURE DURING MEMBRANE TRANSPORT

Is there a correlation between the requirement for ATP cleavage and the change in the protein's structure during transport across the membrane? It is tempting to speculate that ATP may be needed for changing the compact DHFR molecule into a transport-competent structure. To test this hypothesis, the pCOX IV(1–22)-DHFR protein was synthesized in a cell-free protein-synthesizing system and cycloheximide was added after a few minutes of protein synthesis to generate a collection of growing peptide chains. These polypeptides were shown to be nascent chains of the pCOX IV(1–22)-DHFR fusion protein [27]. The nascent chains were separated from the cell-free system (which contains high concentrations of ATP to support protein synthesis) by pelleting the ribosomes, washing them once in the presence of an ATP trap, and then releasing the nascent chains by dissociation of the ribosomes with EDTA. The ATP-depleted nascent chains were then mixed with the purified pCOX IV(1–22)-DHFR precursor, and import of the ATP-depleted protein mixture into isolated mitochondria was studied in the presence or absence of added ATP [27]. Figure 5 shows that import of the full-length precursor is highly ATP-dependent: imported, mature-size protein is formed only if ATP was present during protein import. In contrast, most but not all of the lower-molecular-weight polypeptides were still imported into mitochondria if no ATP was added. The import efficiency of nascent chains, irrespective of whether ATP was added or not, was much higher than that of the full-length precursor protein: for the precursor protein 5–10% but for the nascent chain polypeptides much more than 20% of the total amount added to mitochondria was imported. Thus, import of nascent precursor chains into mitochondria is highly efficient and only slightly, if at all, ATP-dependent. Additional experiments [27] showed that the nascent chains, in contrast to the completed protein, were highly sensitive to protease in the absence of mitochondria. This suggests that they did not have the compact, protease-resistant structure characteristic of the completed pCOX IV(1–22)-DHFR precursor. These experiments demonstrate a correlation between lack of stable structure and lower ATP requirement for protein import into isolated mitochondria, suggesting that ATP is needed for protein unfolding.

PROTEIN IMPORT INTO ISOLATED MITOCHONDRIA IS INHIBITED BY ANTIBODIES RAISED AGAINST A SPECIFIC SUBSET OF OUTER MEMBRANE PROTEINS

Are accessory proteins required for transport of precursors into mitochondria? The existence of such proteins has been inferred from observations that treatment of the mitochondrial surface with different proteases abolished protein import without disrupting the outer membrane [28–30]. This approach, however, is not suited for isolation proteins of a putative translocation machinery. Ohba and Schatz [31] recently showed that the combination of a mild protease pretreatment of mitochondria and

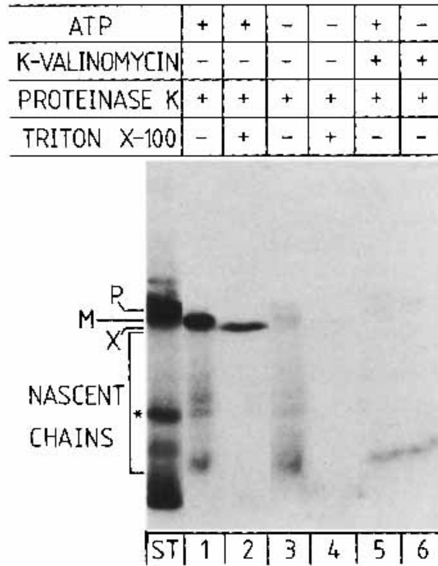

Fig. 5. Only completed, but not nascent chains of the pCOX IV(1–22)-DHFR fusion protein require added ATP for import into isolated yeast mitochondria. Radiolabeled nascent chains of the pCOX IV(1–22)-DHFR precursor were isolated and mixed with the radiolabeled, purified, completed pCOX IV(1–22)-DHFR precursor. The mixture was incubated with energized yeast mitochondria (lanes 1–4) or mitochondria deenergized with valinomycin and KCl (lanes 5, 6). All incubations were done in a shaking waterbath in the presence of the respiratory substrate succinate to generate a membrane potential. Efrapeptin (an inhibitor of the mitochondrial ATPase) was added to each incubation to inhibit formation of ATP by oxidative phosphorylation. Mitochondria were then incubated with proteinase K to remove all proteins bound to the mitochondrial surface (lanes 1, 3, 5, 6). Protease treatment was sometimes done in the presence of Triton X-100 (lanes 2, 4). Each sample was analyzed by SDS polyacrylamide gel electrophoresis and fluorography. ST, 20% of the amount of nascent chains and completed presursor protein added to each of the other incubations; P, precursor; M, mature-size protein; X, proteinase K-resistant digestion product of DHFR (see legend to Fig. 4); band marked with (*), radiolabeled contaminant present in this preparation of the purified full-length precursor protein. The nature of the lower-molecular-weight band present in lanes 5 and 6 is not known. This figure summarizes data presented in reference [27].

incubation with Fab fragments of an antiserum directed against the 45-kd proteins of the mitochondrial outer membrane virtually abolished import of the purified pCOX IV(1–22)-DHFR protein into isolated mitochondria. This import block was not observed with Fab fragments of antibodies directed against other outer membrane proteins. The import block was not specific for the pCOX IV(1–22)-DHFR fusion protein; it was also observed for the pADHIII(1–27)-DHFR precursor or the authentic subunit IV precursor. The inhibitory effects of anti-45-kd antibodies was abolished by preincubating them with purified yeast outer membranes. These experiments suggest that a 45-kd protein of the yeast mitochondrial outer membrane participates in the translocation of proteins into mitochondria. This observation now makes it possible to start a detailed study of the putative protein receptor(s) and translocation machinery. The machinery responsible for the transmembrane transport still is the major "black box" in the study of protein translocation into mitochondria. It seems reasonable to expect that our knowledge of the machinery will develop rapidly in the

next few years, especially since several yeast mutants have been isolated [32,33] which are (partially) blocked in protein transport into mitochondria.

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