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Mutations in a 19-Amino-Acid Hydrophobic Region of the Yeast Cytochrome c_1 Presequence Prevent Sorting to the Mitochondrial Intermembrane Space

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Most mitochondrial proteins destined for the intermembrane space (IMS) carry in their presequence information for localization to the IMS in addition to information for their import. By selecting for mutants in the yeast *Saccharomyces cerevisiae* that mislocalize an IMS-targeted fusion protein, we identified mutations in the IMS sorting signal of the cytochrome c_1 protein. Amino acid substitutions or deletions in a stretch of 19 hydrophobic amino acids of the cytochrome c_1 presequence resulted in accumulation of the intermediate form of the cytochrome c_1 protein in the matrix. In some cases, the accumulated intermediate appeared to be slowly exported from the matrix, across the inner membrane to the IMS. Our results support the hypothesis that the cytochrome c_1 precursor is normally imported completely into the matrix and then exported to the IMS.

Most mitochondrial proteins are encoded by nuclear genes, synthesized in the cytoplasm, and imported into the mitochondria (1, 13). Proteins imported into mitochondria are specifically targeted to one of the four mitochondrial compartments: matrix, inner membrane, intermembrane space (IMS), and outer membrane. Most proteins destined for the three interior compartments of the mitochondria are synthesized with amino-terminal presequences, which contain the information necessary to target proteins to the mitochondria (18, 20, 21, 44).

Mitochondrial precursors are imported into the matrix via a multistep pathway that includes binding to specific receptors on the mitochondrial surface, translocation across both mitochondrial membranes, and removal of the presequence by a processing protease located in the matrix. Proteins destined for mitochondrial compartments other than the matrix often carry additional information at their amino termini. For example, the cytochrome c_1 protein is exposed to the IMS and anchored in the inner membrane by a carboxy-terminal hydrophobic domain (25). The cytochrome c_1 precursor carries a 61-amino-acid presequence that is processed twice during its import into mitochondria (10, 31, 36). The first cleavage is catalyzed by the matrix-localized processing protease; the second cleavage, which depends on prior addition of heme to the cytochrome c_1 protein, is catalyzed by a protease presumably located in the IMS (10, 31).

Two models have been proposed for the import of cytochrome c_1 (Fig. 1). Model A was based on the finding that full-length precursor and intermediate forms of cytochrome c_1 fusion proteins were never found completely in the matrix during import into isolated mitochondria (45). Furthermore, deletion of the carboxy-terminal third of the presequence, which includes 19 uncharged amino acids, caused cytochrome c_1 fusion proteins to accumulate in the matrix (43). Hence, van Loon and Schatz (45) proposed that the hydrophobic amino acids of the cytochrome c_1 presequence act as a stop-transfer sequence, preventing complete translocation of the cytochrome c_1 protein across the inner membrane. In contrast, model B represents a fundamentally different mechanism for sorting. Hartl et al. (14) found that the entire *Neurospora* cytochrome c_1 precursor accumulated inside the inner membrane if cytochrome c_1 was imported into mitochondria incubated at low temperatures and if the matrix-localized processing protease was inhibited. By raising the temperature, the accumulated precursor was chased to the mature form in the IMS. In model B, the second part of the cytochrome c_1 presequence is proposed to act as an export signal that directs the cytochrome c_1 protein across the inner membrane to the IMS (14, 15).

To distinguish between these two models and to dissect the IMS sorting signal, we have isolated yeast mutants that are defective in targeting a cytochrome c_1 fusion protein. By using a protein that carries the presequence of cytochrome c_1 fused to the mature portion of a matrix protein, cytochrome oxidase subunit IV, we have isolated mutants defective in IMS sorting. Mutations in a stretch of 19 hydrophobic amino acids of the cytochrome c_1 presequence cause accumulation of the fusion protein as an intermediate in the matrix. These mutations also prevent the sorting of the bona fide cytochrome c_1 protein. The intermediate forms of some of these altered cytochrome c_1 proteins appeared to be slowly exported across the inner membrane to the IMS. Hence, our results support the hypothesis that cytochrome c_1 is normally imported completely into the matrix and subsequently sorted to the IMS as diagrammed in Fig. 1, model B.

MATERIALS AND METHODS

Yeast strains, media, and genetic techniques. Strains RM2-1b (*MAT***a** *ura3 leu2 trp1 cox4::LEU2*), RM2-2c (*MAT***a** *ura3 leu2 trp1 cox4::LEU2*), and RM4-12d (*MAT*α *ura3 leu2 his3 cox4::LEU2*) were obtained by crossing strain WDIV (27) with strain SM1058 (19). In strain GDIV, the coding sequences for COX4 were replaced by LEU2 sequences. RJ300-1D (*MAT***a** *ura3 lys2 trp1 his3 leu2 cyt1::LEU2*) was constructed as follows. A 4-kbp *Bam*HI-*Sal*I fragment carrying the cytochrome c_1 gene was excised from plasmid

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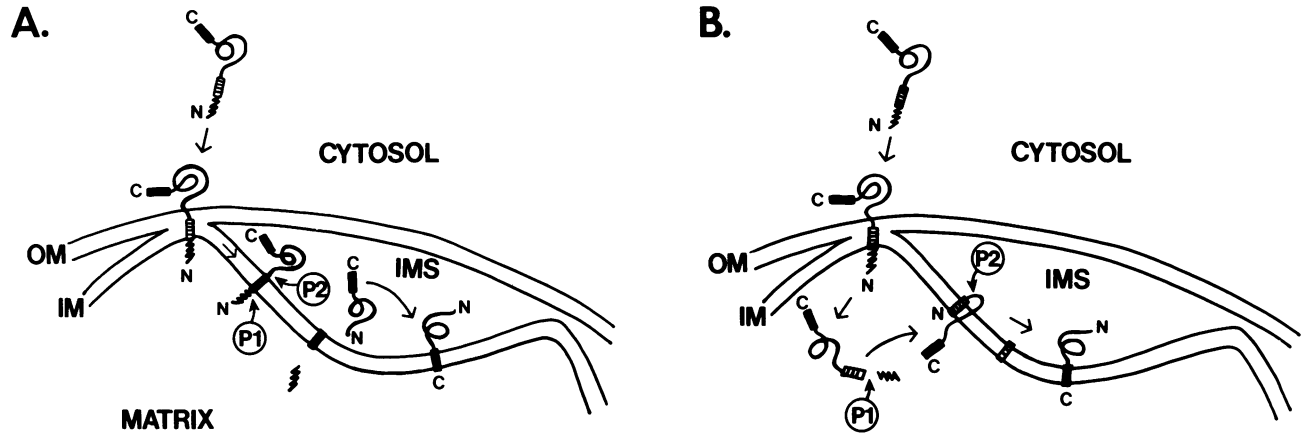

FIG. 1. Two models for sorting cytochrome c_1 to the IMS. The zigzag line denotes the matrix targeting signal, the hatched box indicates the IMS sorting signal, and the black box denotes the membrane anchor of the cytochrome c_1 protein. (A) The cytochrome c_1 protein is prevented from complete import into the matrix by a stop-transfer sequence (45). Processing by the IMS-localized processing protease (P2) releases the cytochrome c_1 protein into the IMS. After sorting to the IMS, the carboxy terminus of cytochrome c_1 inserts into the inner membrane. (B) Cytochrome c_1 is imported into the matrix, processed to the intermediate form by the matrix-localized processing protease (P1), and exported across the inner membrane to the IMS, where the IMS-localized protease (P2) acts (14). Complete translocation into the IMS is prevented by a stretch of hydrophobic amino acids in the carboxy terminus of cytochrome c_1.

YEp13-C1 (a gift from I. Sadler) and subcloned into vector pEMBL8 (7) to form plasmid pEMBL8-C1. Cytochrome c_1 coding sequences were removed by digesting pEMBL8-C1 with *Eco*RV and *Bgl*II. After the DNA ends were filled in with DNA polymerase, a 2.2-kbp DNA *Sal*I-*Xho*I fragment carrying the yeast *LEU2* gene (whose DNA ends were also filled in with DNA polymerase) was blunt-end ligated to form pEMBL8-C1::LEU2. A 5-kbp *Bam*HI-*Sal*I DNA fragment, carrying the *cyt1::LEU2* gene, was used to replace the wild-type cytochrome c_1 gene of strain YPH250 (40) as described previously (34). Yeast medium, genetic techniques, and ethyl methanesulfonate mutagenesis were as described previously (39).

Construction of plasmids that carry C1-COX4 and cytochrome c_1. Three different plasmids capable of expressing a C1-COX4 fusion protein in yeast cells were constructed as follows. For construction of pRJ618, a 0.7-kbp *Xba*I-*Hin*dIII fragment containing the cytochrome oxidase subunit IV (COX4) coding sequence, but lacking the first 24 amino acids of the presequence, was isolated from pSP65-COX4 (obtained from D. Allison) and subcloned into vector pUC18 (50) to form pUC18-mCOX4. The *COX4* gene was excised from PUC18-mCOX4 on a 0.7-kbp *Eco*RI-*Hin*dIII fragment and inserted into *URA3 CEN3* vector pAC1 (43), downstream of the *ADH1* promoter region (pAC1-mCOX4). A 260-bp *Dde*I-*Pvu*II fragment, containing the presequence of cytochrome c_1, was excised from YEp13-C1, the DNA ends were filled in with DNA polymerase, and the fragment was inserted into *Eco*RI-*Bam*HI-cut pAC1-mCOX4, whose ends were also filled in with DNA polymerase. Blunt-end ligation of a filled-in *Dde*I site to a filled-in *Eco*RI site regenerated the *Eco*RI site. Similarly, a *Pvu*II site ligated to a filled-in *Bam*HI site regenerated the *Bam*HI site. The final construction, called pRJ618, contains the first 64 amino acids of cytochrome c_1 fused in frame to the COX4 protein. Two amino acids (Glu and Pro) are inserted between cytochrome c_1 and COX4 sequences because of the polylinker. The C1-COX4 fusion protein is expressed from the yeast *ADH1* promoter. For construction of pRJ619, a 1-kbp *Hin*dIII-*Dra*I fragment was isolated from pRJ618 and subcloned into *Sma*I-*Hin*dIII-cut vector pRS316 (40) to form pRJ617. pRS316 carries the yeast *URA3* gene and contains a centromere, *CEN6*. Subsequently, a 0.6-kbp *Hin*dIII fragment containing the first 20 amino acids of cytochrome c_1 and 400 bp of upstream sequences was inserted into the *Hin*dIII site of pRJ617 to form vector pRJ619. The *C1-COX4* gene is expressed in pRJ619 from cytochrome c_1 sequences. For construction of pRJ620, a 2.4-kbp *Sal*I-*Not*I fragment containing the *ADH1-C1-COX4* gene was isolated from pRJ618 and inserted into *Sal*I-*Not*I-cut vector pRS416 (40) to form pRJ620. pRS416 contains the yeast *URA3* gene and a replication origin from the yeast 2μm plasmid. Consequently, pRJ620 expresses C1-COX4 from the strong *ADH1* promoter, and the plasmid is present in multiple copies in yeast cells. Standard methods were used for restriction enzyme digests, DNA isolations, plasmid constructions, transformation of bacteria with plasmid DNA, and plasmid DNA isolations (26). Lithium acetate yeast transformations were as described previously (38).

For in vitro transcriptions, the wild-type and mutant *C1-COX4* genes were subcloned into pSP65 (28) as follows. First, plasmid pRJ618 was digested completely with *Eco*RI and partially with *Hin*dIII. A 1.2-kbp *Eco*RI-*Hin*dIII fragment containing wild-type C1-COX4 was isolated and inserted into *Eco*RI-*Hin*dIII-cut pSP65 to form pSP65-C1-COX4. All of the mutations identified in this study were found to reside within a 1-kbp *Hin*dIII fragment that carries *COX4* and 22 amino acids of cytochrome c_1. Therefore, pSP65 constructs capable of expressing the different *C1-COX4* mutations were constructed by replacing the 1-kbp *Hin*dIII fragment from pSP65-C1-COX4 with the 1-kbp *Hin*dIII fragment isolated from the *C1-COX4* mutations carried on pRJ618 or pRJ619.

Cytochrome c_1 constructions. A plasmid capable of expressing the intact cytochrome c_1 gene from the *ADH1* promoter was constructed as follows. A 1.2-kbp *Hin*dIII fragment containing COX4 sequences (and 22 amino acids of cytochrome c_1) was replaced in pRJ618 by a 3-kbp *Hin*dIII fragment from YEp13-C1, carrying all but the first 42 amino acids of cytochrome c_1. This plasmid, pAC1-C1, expressed

wild-type cytochrome c_1 in yeast cells and complemented the *cyt1*::*LEU2* mutation described above. Mutations identified in *C1-COX4* were reconstituted onto the intact cytochrome c_1 gene as follows. The C1-COX4 presequence carried on pSP65-C1-COX4 was amplified by using specific oligonucleotides and the polymerase chain reaction (PCR) (37). In particular, with oligonucleotide 1 (5'-CGATTTAG GTGACACTATAG-3') and oligonucleotide 2 (5'-CTGCG GTCATAGCTTCGG-3'), a 365-bp fragment was synthesized from different *C1-COX4* genes with an AmpliTaq polymerase kit (Perkin-Elmer Cetus) by following manufacturer's instructions. The DNA fragments were isolated, digested with *Eco*RI, and inserted into vector pAC1-C1, which was digested with *Eco*RI and *Pvu*II. In this way the G45D, A42P, A48P, Δ2, and Δ10 mutations were reconstituted onto the cytochrome c_1 gene. ΔC, a precise deletion of the 19 hydrophobic amino acids in the cytochrome c_1 presequence, was constructed as follows. Oligonucleotide 1 and oligonucleotide B (5'-CTTCGGCAGTTAATGAGTCCTTT TGAGTAAGCTTGC-3') were used to amplify the amino-terminal region of the cytochrome c_1 gene (lacking the 19 hydrophobic amino acids) from pSP65-C1 by PCR. Oligonucleotide C (5'-GGACTCATTAACTGCC-3') and oligonucleotide D (5'-GGTCTAGACAGCAGTATCTCAGTAC-3') were used to amplify the carboxy-terminal portion of cytochrome c_1. Aliquots of the two PCR products were mixed, the entire cytochrome c_1 gene was amplified with oligonucleotides 1 and D. The PCR product was digested with *Eco*RI and *Pvu*II and inserted into pAC1. For in vitro transcriptions, the different cytochrome c_1 genes were isolated on 2.5-kbp *Eco*RI-*Bgl*II fragments and inserted into the *Eco*RI-*Bam*HI site of pSP65.

DNA sequencing of C1-COX4 plasmids. Plasmid DNA was isolated from yeast cells and used to transform bacteria as described previously (16). Small-scale plasmid DNA preparations from bacteria were as described previously (17), except that the DNA pellets were resuspended in 20 μl of water. Double-strand sequencing of the cytochrome c_1 region of different plasmids was as follows. Eighteen microliters of plasmid DNA was denatured with 2 μl of 2 N NaOH for 5 min at room temperature. Denatured DNA was precipitated by addition of 20 μl of 5 M ammonium acetate, pH 5.4, and 200 μl of ethanol. Precipitated DNA was collected by centrifugation in a microcentrifuge for 10 min. The DNA pellet was resuspended in 8 μl of annealing buffer (50 mM NaCl; 10 mM Tris-HCl, pH 7.5; 5 mM $MgCl_2$), and 2 μl (approximately 50 ng) of a single-stranded sequencing primer was added to the DNA. The primer was annealed to the plasmid DNA by heating the reaction mix at 65°C for 5 min and then incubating it at 37°C for at least 30 min. DNA sequencing was carried out by using a Sequenase kit (U.S. Biochemicals) according to manufacturer's instructions. Two different oligonucleotides, COX4#1 (5'-GCTGCCGCA GATATC-3') and COX4#2 (5'-GCACCAGGACCAA TC-3'), were used to sequence the entire cytochrome c_1 presequence and the amino-terminal region of the *COX4* gene.

Cellular labeling and immunoprecipitation. Yeast cells were labeled and extracted proteins were immunoprecipitated as described previously (49), with the following modifications. Trichloroacetic acid pellets were resuspended in 100 μl of STE buffer (1% sodium dodecyl sulfate [SDS]; 50 mM Tris-HCl, pH 7.5; 1 mM EDTA) and heated to 100°C for 5 min. Supernatants were cleared by centrifugation at 12,500 × *g* for 10 min and diluted into 1 ml of TNET solution (1% Triton X-100; 150 mM NaCl; 25 mM EDTA; 50 mM Tris-HCl, pH 7.5). One hundred microliters of a 3.5% solution of preswelled protein A-Sepharose CL4-B (Pharmacia) in TBA buffer (10 mM Tris-HCl, pH 7.5; 1% bovine serum albumin; 1 mM sodium azide) was added to the supernatant, and the supernatant shaken for 30 min at room temperature. Protein A beads were removed by centrifugation, and 10 μl of specific antiserum was added to the supernatant. After incubation for 1 h at 23°C, 100 μl of protein A beads was added and the tube was shaken at 23°C for 30 min. The protein A beads were collected by centrifugation, and the immunoprecipitated proteins were extracted twice in 75 μl of 2× sample buffer (24) for 5 min at 100°C. Immunoprecipitates were separated by SDS-polyacrylamide gel electrophoresis (PAGE) (11), and labeled proteins were quantitated by scanning fluorographs (3) with a model SZR-1 densitometer from BioMed Instruments, Inc.

Import into isolated mitochondria. Mitochondria were isolated from D273-10D as described previously (6). Mitochondrial genes in pSP65 plasmids were transcribed by using SP6 polymerase (28), and RNA was translated by using rabbit reticulocyte lysate (Promega Biotech) according to manufacturer's instructions in the presence of 10 mCi of [^{35}S]methionine (New England Nuclear) per ml. Imports of radiolabeled precursors into mitochondria were as described previously (20, 22), except that 1 mM potassium succinate, 1 mM potassium malate, and 1 mM NADH were added to the import buffer. After import, mitochondrial pellets were isolated by centrifugation (12,500 × *g* for 10 min). To examine the location of the imported proteins, mitochondria were converted to mitoplasts by addition of 9 volumes of 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-KOH, pH 7.4; addition was followed by incubation on ice for 20 min. Proteinase K digestions (250 μg/ml) were carried out for 30 min, and then 1 mM phenylmethylsulfonyl fluoride was added to inhibit the protease. Mitochondria or mitoplasts were solubilized in 2× sample buffer and heated to 100°C for 5 min prior to SDS-PAGE.

Miscellaneous. Antiserum to subunit IV of cytochrome oxidase was obtained as follows. A 1.0-kbp *Xba*I-*Hin*dIII fragment from pSP65-COX4 containing most of the COX4 coding sequences was inserted into the *lacZ* expression vector, pTRB1 (2). The lacZ-COX4 fusion protein was expressed in bacteria and purified by SDS-PAGE as described previously (48). Antiserum to the fusion protein was obtained from Hazleton Custom Antiserum, Inc. (Denver, Pa.). Oligonucleotides were synthesized on an Applied Biosystems synthesizer at a facility at The Johns Hopkins University School of Medicine.

RESULTS

Isolation of mutations in the signal that targets cytochrome c_1 to the IMS. We established a genetic selection for IMS sorting mutants based on the work of van Loon et al. (43, 45). Specifically, when the 61-amino-acid presequence of the cytochrome c_1 protein (C1) is fused to the mature portion of the matrix protein, cytochrome oxidase subunit IV (COX4), the COX4 subunit is targeted to the IMS. *cox4*::*LEU2* strains expressing this fusion protein are unable to grow on glycerol as the sole carbon source because COX4 is mislocalized to the IMS. We reasoned that mutations in the IMS sorting signal (or in the IMS sorting machinery) would prevent translocation of the fusion protein out of the matrix, allowing the COX4 to assemble with other cytochrome oxidase subunits on the inside of the inner membrane, and would result in production of an active enzyme complex. Since cy-

TABLE 1. Overexpression of C1-COX4 prevents sorting to the IMS

C1-COX4 plasmid	Expression of C1-COX4[a]	Plasmid copy no.[b]	Growth on glycerol[c]
pRJ619	Cytochrome c_1	1–2	−
pRJ618	*ADH1*	1–2	−/+
pRJ620	*ADH1*	20–40	+

[a] The C1-COX4 fusion protein was placed under the control of either the yeast *ADH1* gene or cytochrome c_1 sequences as described in Materials and Methods.

[b] C1-COX4 constructs were subcloned into centromere-containing plasmid pRS316 (40) or pAC1 (43) or pRS416 (40), which contains the replication origin from the yeast 2μm plasmid.

[c] Plasmids were introduced into *cox4*::*LEU2* strain RM2-2c and tested for growth on medium containing glycerol as the sole carbon source. Symbols: −, no growth after 12 days; −/+, no growth after 4 days but detectable growth after 12 days; +, detectable growth after 4 days. Wild-type cells were scored as ++ for growth on YEP-glycerol.

tochrome oxidase activity is required for cell growth on nonfermentable carbon sources (e.g., glycerol), IMS sorting mutants could be isolated by selecting for growth on glycerol. Conceptually similar genetic approaches have proved successful for isolation of yeast mutants defective in secretion and vacuolar targeting (8, 32, 33).

We constructed several plasmids carrying the C1-COX4 fusion protein and transformed these plasmids into *cox4*::*LEU2* strain RM2-1b as described in Materials and Methods. We first observed that different levels of expression of the C1-COX4 fusion protein influenced the ability of *cox4*::*LEU2* strains to grow on glycerol medium. As shown in Table 1, when the *C1-COX4* gene is carried on a plasmid present in one to two copies per cell and expressed from cytochrome c_1 upstream sequences (pRJ619), transformants are unable to grow on medium with glycerol as the sole carbon source, even after 12 days. In contrast, when upstream cytochrome c_1 sequences are replaced with the yeast *ADH1* promoter (pRJ618), expression of C1-COX4 from a low-copy-number plasmid allowed very slow growth on glycerol. Small colonies would appear after 12 days on glycerol medium. Increasing the expression of C1-COX4, by placing the *ADH1-C1-COX4* gene on a multicopy plasmid (pRJ620), led to almost wild-type levels of growth on glycerol medium. These results suggest that the IMS sorting machinery may be saturable by increasing the expression of the C1-COX4 fusion protein.

To select for sorting mutants, strain RM2-1b (*MAT***a** *cox4*::*LEU2*) containing pRJ618 or pRJ619 was plated on medium containing glycerol as the sole carbon source. Ninety-eight independent, ethyl methanesulfonate-induced mutants and 45 spontaneous mutants that grew at wild-type levels on the glycerol plates (Gly^+) were isolated. To distinguish between mutations carried on the C1-COX4 plasmid and mutations in the yeast genome, we mated Gly^+ mutants to a *MAT*α *cox4*::*LEU2* strain (RM4-12d) that did not contain a plasmid. Diploids were selected and tested for the ability to grow on glycerol. If the diploid grew on glycerol medium, we presumed that the mutation was carried on the plasmid. Diploids from 89 of the 98 ethyl methanesulfonate-induced mutants and diploids from all 45 of the spontaneous mutants grew on glycerol. Diploids that failed to grow on glycerol identified nine recessive mutants that were potentially defective in the IMS sorting machinery, and these mutants were set aside for future analysis.

We showed that most of the plasmid-borne mutations were located in the cytochrome c_1 presequence. First, plasmid DNA was isolated from each mutant, propagated in bacteria, and transformed into *cox4*::*LEU2* strain RM2-2c. Each plasmid was shown to promote growth of RM2-2c on glycerol. Second, a DNA fragment containing the C1 presequence was isolated from each plasmid and subcloned in front of the *COX4* gene carried on an unmutagenized plasmid. These new C1-COX4 plasmids were transformed into a *cox4*::*LEU2* strain and tested for growth on glycerol. The C1 presequence was isolated as a 365-bp *Eco*RI-*Bam*HI fragment from pRJ618 plasmids or a 560-bp *Hin*dIII fragment from pRJ619 plasmids. The cytochrome c_1 presequences from 78 of the 134 plasmids promoted the ability to grow on glycerol, indicating that the mutation was located in the c_1 presequence. Plasmid-borne mutations that were not localized to the cytochrome c_1 presequence were not studied further.

Sorting mutations are located in a stretch of 19 hydrophobic amino acids of the cytochrome c_1 presequence. We determined the DNA sequences of the cytochrome c_1 presequences from 78 of our mutants (summarized in Fig. 2). The carboxy-terminal region of the wild-type cytochrome c_1 presequence contains a stretch of 19 uncharged amino acids. The mutations from all 78 of the plasmids resulted in amino acid substitutions in, or deletions of, this hydrophobic region. Some of the amino acid substitutions changed nonpolar amino acids to charged residues. In particular, we identified two mutations, G40D and G45D, that changed a glycine at positions 40 and 45 of the cytochrome c_1 presequence to an aspartic acid, and A48E, which changed an alanine to a glutamic acid. Other substitutions replaced threonine with asparagine (T47N) or alanine with proline (A42P and A48P). Many of these mutations were found repeatedly among our independent isolates, presumably because of the specificity of chemical mutagenesis. We also isolated several deletions in the hydrophobic region of the cytochrome c_1 presequence. Δ2 deleted 10 of the nonpolar amino acids from the cytochrome c_1 presequence. Δ1 deleted 16 amino acids, 8 of which were located in the hydrophobic region. Δ10 removed 10 hydrophobic amino acids and 4 additional amino acids distal to the uncharged region. Surprisingly, Δ3 removed 14 hydrophobic amino acids, all of the cytochrome c_1 sequences distal to the uncharged region, and 24 amino acids of the COX4 protein. Since all of our point mutations and deletions have in common defects in the 19-amino-acid hydrophobic stretch of the cytochrome c_1 presequence, we concluded that this region is critical for IMS sorting.

IMS sorting mutants accumulate the intermediate form of the C1-COX4 protein in vivo. We directly showed that IMS sorting was defective in the C1-COX4 proteins carrying mutations in the hydrophobic region of the C1 presequence. Specifically, a *cox4*::*LEU2* strain that contained a C1-COX4 plasmid carrying either the wild-type cytochrome c_1 presequence or the G45D mutations was pulse-labeled for 5 min with [^{35}S]methionine and chased in the absence of labeling. As shown in Fig. 3A, the wild-type C1-COX4 precursor was rapidly converted to the intermediate form (half-time, approximately 30 s) and slowly converted to the mature form (half-time, approximately 10 min). In contrast, proteins carrying the G45D mutation in the cytochrome c_1 presequence are defective in sorting (Fig. 3B). Precursor was converted to intermediate at the same rate as the wild type, but no mature protein was produced even after 60 min. Since the conversion from intermediate to mature form is catalyzed by a protease whose activity functions in the IMS (10, 31), our results indicate that sorting to the IMS is reduced at

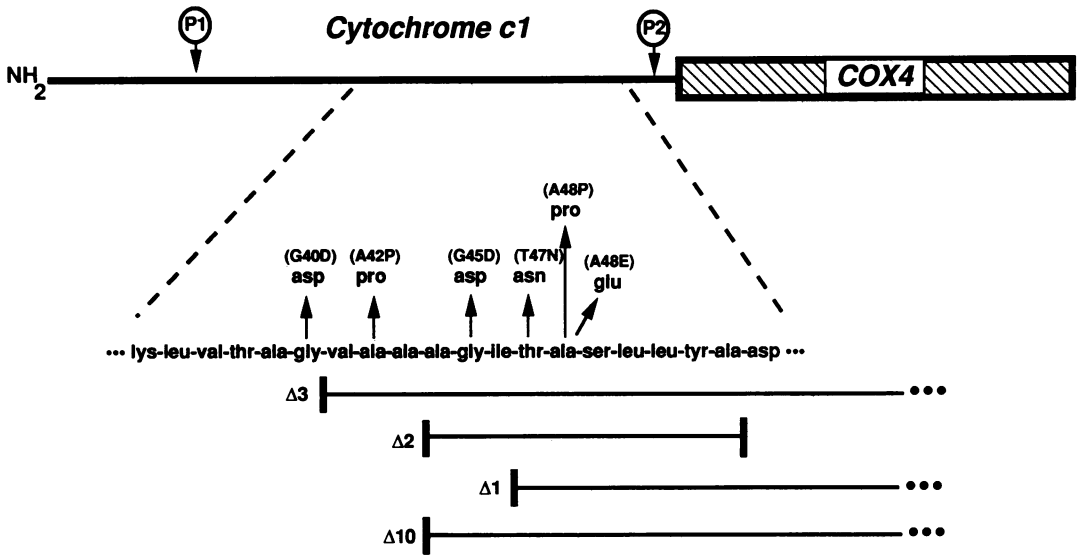

FIG. 2. Mutations in a region of 19 hydrophobic amino acids prevent the sorting of the cytochrome c_1 protein to the IMS. The DNA sequences of mutations that prevent IMS sorting of the C1-COX4 protein were determined as described in Materials and Methods. A diagram depicting the C1-COX4 fusion protein is shown at the top. P1 denotes the approximate cleavage site of the matrix-localized processing protease, and P2 denotes the cleavage site of the IMS-localized protease. A 21-amino-acid sequence from cytochrome c_1 begins at residue 35 of the cytochrome c_1 presequence and ends at residue 55. Amino acid substitutions are indicated in parentheses above the cytochrome c_1 sequence. Deletions that removed parts of the cytochrome c_1 protein are indicated below the sequence. Solid bars indicate the endpoints of the deletions. Dotted lines indicate that the deletion extends into adjacent cytochrome c_1 or COX4 sequences, and these endpoints are described in the text.

least 10-fold by the G45D mutation compared with the wild type. Similar results were seen for C1-COX4 proteins carrying the A48P and Δ2 alterations (data not shown).

Intermediate forms of C1-COX4 proteins carrying mutated IMS sorting signals accumulate in the matrix. C1-COX4 proteins were synthesized in vitro and imported into isolated mitochondria as described in Materials and Methods. As shown in Fig. 4, the wild-type C1-COX4 precursor was efficiently imported into mitochondria and converted to the mature form. Only a small amount of intermediate was detectable. When the mitochondrial outer membrane was disrupted after the import reaction (to form mitoplasts), the mature and intermediate forms of the C1-COX4 protein were digested by trypsin, suggesting that both proteins were located in the IMS. We found that cleavage by the IMS-localized processing protease is inefficient in our isolated mitochondria, especially at temperatures below 30°C (data not shown). Since the intermediate form of wild-type C1-COX4 is present in the IMS, the second cleavage is apparently not required for IMS sorting. Hence, sorting of cytochrome c_1 to the IMS is indicated by its accessibility to trypsin digestion in mitoplasts and not by its cleavage by the IMS processing protease. As a control, we showed that the authentic COX4 protein was imported completely into the matrix and protected from trypsin digestion in mitoplasts.

When the C1-COX4 fusion protein carried a mutation in the C1 presequence (G45D, A42P, A48E, Δ2, or T47N), the precursor was imported into mitochondria and converted to the intermediate form but little or no mature-sized protein was observed. Precursor that pelleted with mitochondria after the import of wild-type and mutant C1-COX4 proteins was bound to the mitochondrial surface and was digested by trypsin added to the outside of intact mitochondria (data not shown). The intermediate produced after import of each of the mutated C1-COX4 proteins was present in the matrix, protected from trypsin digestion in mitoplasts. Hence, the mutations we identified prevent sorting of C1-COX4 to the IMS. Similarly, C1-COX4 proteins carrying the A48P, Δ1, and Δ3 mutations accumulated as intermediates in the matrix (data not shown). The sorting defect of the different substitutions and deletions in the cytochrome c_1 presequence was not limited to a C1-COX4 fusion protein. For example, the G45D and Δ2 alterations blocked IMS sorting of a C1-dihydrofolate reductase fusion protein to the same extent that they blocked sorting of the C1-COX4 protein (data not shown).

In contrast to that of the other mutations, import of C1-COX4 carrying the T47N substitution produced a significant amount of mature-sized protein (Fig. 4). This mature protein was present in the IMS, accessible to trypsin digestion in mitoplasts. All of the intermediate-sized protein, however, was present inside the inner membrane. Hence, the T47N mutation appears to be "leaky"; C1-COX4 that has accumulated in the matrix is slowly exported across the inner membrane to the IMS. In time course studies, we found that C1-COX4 precursor carrying the T47N mutation was efficiently imported into mitochondria and processed to the intermediate form, but this intermediate was converted to the mature form only very slowly (Fig. 5). Our results suggest that if C1-COX4 is accumulated in the matrix by a mutation in its sorting signal, the C1-COX4 intermediate can be exported back across the membrane to the IMS, albeit at a reduced rate.

Mutations in the cytochrome c_1 presequence are defective in sorting the authentic cytochrome c_1 protein to the IMS. To demonstrate that we had identified an IMS sorting signal for the cytochrome c_1 gene, we engineered the mutation from different *C1-COX4* genes onto cytochrome c_1. As described in Materials and Methods, we constructed cytochrome c_1 genes that carried the G45D, A42P, A48P, Δ2, and Δ10

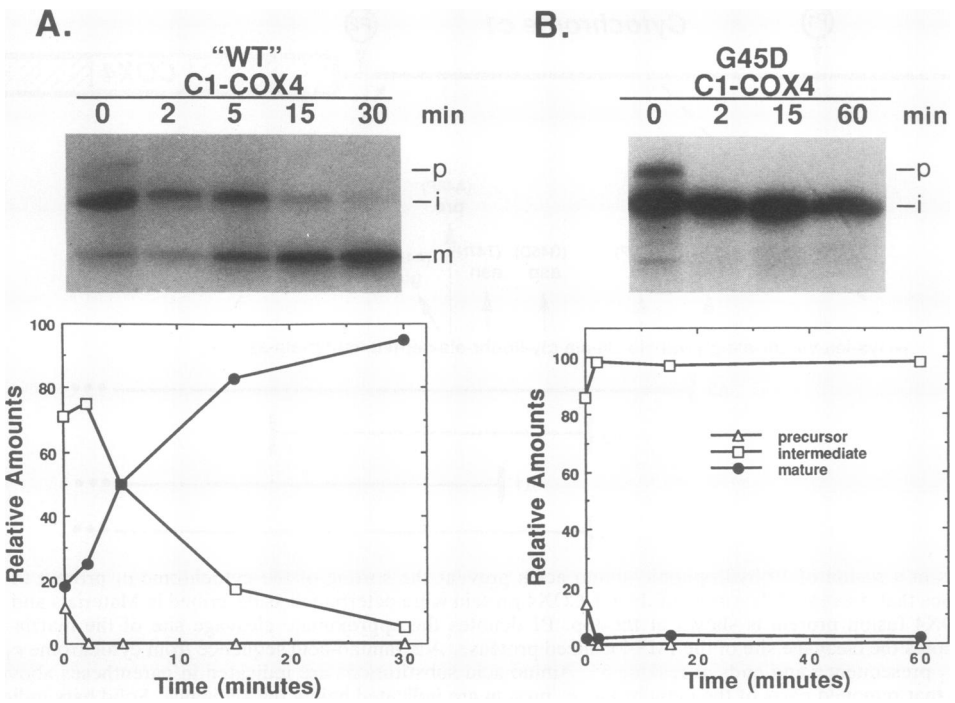

FIG. 3. Cells expressing the G45D mutation accumulate the intermediate form of C1-COX4. Plasmid pRJ618 carrying either wild-type C1-COX4 sequences or the G45D (glycine to aspartate) substitution in C1-COX4 were introduced into *cox4*::*LEU2* strain RM4-12d. Cells were grown to mid-logarithmic phase and labeled with [^{35}S]methionine (0.1 mCi/ml) for 5 min, and labeling was stopped by the addition of cycloheximide (200 μg/ml). At indicated times, aliquots were removed and proteins were extracted and immunoprecipitated with antiserum to the COX4 protein. Precipitated proteins were separated on 15% polyacrylamide gels and fluorographed. Relative amounts of the precursor (p), intermediate (i), and mature (m) forms of C1-COX4 were determined by densitometry. To compensate for differences in recovery, the total amount of precursor, intermediate, and mature forms in each aliquot was adjusted to 100 U. (A) Wild-type ("WT") C1-COX4; (B) C1-COX4 carrying the G45D substitution.

mutations. We also made a cytochrome c_1 gene carrying a precise deletion (ΔC) of the 19 hydrophobic amino acids of the C1 presequence. Constructs were transformed into strain RJ300-1D, in which the chromosomal cytochrome c_1 gene was replaced by the yeast *LEU2* gene, and transformants were assayed for the ability to grow on glycerol as the sole carbon source. As shown in Table 2, strains expressing cytochrome c_1 genes carrying the ΔC, Δ2, and Δ10 mutations failed to grow on glycerol medium, suggesting that these proteins are not properly sorted to the IMS. No growth was detectable even after 12 days. Strains expressing the A48P construct failed to grow after 4 days on glycerol medium but produced small colonies after 12 days. In contrast, strains expressing wild-type cytochrome c_1 and those expressing the A42P and G45D mutations grew at indistinguishable rates on glycerol.

The A48P, Δ2, Δ10, and ΔC mutations caused accumulation of the intermediate form of cytochrome c_1 in the matrix. After import of cytochrome c_1 carrying these alterations into isolated mitochondria, the intermediate form was produced

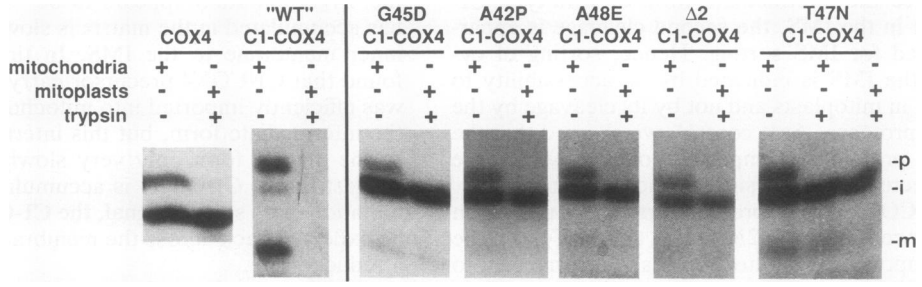

FIG. 4. Altered cytochrome c_1 presequences cause accumulation of the intermediate form of C1-COX4 in the matrix after import into isolated mitochondria. The authentic COX4 protein; the wild-type C1-COX4 protein; and C1-COX4 carrying the G45D, A42P, A48E, Δ2, and T47N alterations were synthesized in vitro and imported into isolated mitochondria as described in Materials and Methods. After import for 30 min at 30°C, mitochondria were recovered by centrifugation. Mitochondria from one-half of each import reaction were converted to mitoplasts, and proteins outside the inner membrane were digested by 200 μg of trypsin per ml for 20 min on ice. Mitochondria from the other half of the import reaction were immediately solubilized in sample buffer. Mitochondrial and mitoplast proteins were separated by 15% polyacrylamide gels and fluorographed. The precursor (p), intermediate (i), and mature (m) forms are indicated.

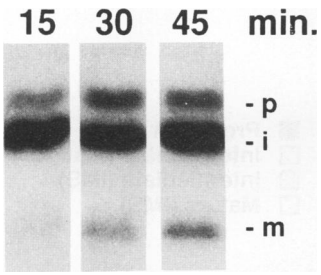

FIG. 5. Import of C1-COX4 carrying the T47N alteration yields the mature form of the protein at a greatly reduced rate. The COX4 protein carrying the T47N alteration was synthesized in vitro and imported into isolated mitochondria as described in Materials and Methods. After 15, 30, or 45 min at 30°C, import was stopped by addition of 0.5 mM valinomycin, followed by incubation on ice. Mitochondria were recovered by centrifugation and solubilized in sample buffer. Mitochondrial proteins were separated on a 15% polyacrylamide gel and fluorographed. The precursor (p), intermediate (i), and mature (m) forms of C1-COX4 are indicated.

but little or no mature-sized protein was observed (Fig. 6). The intermediate form of cytochrome c_1 was not accessible to proteinase K digestion in mitoplasts, suggesting that this protein was located in the matrix. In contrast, import of the wild-type cytochrome c_1, and proteins carrying the G45D and A42P substitutions, produced both the mature and intermediate forms in the IMS. These proteins were accessible to proteinase K digestion in mitoplasts. Hence, neither the G45D nor the A42P mutation affects the sorting of cytochrome c_1 to the same extent as the C1-COX4 fusion protein. In bacteria, signal sequence mutations are often more severe when carried on fusion proteins (5), presumably since the mature protein can carry information important for secretion (42, 47). Our results thus raise the possibility that the mature cytochrome c_1 protein plays a role in IMS targeting.

Cytochrome c_1 carrying the G45D substitution is imported completely into the matrix and then slowly exported to the IMS. Both the G45D and A42P mutations severely affect the IMS sorting of the C1-COX4 fusion protein, but, as shown above, neither mutation appears to have a large effect on the sorting of authentic cytochrome c_1. In time course experiments with isolated mitochondria, however, we found that the G45D mutation affects the rate of IMS sorting of the cytochrome c_1 protein. As shown in Fig. 7, the wild-type cytochrome c_1 precursor was imported into isolated mitochondria and rapidly converted to the intermediate form. Within 10 min, most of this intermediate was converted to the mature protein by the IMS-localized processing protease. At very early times (30 s), a small amount of intermediate appears to be completely inside the inner membrane, protected from proteinase K digestion in mitoplasts. The majority of the intermediate, and all of the mature protein, was located in the IMS, accessible to proteinase K digestion. Import of the cytochrome c_1 protein carrying the G45D substitution gave rise to the intermediate form with nearly wild-type kinetics. However, in contrast to the wild type, most of the G45D intermediate is located in the matrix, protected from protease digestion in mitoplasts. This matrix-localized intermediate appears to be slowly exported to the IMS. By 30 min, the intermediate is gone and wild-type levels of the mature form are found in the IMS (Fig. 6).

TABLE 2. Mutations in the hydrophobic region of the cytochrome c_1 presequence prevent sorting to the IMS

Cytochrome c_1 construct[a]	Growth on glycerol[b]
Wild type	++
Δ2	−
Δ10	−
A48P	−/+
G45D	++
A42P	++

[a] The wild-type cytochrome c_1 gene and cytochrome c_1 genes carrying different mutations in the IMS sorting sequence were subcloned into the yeast expression vector pAC1 and transformed into strain RJ300-1D (*cyt1*::*LEU2*) as described in Materials and Methods.

[b] Transformants were tested for growth on medium containing glycerol as the sole carbon source. Symbols: +, growth after 4 days; −, no growth after 12 days; −/+, no growth after 4 days, but small colonies are visible after 12 days; ++, growth indistinguishable from that of wild-type cells.

DISCUSSION

We have identified mutations in the signal required for sorting the cytochrome c_1 protein to the IMS by isolating mutants that mislocalize a C1-COX4 fusion protein. All of the cytochrome c_1 mutations identified in our studies were localized to a stretch of 19 uncharged amino acids in the carboxy-terminal third of the cytochrome c_1 presequence. Hence, our studies pinpoint this sequence of uncharged amino acids as critical for sorting C1-COX4 to the IMS. We have also shown that this region is necessary for sorting the authentic cytochrome c_1 protein.

Many of our mutations do not allow us to easily distinguish between the two models proposed for IMS targeting (Fig. 1). For example, G40D, G45D, and A48E introduce a charged amino acid into the hydrophobic IMS sorting signal. These substitutions would be expected to disrupt the function of either a membrane stop-transfer sequence or an export signal. Similarly, helix-breaking proline substitutions might perturb either type of sorting signal. The T47N mutation, however, is inconsistent with a simple stop-transfer model. Although asparagine is predicted to be more hydrophilic than threonine (9), asparagine is commonly found in membrane-spanning domains. We therefore suggest that the cytochrome c_1 IMS sorting signal is not a membrane stop-transfer sequence but is instead a signal recognized by a component of the IMS sorting pathway. Supporting this hypothesis is our observation that IMS sorting appears to be saturable: overproduction of the C1-COX4 protein results in its accumulation in the matrix.

Our results support the hypothesis that cytochrome c_1 and C1-COX4 fusion proteins are normally completely imported into the matrix, processed to the intermediate form, and then exported to the IMS (Fig. 1, model B). Mutations in the IMS sorting signal lead to accumulation of the intermediate form of the protein in the matrix. The intermediate form of the C1-COX4 protein carrying the T47N alteration or the cytochrome c_1 protein carrying the G45D substitution appears to be slowly exported across the inner membrane to the IMS and converted to the mature form by the IMS-localized processing protease. Our experiments, however, do not rule out the possibility that cytochrome c_1 is normally sorted to the IMS by the stop-transfer model (Fig. 1, model A). For example, point mutations that do not completely block sorting (e.g., the T47N mutation) may yield two pools of imported protein. The majority of the mutant precursor may be missorted to the matrix and converted to the intermediate

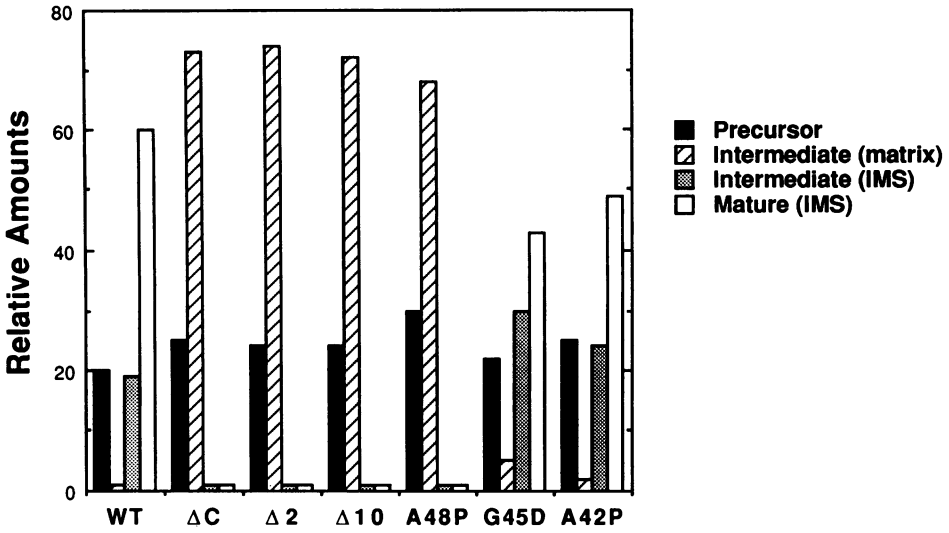

FIG. 6. Cytochrome c_1 proteins carrying presequence alterations are defective in sorting to the IMS. Cytochrome c_1 proteins were produced in vitro and imported into isolated mitochondria as described in Materials and Methods. After 30 min at 30°C, mitochondria were isolated by centrifugation and divided into two aliquots. The mitochondrial outer membrane in one aliquot was disrupted by osmotic shock as described in the legend to Fig. 4 and digested with 250 μg of proteinase K per ml for 20 min on ice. Mitochondria in the other aliquot were immediately solubilized in sample buffer. Proteins from mitochondria and mitoplasts were run on 12.5% polyacrylamide gels and fluorographed. Relative amounts of precursor, intermediate, and mature forms were determined by densitometry. Intermediate (matrix) represents intermediate-sized protein that is protected from proteinase K digestion in mitoplasts. Intermediate (IMS) represents intermediate-sized protein that is digested by proteinase K in mitoplasts.

form, whereas a small portion of the altered protein may be correctly sorted to the IMS via the stop-transfer pathway. Alternatively, it is possible that mutant cytochrome c_1 substrates use sorting machinery not utilized by the wild-type protein. We prefer the simpler hypothesis that all cytochrome c_1 proteins are normally imported into the matrix prior to their export to the IMS. This model for cytochrome c_1 import also explains how the carboxy terminus of cytochrome c_1 becomes anchored in the inner membrane. In particular, the second hydrophobic sequence in the C terminus of the mature cytochrome c_1 protein would function as a stop-transfer sequence during export from the matrix, preventing complete translocation of cytochrome c_1 to the IMS. The machinery importing cytochrome c_1 into the matrix apparently does not recognize either of the two hydrophobic regions of the cytochrome c_1 precursor. Hence, we predict that the import machinery is mechanistically distinct from the IMS export machinery.

The rapid rate of export of cytochrome c_1 may explain why cytochrome c_1 or C1-COX4 fusion proteins are difficult to detect in the matrix. We found that the wild-type cytochrome c_1 and the C1-COX4 proteins are sorted to the IMS with a half-time of less than 2 min. Only at the 30-s time point could a small amount of the wild-type cytochrome c_1 intermediate be detected in the matrix, and we were never able to detect either the precursor or intermediate of the wild-type C1-COX4 fusion protein in the matrix. Mutations in the cytochrome c_1 sorting signal slow the rate of export and allow detection of the intermediate in the matrix. Mutations that reduce the rate of a specific step in a pathway are often useful in identifying short-lived species. For example, the inability to detect the precursor form of secreted bacterial proteins led to the proposal that secretion was obligatorily cotranslational. However, signal sequence mutations in several different bacterial proteins caused precursors to accu-

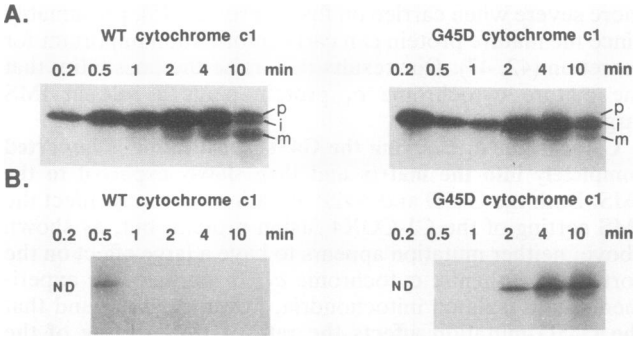

FIG. 7. Cytochrome c_1 that carries the G45D substitution accumulates in the matrix as an intermediate and is converted to the mature form in the IMS at a reduced rate. The wild-type cytochrome c_1 protein and cytochrome c_1 carrying the G45D substitution were synthesized in vitro and imported into mitochondria at 30°C as described in Materials and Methods. At the indicated times, aliquots were isolated and import was stopped by the addition of 0.5 mM valinomycin. Mitochondria were isolated from the import reaction mixture and divided into two aliquots. Mitochondria from one aliquot were solubilized with sample buffer. Mitochondria from the other aliquot were converted to mitoplasts and treated with 250 μg of proteinase K per ml for 20 min on ice, and the protease was inhibited by 10 μM phenylmethylsulfonyl fluoride. Mitoplasts were recovered by centrifugation. Mitochondrial and mitoplast proteins were separated on 12.5% polyacrylamide gels, and radiolabeled proteins were identified by fluorography. The precursor (p), intermediate (i), and mature (m) forms of cytochrome c_1 are indicated. ND, not determined. (A) Import of wild-type (WT) cytochrome c_1 and cytochrome c_1 carrying the G45D substitution into mitochondria. (B) Intermediate forms of cytochrome c_1 and cytochrome c_1 carrying the G45D substitution protected from proteinase K digestion in mitoplasts.

mulate in the bacterial cytosol, after which they were posttranslationally secreted across the plasma membrane (23, 29, 35, 41, 46).

Since all of our mutant C1-COX4 proteins accumulated as intermediates in the matrix, none of our sorting mutants resulted from a mutation in the matrix protease cleavage site. This result suggests that cleavage of the precursor to the intermediate form is not required for export to the IMS. This hypothesis is supported by the observation that cytochrome c_1 is efficiently sorted to the IMS when the activity of the matrix protease is inhibited by chelators (30).

The export of precursors across the inner membrane to the IMS is proposed to be functionally analogous to bacterial secretion (13–15). Since mitochondria are deduced to have arisen from bacteria, the signals and machinery for IMS export may be remnants of the bacterial secretion pathway. Indeed, we find that the signal required to sort cytochrome c_1 to the IMS is very similar to a bacterial signal sequence. The 19-hydrophobic-amino-acid stretch in the cytochrome c_1 presequence is preceded by several basic amino acids and followed by two acidic residues (42, 47). Mutations that disrupt the function of the cytochrome c_1 sorting signal are similar to those that disrupt the function of bacterial signal sequences (23, 29, 35, 41). Furthermore, we have found that C1-COX4 expressed in *Escherichia coli* is secreted into the periplasmic space and that the G45D substitution blocks its secretion (22a). Our data thus strongly support the idea that protein sorting to the IMS is mechanistically derived from bacterial secretion.

By analogy to bacterial signal sequences, we anticipate that the IMS sorting signal may have several roles. For example, bacterial signal sequences help maintain the precursor in an unfolded state (12) and also target the precursor to membrane-bound secretion machinery (47). Hence, the cytochrome c_1 sorting signal may prevent premature folding in the matrix, and the sorting signal may mediate interaction with components of the IMS sorting machinery. In particular, MIF4, a mitochondrial matrix protein homologous to the bacterial GroEL protein, is proposed to be required for the sorting of at least one IMS protein, cytochrome b_2 (4). MIF4 may recognize the hydrophobic sorting signal of IMS-bound proteins and thus may play an active role in export of proteins across the inner membrane. We are currently using our mutations to dissect the role(s) of the IMS sorting signal in export.

Our screen also identified chromosomal mutations that caused defects in IMS sorting. These mutants are potentially defective in the machinery that mediates the IMS sorting pathway, such as receptor proteins, components of the translocation complex, and matrix-localized chaperones. We are presently analyzing these mutants to identify the components required for IMS sorting and to determine their relationship, if any, to bacterial Sec proteins.

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